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MicroRNA-133a-1 regulates inflammasome activation through uncoupling protein-2

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ABSTRACT

Inflammasomes are multimeric protein complexes involved in the processing of IL-1β through Caspase-1 cleavage. NLRP3 is the most widely studied inflammasome, which has been shown to respond to a large number of both endogenous and exogenous stimuli. Although studies have begun to define basic pathways for the activation of inflammasome and have been instrumental in identifying therapeutics for inflammasome related disorders; understanding the inflammasome activation at the molecular level is still incomplete. Recent functional studies indicate that microRNAs (miRs) regulate molecular pathways and can lead to diseased states when hampered or overexpressed. Mechanisms involving the miRNA regulatory network in the activation of inflammasome and IL-1β processing is yet unknown. This report investigates the involvement of miR-133a-1 in the activation of inflammasome (NLRP3) and IL-1β production. miR-133a-1 is known to target the mitochondrial uncoupling protein 2 (UCP2). The role of UCP2 in inflammasome activation has remained elusive. To understand the role of miR-133a-1 in regulating inflammasome activation, we either overexpressed or suppressed miR-133a-1 in differentiated THP1 cells that express the NLRP3 inflammasome. Levels of Caspase-1 and IL-1β were analyzed by Western blot analysis. For the first time, we showed that overexpression of miR-133a-1 increases Caspase-1 p10 and IL-1β p17 cleavage, concurrently suppressing mitochondrial uncoupling protein 2 (UCP2). Surprisingly, our results demonstrated that miR-133A-1 controls inflammasome activation without affecting the basal expression of the individual inflammasome components NLRP3 and ASC or its immediate downstream targets proIL-1β and pro-Caspase-1. To confirm the involvement of UCP2 in the regulation of inflammasome activation, Caspase-1 p10 and IL-1β p17 cleavage in UCP2 of overexpressed and silenced THP1 cells were studied. Suppression of UCP2 by siRNA enhanced the inflammasome activity stimulated by H_2O_2 and, conversely, overexpression of UCP2 decreased the inflammasome activation. Collectively, these studies suggest that miR-133a-1 suppresses inflammasome activation via the suppression of UCP2.

1. Introduction

Inflammasomes are multi-protein structures that regulate the activation of Caspase-1 and the maturation of pro-inflammatory cytokines like IL-1β, IL-18, and IL-33 [1]. Inflammasome activation is a two-step process; the first signal is enabled through the activation of pathogen response receptors (PRRs). Activated PRRs stimulate NF-κB and prime the formation of the inflammasome complex. The second signal comes from a range of stimuli such as ATP, uric acid crystals, hydrogen peroxide, reactive oxygen species (ROS), or intracellular stimuli such as sterile inflammation [2]. Among the wide variety of inflammasomes, the NLRP3 inflammasome complex is well studied [3].

Although the precise mechanisms of activation are not known, studies demonstrate that NLRP3 is activated by a wide range of compounds: they include both exogenous stimuli as well as host ligands including bacterial RNA, ATP, uric acid crystals, antiviral imidazoquinoline compounds, ceramide, and oxygen toxicity [4–7]. So far, based on these findings, three key mechanisms have been described to account for NLRP3 activation [3]. One mechanism for *NLRP3* activation is potassium efflux [8]. External ATP is recognized by the P_2X_7 receptor, a cation channel, which facilitates the activation of potassium efflux that, in turn, triggers NLRP3 activation [8]. The generation of mitochondria-derived ROS plays a critical role in the activation of NLRP3 [9]. Phagolysosomal destabilization also activates NLRP3; it is caused by large crystals and par-

Abbreviations: miR, micro RNA; UCP2, uncoupling protein 2; siRNA, silencing RNA; IL-1β, interleukin 1 beta; THP1, human monocyte cell line.

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ticulates such as monosodium urate (MSU), adjuvant alum, asbestos, and silica [10]. Upon activation of NLRP3, it oligomerizes and recruits the ASC domain which, in turn, recruits pro-Caspase-1.This event leads to auto-proteolytic cleavage of pro-Caspase-1 and formation of active Caspase-1. Active Caspase-1 cleaves pro-IL-1β and leads to secretion of active IL-1β [11].

One defense mechanism of the innate immune system involves inflammasomes which combat invading microbes via activation of Caspase-1 and the production of mature pyrogenic cytokine IL-1β [3]. IL-1β is an essential mediator of the inflammatory response causing fever, hypotension, and production of other pro-inflammatory cytokines [12]. Inflammasomes also take part in a variety of cellular activities including cell proliferation, differentiation, and apoptosis [13]. The synthesis of IL-1β is very tightly regulated by several mechanisms; however, mutations in the NLRP3 gene are associated with a spectrum of auto-inflammatory diseases characterized by excessive production of IL-1β, they include diseases such as cryopyrin-associated periodic syndrome (CAPS), familial cold auto-inflammatory syndrome, Muckle–Wells syndrome, and chronic infantile cutaneous neurological articular syndrome [14–16]. Inflammasomes also aggravate gout [17], asbestosis, silicosis [10,18], and Alzheimer's disease [19].

Recently, microRNAs (miRs) have emerged as a discrete class of biological regulators with conserved functions that have the potential to control IL-1β generation [20]. miRs play a crucial role in cell proliferation, cell differentiation, apoptosis, developmental timing, and other regulatory pathways [21–26]. Increasing evidence in the involvement of miRs in clinical disease models demonstrate that miRs are considered promising agents in clinically- associated research [22–26]. In general, miRs are short, single-stranded, non-protein coding RNA molecules [26,27]. miRs bind to the complementary untranslated region (UTR) of the target mRNA and cleaves the segment: resulting in instability and subsequent degradation of the mRNA [22,28].

In the present study, we investigate the role of miR-133A in inflammasome activation and IL-1β production. miR-133-a-1 was first characterized in mice; it is homologous to some other species, including invertebrates [27]. There are three miR-133 genes identified in the human genome: miR-133a-1, miR-133a-2, and miR-133b [28]. Some in vitro studies suggest that upregulation of miR-133a-1 suppresses UCP2 expression [29]. UCP2 is an integral membrane protein and a member of the large UCP family; it plays a role in ATP synthesis and ROS production [30,31]. UCP2 is widely expressed in several tissues compared to the other UCP family members including the spleen, kidney, pancreas, and immune system [32]. A previous study shows UCP2 negatively regulates ROS and induces an anti-inflammatory response [31]. It plays a critical role in UCP2-mediated mitochondrial function in phagocytosis [33].

In this report, we demonstrate the activation of Caspase-1 p10 and IL-1β p17 by miR-133a-1-induced suppression of UCP2 in inflammasome-stimulated THP1 cells.

2. Materials and methods

2.1. Cells and reagents

THP1 cells derived from human lung adenocarcinoma were obtained from American Type Culture Collection (ATCC, Manassas, VA) and were maintained in RPMI-1640 medium as per vendor instructions. PMA (Sigma–Aldrich, St. Louis, MO, USA), anti-mouse (Cell Signaling Technology, Danvers, MA), anti-goat (Santa Cruz, Inc., CA, USA), or anti-rabbit (Cell signaling) HRP conjugated secondary antibodies were bought from its listed companies. Primary antibodies specific to NLRP3 (Enzo Life Sciences, Inc. Farmingdale, NY, USA), ASC (Enzo) Caspase-1 (Santa Cruz), UCP2 (R&D systems, MN, USA), IL-1β (Cell signaling), β-Actin (Cell signaling), and GAPDH (Cell signaling) were used in Western blot.

2.2. Inflammasome activation

Inflammasome was stimulated in THP1 cells as previously mentioned [34]. In brief, THP1 cells were differentiated by treating it with 0.5 μg/ml of PMA for 3 h, and the medium was replaced with complete RPMI 1640 overnight. After 24 h, cells were transfected with miR-133-a1 or miR vector control. After 24 h, cells were briefly washed with PBS and stimulated with 5 mM H_2O_2 for 3 h or 2.5 mM ATP for 1 h. The activation of inflammasome was confirmed by measuring the IL-1β secretion and comparing the expression of mature IL-1β and Caspase-1 expression by ELISA and Western blot analysis.

2.3. Micro RNA and SiRNA transfection

THP1 cells were seeded at 0.5 million cells per well in a six-well plate, and differentiated with 0.5 μg/ml of PMA for 3 h. To remove the residual effect of PMA, the medium was replaced with complete RPMI 1640 overnight. After 24 h, the medium was replaced by serum-free RPMI-1640 and cells were transfected with 2.5 μg/well DNA of pCMV-miR control (OriGene, Rockville, MD) and miR-133a-1 (Origene) plasmid using Lipofectamine® LTX plus Reagent (Invitrogen, CA, USA) as per manufacturer instructions. After 4 h of transfection, the medium was replaced with complete RPMI-1640. After 24 h, cells were washed with PBS and medium was replaced by serum-free RPMI-1640 for 2 h, then cells were treated with 5 mM H_2O_2 for 3 h.

2.4. Western blot

Cells were lysed with 150 μl of RIPA buffer and 10 μl/ml of protease and phosphatase inhibitors; protein quantification was performed using the Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). Equal quantities of protein were loaded into SDS–PAGE, and protein was transferred to PVDF membrane. The membrane was blocked in 5% nonfat dry milk, and probed with primary and secondary antibodies diluted in blocking buffer. A Pierce ECL Western blotting substrate kit (Thermo scientific Rockford, IL, USA) was used to detect the protein under ChemiDoc XRS (Bio-Rad, Hercules, CA, USA). Bands were quantified and densitometric analysis was performed using ImageJ (Data not shown).

2.5. Immunofluorescence and immunocytochemistry

Differentiated THP1 cells in 35 mm, 4 compartment, and glass-bottom dish (Greiner bio-one, Maybachstr, Germany) were transfected with pCMV-miR control and pCMV-miR-133 plasmid containing IRES-tGFP for 24 h. Localization of GFP was observed under the Olympus IX81 inverted microscope equipped with the 3i Yokogawa spinning disk scanner and CCD cameras with revolving laser arrays. Cy3-labeled siRNA scramble was transfected into differentiated THP1 cells. At 48 h post-transfection, the cells were fixed with 4% paraformaldehyde; the nuclei were stained with DAPI, and examined for Cy3 localization under the microscope.

2.6. Bone marrow macrophage isolation from knockout mice

Approval of the study protocol was obtained from the University of South Florida Institutional Animal Care and Use Committee. All mice were maintained in a specific-pathogen-free animal facility at the University of South Florida and animal experiments were carried out according to the provisions of the Animal Welfare Act,

PHS Animal Welfare Policy, and the principles of the NIH Guide for the Care and Use of Laboratory Animals. Bone marrow cells were obtained from $ASC^{-/-}$, $NLRP3^{-/-}$ and wild type (WT) mice (The Jackson Laboratory, Bar Harbor, ME) by dissecting the leg bones at the thigh bone, then flushing the marrow from the leg bones with a 23-gauge needle and L-conditioned DMEM medium (M.A. Bioproducts, Walkersville, MD) supplemented with penicillin/streptomycin (P/S: 100 IU/ml and 100 pg/ml, respectively). The cells were dissociated by gentle vortexing, centrifuged at 1000 rpm, and resuspended in medium. The cell suspension was seeded at 1 million cells per well in a 12-well plate. Medium was replaced once in every 3 days and cells were used for further experiments.

2.7. Statistical analysis

All experiments were performed in triplicate. A paired *t*-test was used to determine the statistical significance. A *p*-value of $p < 0.05$ was accepted as statistically significant.

3. Results

3.1. miR-133a-1 enhances Caspase-1activation and IL-1β processing

To understand the role of miR-133a-1 in the activation of NLRP3 inflammasome, THP1 cells were transfected with pCMV-miR (Vector control) or pCMV-miR-133a-1, followed by stimulation with an inflammasome activator, H_2O_2, as mentioned in the Section 2. After transfection and post-inflammasome stimulation, supernatants were used to check Caspase-1 p10 and cleaved IL-1β p17 expression by Western blot. Our results indicate that in pCMV-miR-133a-1, the transfection of miR-133a-1 itself increases Caspase-1p10 compared to miR vector control (Fig. 1A). In addition, inflammasome stimulation by H_2O_2 also further increases Caspase-1 p10 release in supernatants. This data demonstrates that miR-133a-1 enhances Caspase-1 p10 activation. Since inflammasome stimulation and Caspase-1 activation process mature IL-1β, secretion of mature IL-1β p17 was analyzed in vector control and miR-133a-1 transfected THP1 cells. In miR vector control-transfected cells, the activation of IL-1βp17 was higher in H_2O_2 stimulated cells (Fig. 1A). This confirms the inflammasome activation by H_2O_2. In miR-133a-1 transfected cells, the transfection of miR-133a-1 increase IL-1β p17 release when compared to miR control vector. In addition, inflammasome stimulation by H_2O_2 also further increases IL-1β p17. This data demonstrates that miR-133a-1 enhances IL-1β p17 processing and secretion. Since the plasmids encoding precursor microRNA also possess internal ribosome entry site (IRES)-driven tGFP the transfection was confirmed by checking the tGFP expression in a fluorescence microscope at 24 h post-transfection (Fig. 1B). In order to understand whether miR-133a-1 only controls inflammasome activation or if it affects the basal expression of inflammasome components and its effector proteins, the expression of NLRP3, ASC, pro IL-1β and pro-Caspase-1 were analyzed by Western blot. The expression pattern of inflammasome components in miR-vector control and miR-133a-1 transfected cells under untreated and H_2O_2-treated conditions remain unchanged (Fig. 1C). These results suggest that miR-133a-1 is involved in inflammasome activation that can lead to Caspase-1 activation as well as IL-1β processing and secretion.

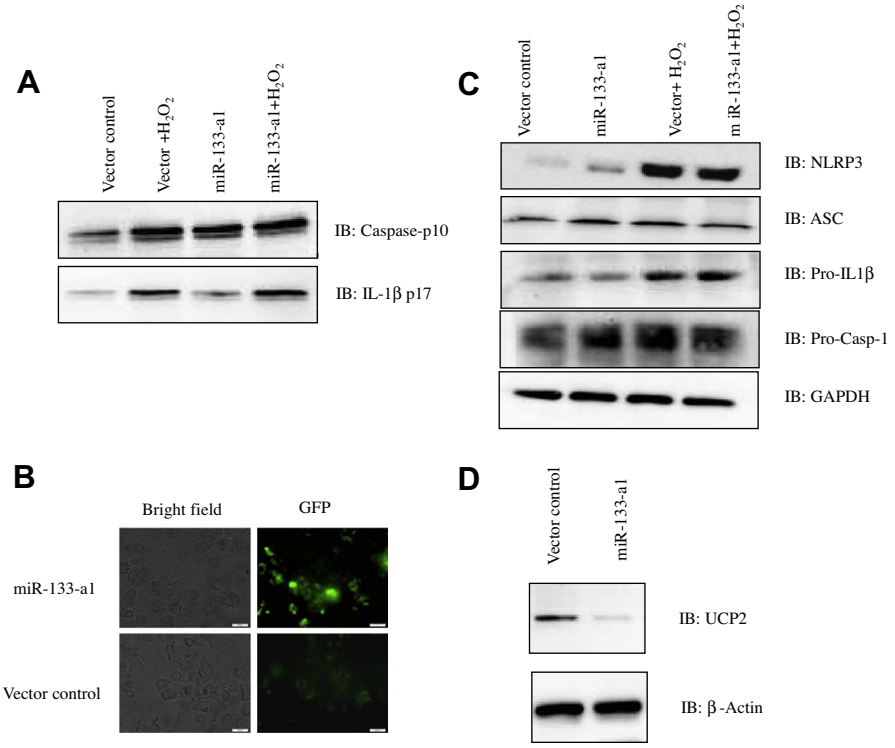

Fig. 1. miR-133-a1 enhances H_2O_2-mediated inflammasome activation and IL-1β production in THP1 cells and suppresses its target UCP2. Differentiated THP1 cells were transfected with miR-133-a1 or vector control (miR control). At 48 h post-transfection, inflammasomes were stimulated by adding H_2O_2 at 5 mM for 4 h. (A) Expression levels of Caspase-1 p10 and cleaved IL-1β p17 in the supernatants were measured by Western blot analysis. (B) Transfection efficiency of micro RNA plasmids (vector control and miR-133-a1) was confirmed by examining the localization of IRES-GFP under bright field and fluorescence microscopy. (C) Expression levels of NLRP3, ASC, pro-IL1β, pro-Caspase-1, and GAPDH in lysates were observed under immunoblot. (D) Differentiated THP1 cells were transfected with miR-133-a1 or vector control (miR control) plasmids. Expression levels of UCP2 at 48 h post-transfection were analyzed by Western blot.

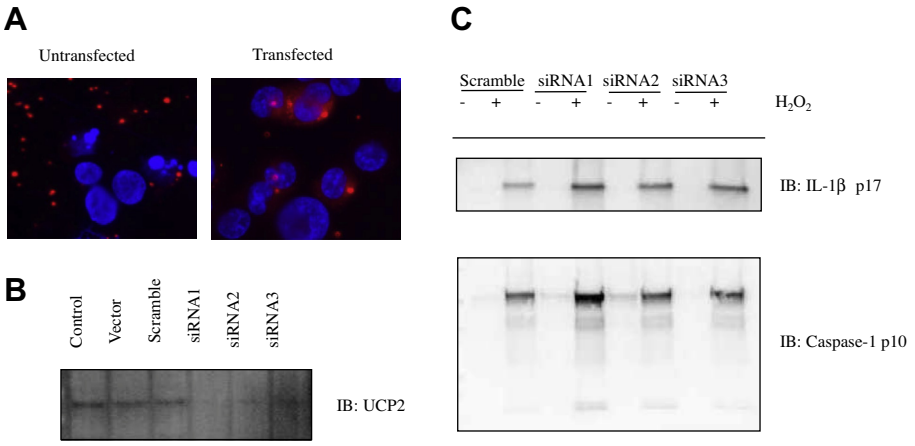

Fig. 2. UCP2 suppression augments inflammasome activation. siRNA specific to UCP2 (siRNA1, 2, 3), Cy3-labeled siRNA control, and scrambled siRNA control were transfected into differentiated THP1 cells. (A) At 48 h post-transfection, expression of Cy3 was examined under fluorescence microscope. siRNA (in red) localization with nucleus, in blue, can be seen in the transfected cells. The left image shows THP1 cells exposed to siRNA without transfection reagent. (B) Western blot analysis of UCP2 was utilized to confirm the silencing of UCP2 in transfected THP1 cells. (C) After transfection with siRNAs and controls, THP1 cells were stimulated with 5 mM H_2O_2 and secretion of mature IL-β p17 and Caspase-1 p10 in the supernatants was compared by Western blot analysis. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. miR-133a-1. suppress UCP2 expression

The miRNA database shows that the miR-133-a1 targets the untranslated region (UTR) of UCP2. To confirm that miR-133a-1 controls UCP2, the expression of UCP2 was checked by Western blot in miRNA-overexpressed THP1 cells. The expression of UCP2 was downregulated in miR-133a-1-transfected THP1 cells (Fig. 1D). This data demonstrated miR-133a-1-mediated down-regulation of UCP2.

3.3. Silencing UCP2 enhances inflammasome activation

To understand the direct influence of UCP2 on inflammasome activation, Caspase-1 and IL-1β secretion levels were examined in the UCP2 silenced cells. Human UCP2 in siRNA's were transfected into THP1 cells by using Lipofectamine as per manufacturer instructions. The transfection efficiency in 48 h post-transfected cells were confirmed by examining the Cy3-labeled siRNA-transfected cells under microscope and Western blot analysis (Fig. 2A, B). Compared to the scrambled siRNA control, UCP2 siRNA-transfected cells exhibited a significant increase in Caspase-1 and IL-1β secretion levels in response to H_2O_2 (Fig. 2C). These results indicate that UCP2 suppression leads to activation of the inflammasome.

3.4. UCP2 overexpression limits Capase-1 and IL-1β production

Since UCP2-silenced cells enhanced inflammasome activation, Caspase-1 and IL-1β secretion were compared in UCP2-overexpres-

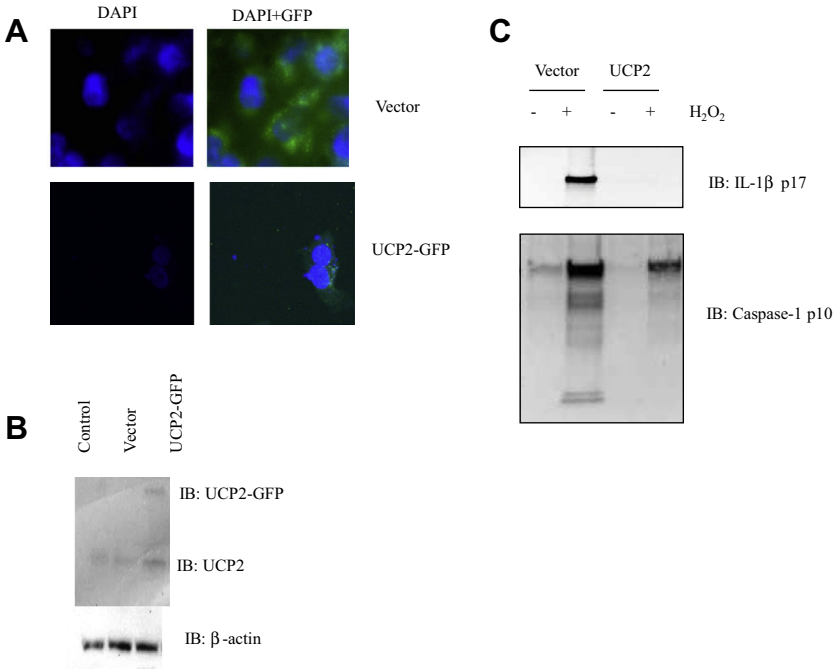

Fig. 3. UCP2 overexpression suppressed IL-1β and Caspase-1p10 production in THP1 cells. pCMV-UCP2-GFP or pCMV-GFP (vector) was transfected into differentiated THP1 cells seeded at 0.5 million cells/well in a six-well plate with coverslip. Untransfected cells acted as controls. (A) 48 h post-transfection, cells were fixed in 4% paraformaldehyde, and the expression of GFP was examined under fluorescence microscope. (B) Expression of UCP2 in cell lysates transfected with UCP2-GFP or vector was analyzed by Western blot to confirm transfection. (C) After post-transfection with plasmids overexpressing UCP2 and controls, THP1 cells were stimulated with 5 mM H_2O_2 and secretion of mature IL-β p17 and Caspase-1 p10 in the supernatants was compared by Western blot analysis.

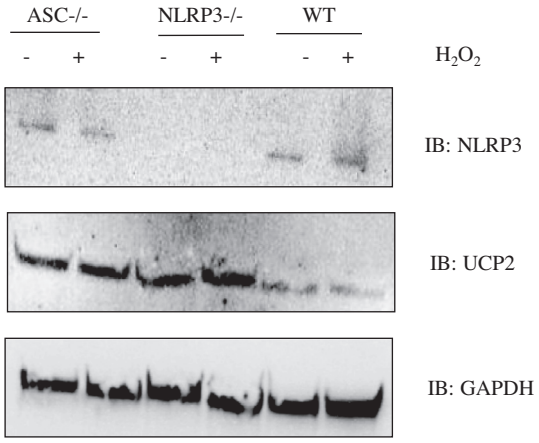

Fig. 4. UCP2 is upregulated in bone marrow macrophages of NLRP3 and ASC deficient mice. Bone marrow macrophages from wild type, NLRP3$^{-/-}$, and ASC$^{-/-}$ knockout mice were isolated, inflammasome was stimulated using H_2O_2, and the expression levels of UCP2, NLRP3, and GAPDH were compared by Western blot analysis.

sed THP1 cells. Human UCP2 cDNA tagged with GFP was transfected into THP1 cells; the transfection efficiency was confirmed by examining the GFP expression under microscope and by checking UCP2 levels by Western blot analysis (Fig. 3A, B). The H_2O_2 mediated activation of Caspase-1 and IL-1β decreased more significantly in UCP2-overexpressed cells than the vector control (Fig. 3C). This demonstrated that UCP2 may act as a negative regulator of inflammasome activation.

3.5. UCP2 is upregulated in the absence of NLRP3 and ASC

To define the role of inflammasome components, NLRP3 and ASC, in the regulation of UCP2, bone marrow macrophages were derived from NLRP3 and ASC knockout mice (NLRP3$^{-/-}$, ASC$^{-/-}$), and the expression of UCP2 was compared with wild type controls (WT). UCP2 levels were increased in NLRP3$^{-/-}$ and ASC$^{-/-}$ cells when compared to the wild type controls (Fig. 4). This showed that UCP2 is negatively regulated by NLRP3 and ASC.

4. Discussion

In this study, we demonstrated the involvement of miR-133a-1 in the regulation of inflammasome effector proteins Caspase-1p10 and IL-1β p17 by suppressing its target UCP2. Inflammasomes are multiprotein complexes that are involved in IL-1β processing and secretion through a tightly regulated process. Since IL-1β is directly correlated with various disorders that include autoinflammatory, autoimmune, and infectious diseases; approaches to inhibit IL-1β production should gain priority in clinical research. Recent studies are focusing on cellular mechanisms regulating IL-1β processing and secretion. Since the discovery of inflammasomes, many molecular mechanisms have been explained to present the activation and processing of IL-1β. However, there is little evidence that link microRNA-mediated regulation of inflammasome activation and IL-1β processing. Recent studies with miR-155 unlocked the possibilities of IL-1β regulation through the modulation of miRNA expression [35]. For the first time, we have presented the involvement of miR-133-a1 in modulating the activation of NLRP3 inflammasome.

Our results indicate that overexpression of miR-133a-1 increases Caspase and IL-1β p17 levels in response to inflammasome stimuli: H_2O_2 (Fig. 3). In addition, miR-133a-1-mediated suppression of UCP2 (Fig. 1D) observed in this report was in accordance with previous studies [29]. Studies with UCP2 overexpression and siRNA show a direct relationship between UCP2 and the activation of Caspase-1 and processing of IL-1β (Figs. 2 and 3). Inhibition of UCP2 has been previously reported to elevate ROS and not complete phagocytosis. UCP2 may regulate inflammasome activation by modulating cellular ROS and phagocytosis. To further check the role of inflammasome components in regulating UCP2, we checked UCP2 levels in inflammasome component knockout mice NLRP3$^{-/-}$ and ASC$^{-/-}$. Our results reveal that UCP2 is negatively regulated by NLRP3 and ASC (Fig. 4).

The inflammasome activation is often a two-step process, and the basal expression levels of individual inflammasome components are not necessarily modulated to regulate inflammasome activation. Our present data demonstrated that miR-133a-1 did not alter the basal expression of individual components of the NLRP3 complex (Fig. 1C), however, it regulated IL-1β processing and expression levels of its effector protein Caspase-1 (Fig. 1A). Hence, miR-133a-1 may regulate inflammasome activation by disturbing NLRP3 complex formation. Additional studies comparing the co-localization of ASC, NLRP3, and Caspase-1 will address this phenomenon. In conclusion, our results demonstrate the novel role of miR-133a-1 in enhancing inflammasome activation and secretion of Caspase-1 and IL-1β in human macrophages.

Acknowledgments

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