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ORIGINAL PAPER

Melatonin Alters the miRNA Transcriptome of Inflammasome Activation in Murine Microglial Cells

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Abstract

Systemic inflammation can have devastating effects on the central nervous system via its resident immune cells, the microglia. One of the primary mediators of this inflammation is inflammasomes, multiprotein complexes that trigger a release of inflammatory proteins when activated. Melatonin, a hormone with anti-inflammatory effects, is an attractive candidate for suppressing such inflammation. In this study, we have investigated how melatonin alters the microRNA (miRNA) transcriptome of microglial cells. For that purpose, we have performed RNA sequencing on a lipopolysaccharide and adenosine triphosphate (LPS + ATP) induced NLR family pyrin domain containing 3 (NLRP3) inflammasome activation model in the N9 mouse microglial cell line, with and without melatonin pre-treatment. We have identified 136 differentially expressed miRNAs in cells exposed to LPS + ATP compared to controls and 10 differentially expressed miRNAs in melatonin pre-treated cells compared to the inflammasome group. We have identified miR-155-3p as a miRNA that is upregulated with inflammasome activation and downregulated with melatonin treatment. We further confirmed this pattern of miR-155-3p expression in the brains of mice injected intraperitoneally with LPS. Moreover, an overexpression study with miRNA-155-3p mimic supported the idea that the protective effects of melatonin in NLRP3 inflammasome activation are partly associated with miRNA-155-3p inhibition.

Keywords Melatonin · Microglia · NLRP3 inflammasome · microRNA · LPS · ATP

Abbreviations

ATP	Adenosine triphosphate
ASC	Apoptosis-associated speck-like protein containing a caspase activation and recruitment domain
CNS	Central nervous system
Ctrl	Control
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GSDMD	Gasdermin D
IL	Interleukin
IP	Intraperitoneal
KEGG	Kyoto encyclopedia of genes and genomes
LA	LPS and ATP
LPS	Lipopolysaccharide
miRNA	MicroRNA
MLA	Melatonin and LPS and ATP
MT	Melatonin
NLRP3	NLR family pyrin domain containing protein 3
PBS	Phosphate-buffered saline
qPCR	Quantitative real-time PCR
TLR	Toll-like receptor

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Introduction

Microglia are the primary immune cells in the central nervous system (CNS), responsible for or auxiliary to the inflammatory processes that protect, regulate, and occasionally damage the neuronal milieu [1]. Inflammation in the brain involves a significant degree of inflammasome

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activation [2]. Inflammasomes are multiprotein complexes activated in response to certain pathogenic or otherwise harmful molecules and initiate various inflammatory and secretory pathways. NLR Family Pyrin Domain Containing Protein 3 (NLRP3; also known as NALP3) is a well-characterized inflammasome implicated in the pathogenesis of many CNS diseases and injuries [3–5].

Various molecular signals can activate NLRP3, including pathogen-associated molecular patterns, extracellular adenosine triphosphate (ATP), potassium efflux, particulate aggregates, lysosomal or mitochondrial destabilization, and reactive oxygen species [6]. Upon activation on its ligand-sensing leucine-rich repeat domain, NLRP3 self-oligomerizes via its NACHT domain and recruits an adaptor protein called Apoptosis-associated Speck-like Protein containing a caspase activation and recruitment domain (ASC) on its pyrin domain [7]. The ASC proteins clustered on the NLRP3 complex then recruit the inactive enzyme Pro-caspase-1 on their caspase recruitment domains, resulting in proximity-induced cleavage of this enzyme into active caspase-1 [8]. Caspase-1 activates (by cleaving) the inactive forms of inflammatory cytokines Interleukin (IL)-1β and IL-18 and the pore-forming protein Gasdermin-D (GSDMD). Cell contents are secreted outside through pores made by active GSDMD and cause paracrine inflammatory effects [7, 8].

Melatonin is a hormone produced mainly by the pineal gland and conveys light information from the retina to the rest of the body, mediating the circadian rhythm [9]. While it is generally recognized as a hormone, melatonin also functions as a potent antioxidant; and is thought to be produced by any animal cell with mitochondria [10, 11]. In addition to its antioxidant activities, melatonin also has anti-inflammatory, anti-cancer, immune-regulatory, and neuroprotective effects [12]. The anti-inflammatory properties of melatonin also involve inhibition of the NLRP3 inflammasome [13, 14], making melatonin-associated pathways intriguing drug targets for regulation of inflammasome-mediated inflammation.

microRNAs (miRNAs) are small noncoding RNAs involved in the regulation of gene expression and consequently in many inter- and intracellular processes [15]. Several miRNAs have been investigated in the context of inflammation and inflammasomes. Among them, miR-155 and miR-146a have been observed multiple times as being upregulated in response to Toll-like receptor (TLR) signaling, with miR-155 either exacerbating or reducing, and miR-146a reducing inflammation [16]. miR-223, a miRNA that targets NLRP3, is also a crucial player in measures against inflammation [17, 18]. As a highly multifaceted molecule, melatonin influences the miRNA expression of cells and mediates part of its anti-inflammatory and anti-cancer effects through miRNAs [19–22]. Learning how melatonin changes the miRNA repertoire of microglial cells would shed light on how melatonin drives its neuroprotective and anti-inflammatory effects in microglia.

We have previously shown in the N9 microglial cell line that Lipopolysaccharide (LPS) and ATP induce activation of NLRP3 and that melatonin inhibits this induction, as demonstrated by altered levels of NLRP3 and mature caspase-1, IL-1β, and IL-18 [14]. In the current study, we aim to identify how the miRNA transcriptome of microglia changes in response to inflammasome activation and how this change is further altered in melatonin treatment.

Materials and Methods

Cell Culture

The N9 mouse microglial cell line was provided by Dr. Paola Ricciardi-Castagnoli (Cellular Pharmacology Center, Milan, Italy) [23]. Cells were cultured in a growth medium composed of RPMI 1640 with 10% fetal bovine serum, 2 mM L-glutamine, 100 u/ml penicillin, and 100 µg/ml streptomycin (all purchased from Thermo Fisher Scientific) at 37 °C with 5% CO_2. An experiment medium composed of RPMI 1640 with 2 mM L-glutamine (without serum or antibiotics) was used in experimental setups.

In Vitro NLRP3 Inflammasome Activation

Canonical NLRP3 inflammasome activation model was implemented in the study. NLRP3 inflammasome activation was induced by LPS (priming) and ATP (activation), as performed previously [14, 24, 25]. N9 cells were plated on 10 cm cell culture dishes at 3×10^6 cells/dish one day before the experiment. Before the experiment, the growth medium was removed and replaced with the experiment medium. Cells were divided into the following groups: control, LPS+ATP, and melatonin+LPS+ATP (hereafter referred to as Ctrl, LA, and MLA).

The MLA group was pre-treated with 0.5 mM melatonin (Sigma-Aldrich M5250; from an ethanol-dissolved stock of 200 mM), the other groups were incubated with medium without serum or antibiotics for 1 h. Post-incubation, LPS (InvivoGen, LPS from *E. Coli* 0111:B4) was applied at a concentration of 1 µg/ml (from a water-dissolved stock of 5 mg/ml) to the LPS+ groups, and cells were further incubated for 4 h. Finally, ATP (Sigma-Aldrich, A6419) was applied to the ATP+ groups at a concentration of 5 mM (from a Phosphate-buffered Saline (PBS, purchased from

Sigma-Aldrich) dissolved stock of 100 mM), and cells were incubated for 1 h. In all groups, previously added molecules were kept at a constant concentration throughout the experiment.

RNA Isolation

At the end of treatments, cells were scraped and centrifuged at 500 × *g*. Cell pellets were washed with PBS, and total RNA was isolated using a miRNeasy Mini Kit (QIAGEN). Brains from the animal experiment were placed in QIAzol lysis solution (QIAGEN) and homogenized on ice. Total RNA from the brains was also isolated with miRNeasy Mini Kit, according to kit instructions.

RNA Sequencing

Small RNA-seq and the analysis and annotation of the acquired data were performed by Novogene (Novogene (HK) Company Limited, Hong Kong). After passing quality control performed via Agilent 2100 Bioanalyzer (with an average RNA integrity number of 9.99), 3 µg of RNA per sample was used to prepare a small RNA library, using NEBNext Multiplex Small RNA Library Prep Set for Illumina. RNA was sequenced using Illumina SE50, with 10 million reads per sample. The raw data were mapped to the mouse genome using Bowtie [26], and miRNA alignment and annotations were performed via miRDeep2 [27] using miRBase 20 as reference. Expression comparison of groups was performed between normalized read counts of transcripts per million (TPM), where normalized read count is calculated as "mapped read count/total reads* 1,000,000". Differential expression analysis between groups was performed using the DESeq R package (1.8.3). The p-values were adjusted with the Benjamini & Hochberg method [28]. Corrected p-value of 0.05 was set as the threshold for significantly different expression.

Pathway Enrichment Analysis

Kyoto Encyclopedia of Genes and Genomes (KEGG) is a collection of manually curated databases related to genomes, biological pathways, diseases, drugs, and chemical substances [29]. In RNA-seq, KEGG analysis can reveal the main pathways associated with the target gene candidates. We have performed a pathway enrichment analysis using DIANA miRPath v.3 (https://dianalab.e-ce.uth.gr/html/mirpathv3/index.php) [30], which identifies significantly enriched metabolic pathways and signal transduction pathways associated with differentially expressed miRNA target genes, retrieved from TarBase [31]. Genes with adjusted p values smaller than 0.05 were considered significantly enriched.

Quantitative RT-PCR

cDNA for Quantitative real-time PCR (qPCR) was prepared via miScript II RT Kit (QIAGEN), and qPCR was performed using miScript SYBR-Green I kit (QIAGEN) and LightCycler 480 Instrument II (Roche Life Science, USA) according to the manufacturer's recommendations. Relative expression levels were measured by the $2^{-\Delta\Delta ct}$ method [32]. For normalization, U6 small nuclear RNA (Rnu6) was used. As primers, miScript Primer Assays (QIAGEN) were used (No. MS00024255 for miR-155-3p and No. MS00033740 for Rnu6).

In Vivo NLRP3 Inflammasome Activation

Male BALB/c mice, aged 12–16 weeks, were provided by Izmir Biomedicine and Genome Center Vivarium. All animals were maintained and housed in the vivarium under controlled conditions (22 ± 2 °C, 55 ± 10% humidity, 12 h light/dark periods) with access to food and water ad libitum. Mice were randomly divided into three groups with 7 animals per group: Ctrl, LPS, Melatonin + LPS. 5 mg/kg doses of LPS (LPS from *E. Coli* 055:B5) were injected intraperitoneally (IP). Melatonin was injected IP at a 30 mg/kg dose 4 times with 6 h intervals starting 2 h before LPS injection. The LA and Ctrl groups also received equivalent IP PBS injections at corresponding times. 24 h after LPS injection, the mice were sacrificed by decapitation; their brains were collected, flash-frozen in liquid nitrogen, and stored for analysis.

All animal studies and animal care were approved by Dokuz Eylul University Izmir International Biomedicine and Genome Institute Animal Experiments Local Ethics Committee (IBG-AELEC) (Protocol Number: 03/2018).

Transfection

miR-155-3p mimic transfection was performed using products and guidelines by QIAGEN. As mimic, Syn-mmu-miR-155-3p miScript miRNA Mimicy (No. 219600, MSY0000165) was used and as negative control (scramble-miRNA) miScript Inhibitor Negative Control (No. 1027272) was used; both at a final concentration of 20 nM. HiPerFect Transfection Reagent (No. 1029975) was used as the reagent, at double the volume of transfected miRNA. The miRNA-reagent mixture was prepared in growth medium and added dropwise to the growth medium of cells. Cells were then incubated at 37 °C for 48 h. At the end of this procedure, the medium was replaced and further experiments were performed.

ELISA

Secreted IL-1β levels in supernatants of NLRP3 inflammasome induction experiments were determined by mouse IL-1β enzyme-linked immunosorbent assay (ELISA) kits

(No. 88-7013-88, Thermo Fisher Scientific), using F96 Nunc™ Immuno Plates (Thermo Fisher Scientific), according to the manufacturer's instructions.

Statistical Analyses

Statistical analyses of non-RNA-seq data were performed on GraphPad Prism (version 8). Data are presented as mean ± SEM. Normality of the data was assessed by Shapiro–Wilk test. For parametric data with homogeneous variance, comparisons between two groups were analyzed by Student's *t*-test; and comparisons between multiple groups were analyzed by one-way ANOVA with Bonferroni multiple comparison corrections. For parametric data with heterogeneous variance, comparisons between multiple groups were analyzed with Brown-Forsythe ANOVA, with Games-Howell multiple comparison corrections. For non-parametric data, comparisons between two groups were analyzed with the Mann–Whitney *U*-test; and comparisons between more than two groups were analyzed with the Kruskal–Wallis test with Dunn's multiple comparisons. Statistical significance was set at $p < 0.05$.

Results

For miRNA transcriptome data of inflammatory N9 cells treated with melatonin, we performed the LPS+ATP induced NLRP3 inflammasome activation model from our previous study [14]. In brief, N9 cells were pre-treated with 0.5 mM melatonin for 1 h, then with 1 µg/ml LPS for 4 h, and finally with 5 mM ATP for 1 h (while keeping the concentrations of agents added at previous steps constant).

RNA sequencing performed on these cells identified various up- and down-regulated miRNAs in the inflammasome and the melatonin treated groups (Tables 1, 2). In the inflammasome activation group LA, we identified 58 up- and 78 down-regulated miRNAs compared to Ctrl. In contrast, in the melatonin pre-treated group MLA, we identified 43 up- and 55 down-regulated miRNAs compared to Ctrl, and 7 up- and 3 down-regulated miRNAs compared to LA (Fig. 1a). Among the significantly altered miRNAs in the inflammasome group, only one was seen to be significantly altered in the opposite direction in the melatonin group: miR-155-3p, which was upregulated with LPS+ATP treatment and downregulated with the addition of melatonin. The number of uniquely expressed miRNA in the Ctrl, LA, and MLA groups were 41, 5, and 14, respectively (Fig. 1b). A cluster analysis of the miRNA reads of the three groups shows that about 45% of miRNAs that are altered with LPS+ATP (bottom of the chart) are partially reverted to control levels with melatonin treatment (Fig. 1c).

To obtain a broad picture of pathways associated with altered miRNA in the LA and MLA groups (versus Ctrl and LA, respectively), we have performed a KEGG analysis on the RNA-seq results. The KEGG pathway analysis of miRNA reveals multiple common pathways that differentially expressed miRNAs in both LA vs. Ctrl and MLA vs. LA appear in; including inflammation-associated pathways like MAPK and FoxO signaling pathways (Fig. 2).

Table 1 The top ten most up-and down-regulated miRNAs in the LA (inflammasome) group versus the control group, sorted by fold change (n = 4)

miRNA	Fold change	Adjusted p-value
Upregulated in the inflammasome group vs. the control group		
mu-miR-378d	3.72	0.002568
mmu-miR-145a-5p	3.43	0.010218
mmu-miR-155-3p	3.01	7.75E-29
mmu-miR-215-5p	2.89	0.041301
mmu-miR-202-5p	2.88	0.030084
mmu-miR-7012-3p	2.79	0.04497
mmu-miR-215-3p	2.79	0.049725
mmu-miR-3058-3p	2.74	0.04714
mmu-miR-155-5p	2.56	1.96E-13
mmu-miR-96-5p	2.27	0.009007
Downregulated in the inflammasome group vs. the control group		
mmu-miR-27a-5p	0.05	7.32E-272
mmu-miR-6481	0.06	4.38E-29
mmu-miR-26a-2-3p	0.09	6.89E-12
mmu-miR-181b-1-3p	0.11	2.15E-29
mmu-miR-148a-5p	0.12	1.00E-144
mmu-miR-467b-3p	0.13	1.04E-07
mmu-miR-92a-1-5p	0.19	1.07E-43
mmu-miR-30b-3p	0.20	5.28E-46
mmu-miR-16-1-3p	0.21	1.46E-132
mmu-miR-703	0.21	0.000965

Table 2 Significantly up- and down-regulated miRNAs in the MLA (melatonin treatment group) versus the LA (inflammasome) group, sorted by fold change (n = 4; significance cut-off value for p-values is p = 0.05)

miRNA	Fold change	Adjusted p-value
Upregulated in the melatonin group vs. the inflammasome group		
mmu-miR-486a-5p	2.99	0.00013129
mmu-miR-486a-3p	2.98	0.00013129
mmu-miR-486b-3p	2.98	0.00013129
mmu-miR-1a-3p	2.89	0.041575
mmu-miR-1b-5p	2.87	0.041575
mmu-miR-451a	1.95	0.00057708
mmu-miR-363-3p	1.93	0.0051
Downregulated in the melatonin group vs. the inflammasome group		
mmu-miR-155-3p	0.64	4.91E-06
mmu-miR-27b-5p	0.67	0.0051
mmu-miR-21a-3p	0.76	0.04186

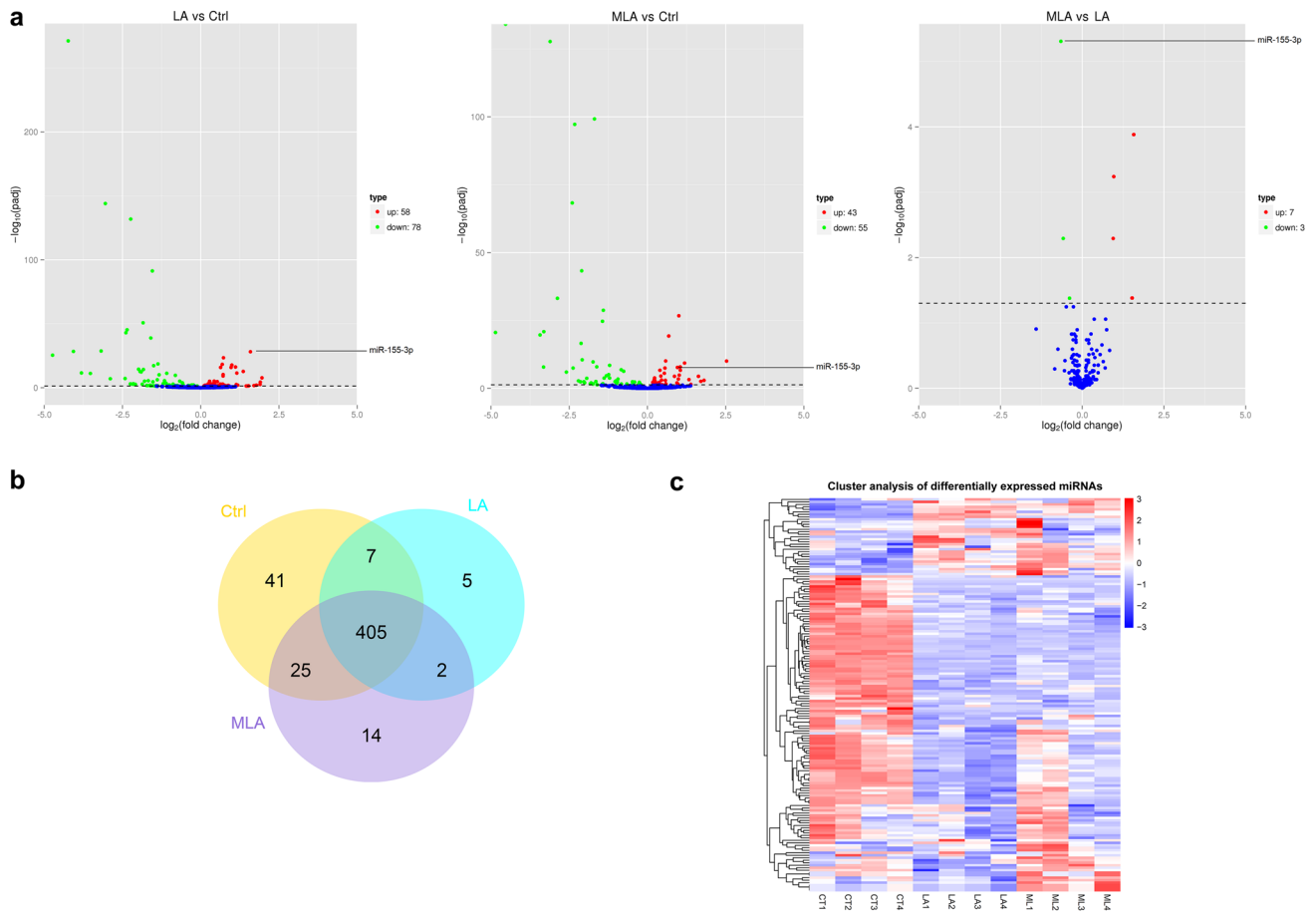

Fig. 1 Differential Expression and clustering analyses from the RNA-seq data of melatonin treatment in NLRP3 inflammasome activation model in N9 microglial cells. **a** Volcano plots of up- (red) and down- (green) regulated miRNAs between the pairs LA vs. Ctrl, MLA vs. Ctrl, and MLA vs. LA. miR-155-3p is indicated in the first and third plots. **b** Venn diagram showing numbers of uniquely expressed miRNAs in each group and co-expressed miRNAs between groups. **c** Cluster analysis of the experimental groups, where each column is a replicate, and each row is a miRNA. miRNAs are hierarchically clustered so that miRNAs that follow similar expression trends are grouped. The spectrum from red to blue represents $\log_{10}$ (TPM+1) values where red indicates upregulation and blue indicates downregulation. (LA: LPS+ATP, Ctrl: Control, MLA: Melatonin+LPS+ATP; cutoff p-value: 0.05)

As miR-155-3p was the only miRNA altered in reverse directions in the LA and MLA groups, we sought to confirm its expression pattern using qPCR. In N9 cells, we observed a rise in miR-155-3p expression in the inflammasome group and the reversal of this rise in the melatonin pre-treated group (Fig. 3a). We also replicated the finding in a mouse model of NLRP3 inflammasome activation: mice that were intraperitoneally injected with LPS had higher levels of miR-155-3p in their brains while this rise was reversed in LPS injected mice that were also injected with four doses of melatonin (Fig. 3b).

To understand the function miR-155-3p in the context of inflammasome activation, we overexpressed miR-155-3p in N9 cells using a miRNA mimic molecule (Fig. 4a). The in vitro NLRP3 activation model was applied post-transfection. At the end of the experiment, supernatants were collected and analyzed by an IL-1β ELISA kit. Overexpression of miR-155-3p reduced the protective effect of melatonin against IL-1β release in a statistically significant manner (from a 55% decrease in IL-1β secretion to 45%; $p = 0.031$), (Fig. 4b).

Discussion

We have shown previously that our LPS and ATP induced inflammasome activation model in N9 cells and LPS-induced in vivo inflammasome model in mice activated the NLRP3 inflammasome at both mRNA and protein levels [14]. In this study, we utilized RNA-seq analysis on the NLRP3 inflammasome activation model in N9 murine microglia to evaluate alterations of miRNA expression profiles. We identified numerous upregulated and downregulated miRNAs in the inflammasome activated group vs. the control group and the

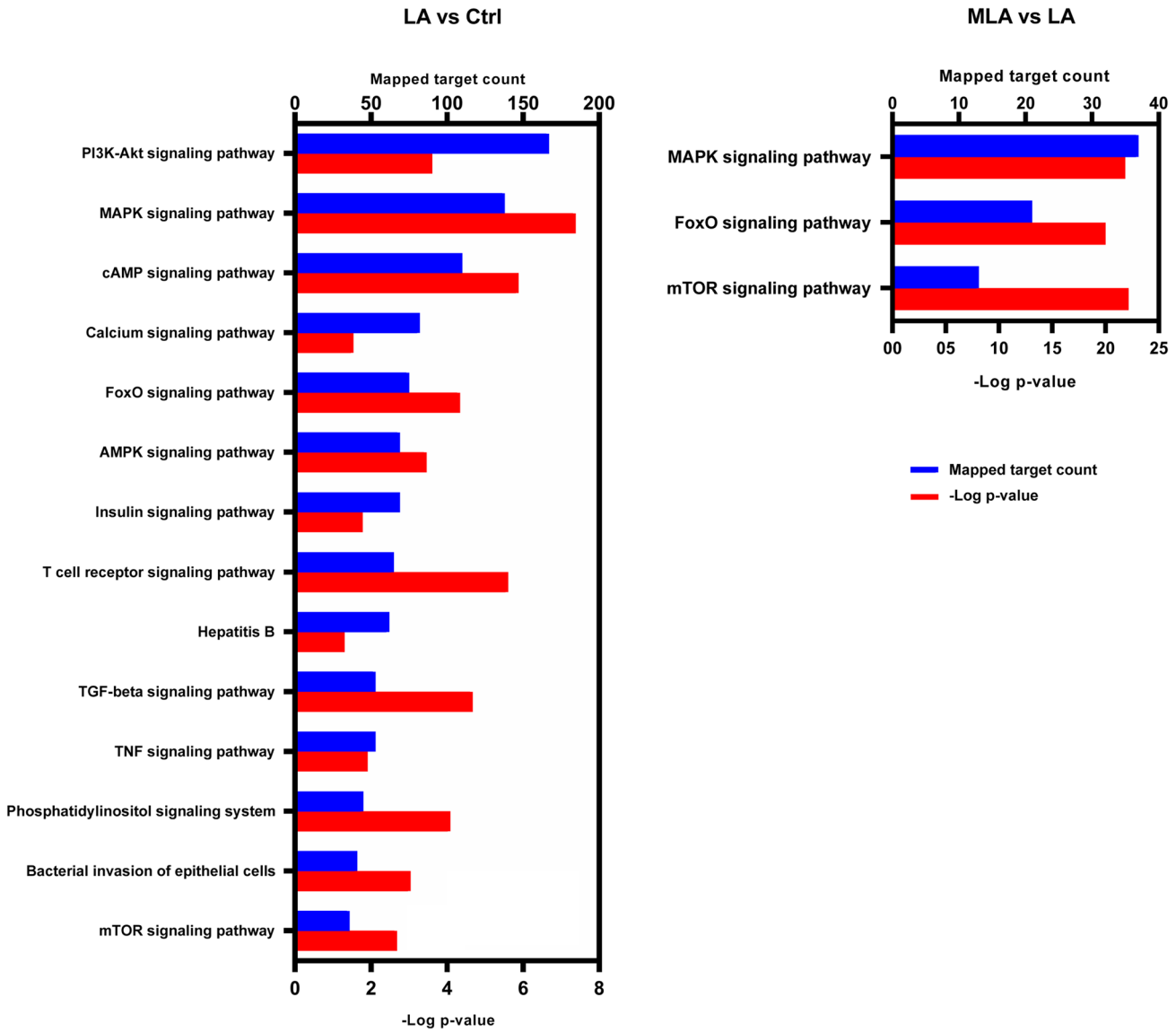

Fig. 2 miRNA target pathway analysis differentially expressed miRNAs in melatonin treatment in NLRP3 inflammasome activation model in N9 microglial cells. An inflammation-related subset of the list of pathways associated with the differentially expressed miRNA in comparisons between LA vs. Ctrl and MLA vs. LA, obtained from KEGG PATHWAY database (14 of 73 pathways in LA vs. Ctrl and 3 of 27 pathways in MLA vs. LA were included). Blue lines indicate the number of associated genes belonging to the corresponding pathway with the differentially expressed miRNAs between the compared groups. Red lines indicate the − log p-value, i.e., the degree of significance. (LA: LPS+ATP, Ctrl: Control, MLA: Melatonin+LPS+ATP; cutoff p-value: 0.05)

melatonin group vs. the inflammasome group (Tables 1, 2). Among them, we identified miR-155-3p as a potent miRNA that was upregulated with inflammasome activation and downregulated with melatonin treatment. Further, we confirmed this NLRP3 inflammasome and melatonin induced deregulation of miR-155-3p in N9 microglial cells and mouse brains via qPCR. Finally, we showed that miR-155-3p overexpression modestly but significantly reversed the protective effect of melatonin treatment against inflammasome activation.

Melatonin, as a multi-functional molecule, is naturally linked with the regulation of numerous miRNAs. Of those, miR-155-3p and miR-155-5p are two different miRNAs, processed from the same primary transcript (pri-mir-155) and generated from the same pre-miRNA molecule. miR-155-5p is more abundant than miR-155-3p in mouse and human tissues, likely due to having a higher affinity to being loaded into Argonaute [33, 34]. This is also the case in our RNA-seq data, where TPM values for miR-155-5p is 30–40 times higher than the values for miR-155-3p. Additionally, both miRs follow an up- and down-regulation pattern with LPS+ATP and melatonin, respectively; but only the changes in miR-155-3p is significant for

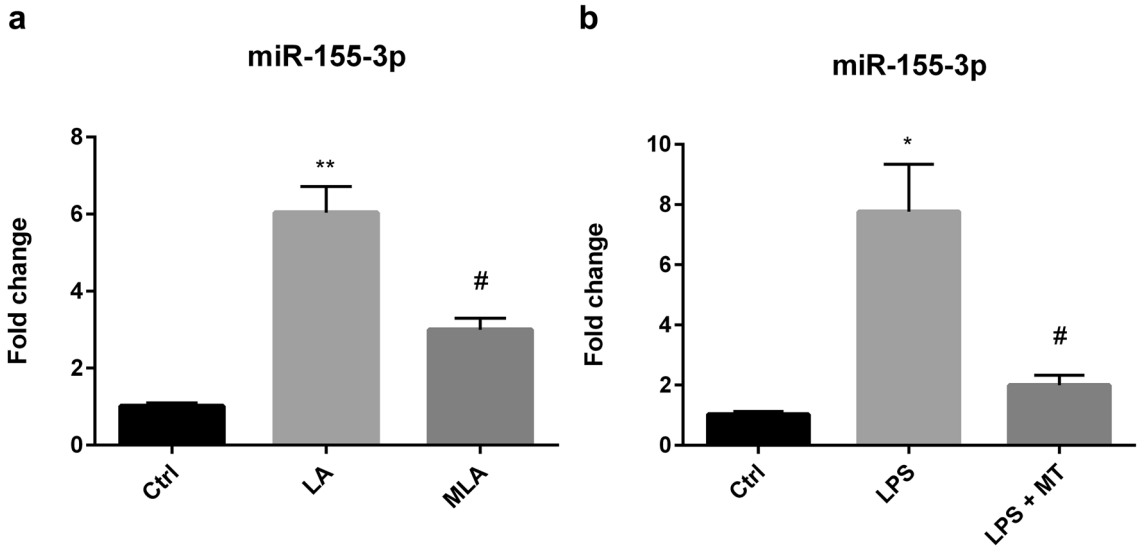

Fig. 3 Confirmation of the protective role of melatonin against LPS-induced miR-155-3p upregulation in N9 cells and mice. **a** Fold change in miR-155-3p expression in N9 cells (n = 7). **b** Fold change in miR-155-3p in brain homogenates of LPS-challenged mice with and without melatonin treatment (n = 7; MT: melatonin). (Data are presented as mean ± S.E.M. *p < 0.05 and **p < 0.01 compared to control; #p < 0.05 compared to the inflammasome group)

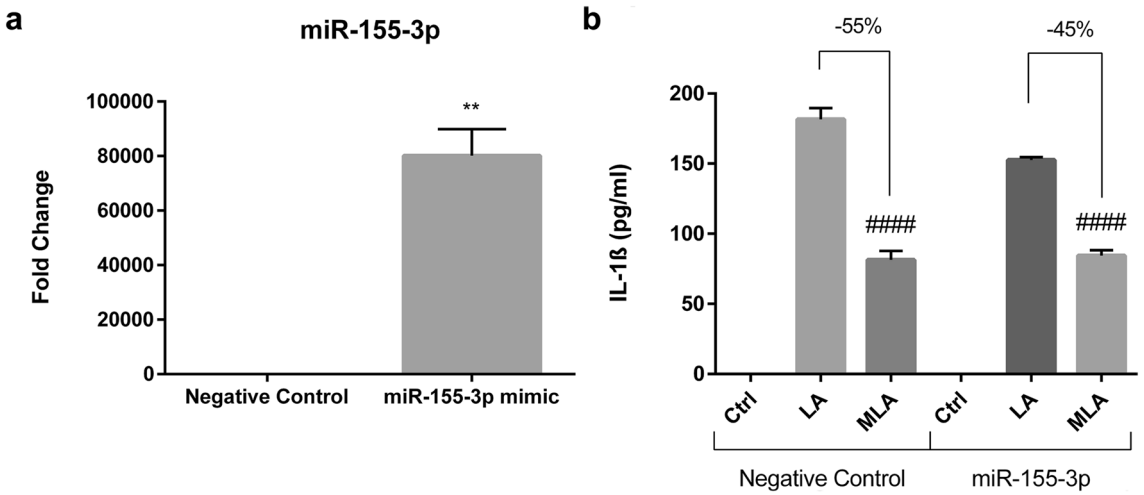

Fig. 4 Protective role of melatonin against inflammasome activation is decreased in N9 cells transfected with a miR-155-3p mimic molecule. **a** Efficiency of miR-155-3p mimic transfection (20 nM, 48 h), as shown by fold change of miR-155-3p in cells transfected with negative control miRNA and miR-155-3p mimic (n = 3). **b** Secreted IL-1β levels in supernatants of N9 cells, as measured by ELISA (n = 7). (Data are presented as mean ± S.E.M. **p < 0.01 compared to negative control; ####p < 0.0001 compared to the inflammasome groups within the respective transfections; control groups in **b** are not included in significance testing since their values are zero)

both interventions. Despite being produced from the same molecule, miR-155-3p is distinct from its partner strand (and not simply a byproduct). Changes in miR-155-3p expression profile can be substantially different from those of miR-155-5p [34]. For example, upon exposure of macrophages to LPS, TNF-α, and IL-1β, miR-155-3p expression peaks about 2–3 h earlier than miR-155-5p. On the other hand, the inflammatory agents poly (I:C) and Pam3Cys induce weaker miR-155 upregulation where both strands increase in a similar fashion[35]. Thus, it can be said that miR-155-3p can act independently from its partner strand. Notwithstanding this distinction, miR-155-5p has been extensively studied as a regulator in inflammatory pathways [36], whereas miR-155-3p remains relatively understudied.

Our data confirm the same LPS-induced expression pattern for miR-155-3p both in vitro and in vivo, pointing to

miR-155-3p as a miRNA potentially responsible for inflammation. Previous literature also lists multiple cases where miR-155-3p's expression is in parallel with induction of inflammation [35, 37–42]. Examples include elevated miR-155-3p in brain tissue in mouse models of multiple sclerosis [37, 38] and higher miR-155-3p expression in M1 vs. M2 polarized macrophages [40]. miR-155-3p is also reported to promote TNF-α expression in human fetal astrocytes and plasmacytoid dendritic cells (PDCs) [39, 41]. Nevertheless, other studies on miR-155-3p have revealed anti-inflammatory roles. In both monocytes and macrophages, it reduces TNF-α expression and upregulates IL-6 [43]. It targets Myd88, a critical player in inflammation via TLR signaling, in breast cancer cells [44]. It also targets lymphotoxin-beta, which contributes to NF-κB mediated inflammation, in mantle cell lymphoma [45]. Moreover, miR-155-3p appears to promote M2-like (anti-inflammatory) polarization in macrophages [46]. It is possible that miR-155-3p acts alongside miR-155-5p as a balancing molecule to the inflammatory effects of the 5p strand. An example of this is seen in, PDCs where the two miRNAs work antagonistically, with miR-155-3p promoting and miR-155-5p suppressing type I interferon production [41]. Ultimately, these contradictory findings in the literature suggest the role of miR-155- in inflammation is dependent on cell type and disease model. To date, no study has shown an effect of melatonin on miR-155-3p in any model. Here, we show for the first time that melatonin downregulates an inflammation-induced increase in miR-155-3p. To examine how miR-155-3p might be affecting the NLRP3 inflammasome model itself, we have performed an overexpression experiment where we measured IL-1β levels in N9 cells transfected with miR-155-3p. The data from this indicate that miR-155-3p reduces the protective effect of melatonin.

The downregulated and upregulated miRNAs in our data were shown in previous studies to be involved in inflammatory pathways in many ways. In a study, small RNA-seq profiling showed that miR-486a-5p may stimulate RAW264.7 murine macrophage cells towards an M2 phenotype [47]. Zhang et al. demonstrated that Kcnq1ot1, a long noncoding RNA, activated pyroptosis in MPC5 mouse podocyte cells by blocking miR-486a-3p, which restrains NLRP3 expression [48]. Moreover, ADAR1 (Adenosine Deaminase RNA Specific)/miR-1a-3p signaling was shown to regulate A20 (TNFα-induced protein 3), which decreases cardiomyocyte inflammation and apoptosis in a viral mouse myocarditis model [49]. A study on miR-451a pointed out that elevated miR-451a levels were detected in the serum of sepsis and septic shock patients, and it was proposed as a sepsis biomarker.[50]. miR-363-3p prevents apoptosis, oxidative stress injury, and inflammatory reactions in a mouse atherosclerosis model and coronary artery endothelial cells [51]. This miRNA is also reported to increase in the plasma exosomes of rats treated with melatonin [52]. In a mouse EAE model, Δ9-Tetrahydrocannabinol and Cannabidiol introduction improved EAE score and repressed neuroinflammation via downregulating miR-27b-5p and other six miRNAs [53]. Lastly, miR-21a-3p was shown to increase the severity of sepsis in a rat CLP model [54].

Melatonin treatment has previously been shown to alter other miRNAs. Its use in non-alcoholic fatty liver disease results in reduced expression of miR-34a, a pro-inflammatory miRNA upregulated in various diseases [22, 55]. Other miRNAs reported to be downregulated by melatonin include miR-23a, miR-155-5p, miR-212 [22]. These three miRNAs, along with miR-34a, suppress the activity of sirtuin-1 (SIRT1), a deacetylase that appears to be a mediator of the anti-inflammatory and antioxidant activities of melatonin [56]. Melatonin also increases miR-223 [57], which targets and downregulates NLRP3 [18, 58].

To attain further insight into the pathways associated with the differentially expressed miRNAs in our study, we performed a KEGG pathway enrichment analysis. The miRNA expression in the LA and MLA groups had significant alterations in the MAPK and FoxO pathways, which are associated with regulation of inflammation [59, 60] and are thus predictable pathways in the context of inflammasome activation or melatonin treatment.

In summary, the role of miR-155-3p in inflammation is ambiguous. In this study, we reproduce its upregulation in response to LPS and we show for the first time its downregulation in response to melatonin. We further show that it reduces, modestly, the protective effect of melatonin against inflammasome activation. Our sequencing has also revealed other, lesser-known miRNAs altered with inflammation or melatonin treatment. Pursuing their targets and functions in the context of inflammation is likely to elucidate the field further.

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Author Contributions KUT and SG designed the study. ET, BT, and BIA designed and performed the experiments. ET, BT, BIA, KUT, and SG analyzed and interpreted the data. ET, BT, BIA, KUT, and SG wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Data Availability The RNA-seq datasets analyzed within this study are available upon request to the corresponding author.

Declarations

Conflict of interest The authors declare no financial or non-financial interest regarding this article.

Ethical Approval In vivo studies were performed under the approval of, and following the regulations set by Izmir International Biomedicine and Genome Institute Local Ethics Committee for Animal Experiments (IBG-AELEC, protocol number: 03/2018).

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