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MICRORNA-200 FAMILY EXPRESSION LEVEL CHANGES IN STIMULATED THP-1 CELLS FOLLOWING NLRP3 INFLAMMASOME ACTIVATION

Pilot Study

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Abstract

Innate immunity is the immune systems rapid responses to infection after being attacked by a pathogen. Inflammatory responses are activated by the detection of pathogen-associated molecular patterns and danger-associated molecular patterns through pattern recognition receptors on inflammatory cells. NLRs are activated by intracellular PAMPs which warn cells of damage and have a major role in initiating the innate inflammatory responses as well as the development of infectious and inflammatory diseases. NLRP3 is a very large multiprotein complex and is the most studied inflammasome. The NLRP3 Inflammasome follows a two-signal model for activation, signal one forms the NLRP3 complex and signal two activates the inflammasome. NLRP3 initiates an inflammatory form of cell death called pyroptosis and triggers the release of pro-inflammatory cytokines IL-1β and IL-18. The miR-200 family has five members, miR-200a, miR-200b and miR-429 located on chromosome 1 and miR-200c and miR-141 located on chromosome 12. In this study, THP-1 cells were differentiated with PMA then stimulated with LPS and ATP. Various time samples were collected and isolated to obtain miRNA. Two-step RT-qPCR was then performed to quantitively monitor the changes in miRNA-200 family expression levels. The purpose of this study was to observe how miRNA-200 family expression levels change in stimulated THP-1 cells as the NLRP3 inflammasome is activated. This became a pilot study as all biological replicates could not be analyzed, miR-200 family is showing a potential response to the activation of the NLRP3 inflammasome and they should be investigated further.

List of abbreviations

3'UTR	3' untranslated region
ASC	Apoptosis-associated Speck-like protein containing a CARD
ATP	Adenosine triphosphate
CARD	Caspase recruitment domain
DAMPs	Danger-associated molecular
EMT	Epithelial-to-mesenchymal transition
GSDMD	Gasdermin-D
IL-18	Interleukin 18
IL-1β	Interleukin 1- βeta
LPS	Lipopolysaccharides
LRR	Leucine-rich repeat
miRNA	microRNA
NF-κB	Nuclear factor-kappa B
NLRP	Nucleotide-binding oligomerization domain, Leucine rich Repeat and Pyrin domain
NLRs	NOD-like receptor
NOD	Nucleotide-binding oligomerization domain
NTC	Non-template control
PAMPs	Pathogen-associated molecular patterns
PMA	Phorbol Myristate Acetate
PMA	Phosphate buffered saline
PRRs	Patterns pattern recognition receptors
PYD	*Pyrin* domains
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
RT	Reverse transcription
TLRs	Toll-like receptors

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Introduction

Innate immunity is one of the immune systems responses to infection within minutes or hours of being attacked by a pathogen, triggered by extracellular or intracellular receptors. Fluid, cells, and molecules go to the site of infection causing swelling and inflammation. This immune response is successful in ridding of pathogens and damaged or dead cells. Antimicrobial molecules such as cytokines engage and activate other cells, enzymes, and proinflammatory mediators (Owen, Punt, Stranford, Jones & Kuby, 2013).

Inflammatory responses are activated by the detection of pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) through pattern recognition receptors (PRRs) on inflammatory cells which can be either extracellular or intracellular PRRs (Yi, 2019). Toll-like receptors (TLRs) are the first PRRs to have been discovered and are widely known for how they bind PAMPs and activate cells. A family of PRRs include NLRs which stands for nucleotide-binding oligomerization domain (NOD)-like receptor. NLRs are activated by intracellular PAMPs which warn cells of damage and have a major role in initiating the innate inflammatory responses but also the development of infectious and inflammatory diseases (Owen et al., 2013). Inflammasomes are multi-protein complexes categorized into canonical inflammasomes that include the NLR inflammasomes and non-canonical inflammasomes which include mouse caspase-11 and human caspase-4 and caspase-5 inflammasomes (Yi, 2019).

NLRs assemble into a complex with other proteins known as an inflammasome which activate proteases necessary for converting procytokines which are the precursor forms of interleukin 1-βeta (IL-1β) and interleukin 18 (IL-18) into their mature forms and are then secreted from active cells (Owen et al., 2013). NLRPs are a specific group of NLR proteins that have *pyrin* domains (PYD) in the N-terminal, a NACHT domain and a C-terminal leucine-rich repeat (LRR) domain (Kelley, Jeltema, Duan, & He, 2019; Owen et al., 2013). NLRP3 is the most studied inflammasome and is a very large protein complex consisting of many copies of NLRP3 bound to the pyrin domain of apoptosis-associated speck-like protein containing a CARD (ASC) and the caspase recruitment domain (CARD) is bound to the CARD of pro-caspase-1 (Tezcan et al., 2019).

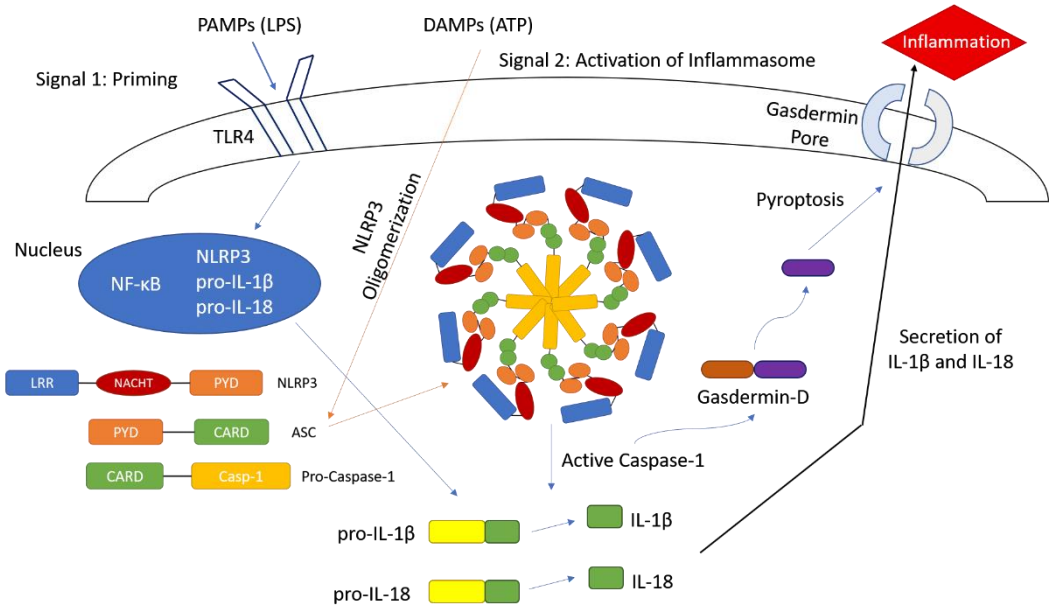

Figure 1. NLRP3 Inflammasome activation. The NLRP3 Inflammasome follows a two-signal model for activation, signal one forms the NLRP3 complex and signal two activates the inflammasome. Pro-inflammatory cytokines IL-1β and IL-18 are secreted. Cleavage of Gasdermin-D by active Caspase-1 results in the inflammatory cell death pyroptosis.

In canonical activation, NLRP3 follows the two-signal model for activation, the priming signal forms the NLRP3 complex and is initiated by the interaction of extracellular PRRs such as TLR4 binds with PAMPs and activates inflammatory signaling pathways like nuclear factor-kappa B (NF-κB) which induces the expression of NLRP3, pro-caspase-1 and pro-IL-1β. The second signal is essential for the activation of NLRP3, NLRs sense DAMPS such as extracellular adenosine triphosphate (ATP) which activates the inflammasome (Kelley et al., 2019; Tezcan et al., 2019; Yi, 2019). During non-canonical activation, detection of lipopolysaccharides (LPS) in the cytosol activates caspase-11 in mice and 4/5 in humans (Yi, 2019).

Active caspase-1 in the inflammasome cleaves the pro-IL-1β and pro-IL-18 into mature IL-1β and IL-18 and the inflammasome secretes them (Kelley et al., 2019; Owen et al., 2013; Yi, 2019). IL-1β is an important proinflammatory cytokine affecting many cells and is responsible for expression of genes causing fever, vasodilation, hypotension, increased acute phase proteins and highly associated with autoinflammatory diseases while IL-18 induces IFN-γ production and mediates adaptive immunity. Caspase-1 also cleaves the gasdermin-D (GSDMD) allowing it to create pores in the plasma membrane triggering pyroptosis where the cell lysis causing cell death and release of its cytoplasmic contents (Kelley et al., 2019). This can all be seen in Figure 1, from the formation of the NLRP3 inflammasome to the activation of the inflammasome and the release of pro-inflammatory cytokines IL-1β and IL-18.

microRNAs (miRNA) are small noncoding ribonucleic acid (RNA) molecules of about 19-24 nucleotides that form complexes with proteins and allows that complex to bind to complementary sequences in mRNA molecules of at least 7 nucleotides. That miRNA-protein complex then degrades the targeted mRNA if it is complementary along the whole sequence or blocks its translation if it is only partially complementary (Owen et al., 2013). miRNA such as miR-223, miR-22 and miR-7 suppress NLRP3 protein translation and inflammasome formation by binding to the 3' untranslated region (3'UTR) of the mRNA from NLRP3 (Tezcan et al., 2019). Due to that, the inflammasome protein complex cannot form during the activation stage therefore inhibiting the inflammasome.

The miR-200 family has five members that share two seed sequences and have a single nucleotide in the seed region that is different among them and different sequences at their 3' end (Bjerke & Yi, 2019). There are two clusters, miR-200a, miR-200b and miR-429 located on chromosome 1 and miR-200c and miR-141 located on chromosome 12 (Magenta et al., 2017; Hoefert et al., 2018). Then there are two functional groups that share the same seed sequence, group I consists of 200b, 200c and 429 that have the seed sequence AA**U**ACUG and group II consists of 200a and 141 which have the seed sequence AA**C**ACUG (Humphries & Yang, 2015). miRNAs have been proven to play important roles in human diseases including cancers and the miR-200 family have been suggested to have the potential to be diagnostic markers or have therapeutic impacts (Bojmar et al., 2013; Dhayat et al., 2015). Bioinformatic studies have predicted that miRNAs could target NLRPs and miR-200 might target NLRP3 and NLRP4 (Glinsky, 2008).

The miR-200 family inhibit epithelial-to-mesenchymal transition (EMT) which is involved in tumor invasion and metastasis, the family has a double-negative feedback loop with the transcriptional factors zinc-finger enhancer binding (ZEB)1 and ZEB2. The miR-200 family repress ZEBs and ZEBs repress the transcription of the two genes that encode the five family members. ZEB1 is important in the activation of EMT in several cancers. ZEBs repress members of miR-200 by binding to recognition sequences in their promoters, inciting tumor formation. Decreased expression of miR-200c and miR-141 increases ZEB1 which induces EMT during tumor transformation by repressing epithelial gene expression such as E-cadherin. On the other hand, the miR-200 family bind to target sites on the 3' UTRs of ZEB1 and ZEB2, inhibiting their expression. Decreasing ZEB1 expression causes E-cadherin to be upregulated, inhibiting EMT. The

transcription factor p53 is a tumor suppressor that has a role in regulating EMT through transactivating miR-200c (Chang et al., 2011).

Alterations to the promoter regions of the miR-200 clusters can result in loss of expression of the miR-200 family in cancer (Humphries & Yang, 2015). While ZEB1 and ZEB2 can inhibit the transcription of miR-200 family members when bound to the promoter region, p53 can activate transcription of the clusters (Humphries & Chang, 2015). Mutations and deletions of p53 are observed in cancer and it plays a role in tumor progression and migration similar to EMT's involvement in those processes (Kim et al., 2011). EMT was found to be inhibited by p53, resulting in reduced tumor invasion and metastasis, p53 also represses ZEB1 and ZEB2 expression levels, when simultaneously repressed, mesenchymal characteristics were decreased significantly. The miRNA-200 family and miR-192 are regulated by p53 and prevent EMT through repression of ZEB1 and ZEB2 (Kim et al., 2011).

The miR-200 family is well-known in cancer studies and its expression has been observed as deregulated in several of those studies. Members of the miRNA-200 family are considered promising biomarkers and may serve as novel targets for therapy of cancer and inflammatory diseases. The miR-200 family has been found to be up-regulated in some cancers but down-regulated in others. Members of the miR-200 family have shown up-regulation in several cancers including, ovarian, cervical, bowel, melanocyte lineage, bile duct, prostate, and cancer models (Elson-Schwab, Lorentzen, & Marshall, 2010). miR-200a and miR-200c have been found to be up-regulated in human melanoma lines and miR-200c was found to be up-regulated in lung cancer (Elson-Schwab et al., 2010; Yin et al, 2018). Following RT-qPCR, all members of the miR-200 family were shown to have significantly lower expression and be downregulated in gastric cancer (Chang et al., 2015). Deregulation of the expression of the miR-200 family has been observed in several cancer studies. The miR-200 family's role in other diseases has also been researched, including metabolic diseases such as diabetes, cardiovascular disease, inflammatory diseases, endometriosis, and others.

The THP-1 cell line used in this study was derived from the blood of a patient with acute monocytic leukemia and resembles primary monocytes and macrophages. The cell line has been used extensively in the study of immune responses, they are easy and safe to use (Chanput, Peters, & Wichers, 2015). Chanput, Mes, & Wichers (2014) found PMA to be the most effective differentiation agent to differentiate THP-1 cells from monocytes to macrophage-like cells. During culturing, they have a high growing rate, much higher than PBMC-derived monocytes, and can be grown longer, up to passage 25 with no changes to cell sensitivity and activity (Chanput, et al., 2014).

The role of microRNA's in cancer has been widely studied, now their role in innate and adaptive immune responses should be more widely researched. There have been many studies into miRNAs, but few have combined that research with the NLRP3 inflammasome or specifically setting up a time series to observe the effect of increasing durations of stimulation and changes in the microRNA's expression levels once the inflammasome has been activated. This research wants to answer the question of how the expression levels of the microRNA-200 family are affected once the NLRP3 inflammasome is activated and if the miR-200 family can be a regulator of the inflammasome. The aim of this study was to observe the changes in miRNA-200 family expression levels of stimulated THP-1 cells before and after the NLRP3 inflammasome is activated. Through the main objectives of isolating total RNA from the stimulated THP-1 cell cultures and measuring the expression levels of the microRNA-200 family with two-step reverse transcription quantitative polymerase chain reaction then evaluating the data. This will lead to learning if the miR-200 family members can be regulators of the NLRP3 inflammasome, potentially preventing

inflammasome activation and inflammation. Therefore, the microRNA-200 family may possibly serve novel therapeutic roles in controlling the causes and formation of different diseases.

Materials and methods

Ethics

There are no ethical considerations as there is no use of patients, there are no risks involved in the research or permissions needed to begin working. The research follows the Swedish Research Councils ethic and good research guidelines as well as the All European Academics (ALLEA) The European Code of Conduct for Research Integrity. The University of Skövde has permission to work with THP-1 cells which require a Biosafety Level 1 and a Biosafety Level 2 laboratory was used, where work was completed in fume and sterile hoods.

Cell Culture

THP1-ASC-GFP cells (Invivogen) at a passage level of five were cultured at 37° Celsius (C) and 5% CO_2 in RPMI 1640 with 2mM L-glutamine, 10% heat inactivated Fetal bovine serum (FBS) (Biowest), 100 mM sodium pyruvate, glucose solution (45%), 10 mM HEPES, and 100 U/mL pen-strep (Sigma). Cells were passaged every other day; the density was kept between 5 x 10^5 and 1.5 x 10^6 cells/mL and 200 µg/mL of Zeocin (Invivogen) was added every other passage. When culturing, the cells were spun down at 1453 x g in the Hettich Universal 32 centrifuge. Three replicates were grown to a total of 50 million cells with a final passage level of seven, then differentiated from monocytes to macrophage-like cells with 0.5 ug/mL Phorbol Myristate Acetate (PMA) (Invivogen) for four hours. Medium was removed by pipette and discarded then washed and replaced with fresh medium without PMA and incubated overnight for 18 hours. Cells were stimulated with 100 ng/mL LPS (Invivogen) diluted in Ultrapure water for 3.5 hours followed by 5 mM of adenosine triphosphate (ATP) (Sigma) for 30 minutes. Each replicate had ten samples taken and pelleted as seen in Table 1, including a pre-PMA sample, pre LPS sample, then the rest taken, 20, 40, 60, 90, 120, 180 minutes, 20, and 24 hours post ATP. The Heraeus Biofuge Fresco centrifuge was used at 380 x g for 5 minutes at 4° C to pellet the cells.

Table 1. Time points for each sample taken of THP-1 cell culture

Sample	Time point	Time Point
1.1	-3h	Post PMA, pre LPS; control
1.2	-30m	Post LPS, pre-ATP; control
1.3	20m	20 minutes post ATP
1.4	40m	40 minutes post ATP
1.5	60m	60 minutes post ATP
1.6	90m	90 minutes post ATP
1.7	120m	120 minutes post ATP
1.8	180m	180 minutes post ATP
1.9	20h	20 hours post ATP
1.10	24h	24 hours post ATP

Isolation of miRNA

The 30 samples that were pelleted and frozen were then isolated following the mirVana™ miRNA Isolation Kit, with phenol (Invitrogen) using 600 uL of lysis/binding buffer for larger numbers of cells, removing 300 uL from the aqueous phase as was found to be enough during testing and ending with a final volume of 100 uL of isolated RNA. After resuspending the pellets from replicate three in phosphate buffered saline (PBS) and centrifuging at 1000 x g there were no visible pellets therefore an additional seven minutes centrifugation at this speed was run then another ten more

minutes at 1500 x g. Samples 3.1-3.5 had 300 uL of lysis/binding buffer added due to a smaller number of cells resulting in the following steps using lower volumes until the aqueous phase was removed, 300 uL was still taken then the rest of the protocol was followed accordingly. During isolation of replicate two, there were still only small pellets smeared on the walls, therefore samples 2.9 and 2.10 were used to test a higher centrifugation speed but there was little change. Therefore, the remaining steps followed the protocol. Samples 2.1-2.5 had 600 uL removed from the aqueous solution mistakenly but the protocol was correctly followed for the remaining steps. Following isolation of RNA, Nanodrop spectrophometer (ThermoFisher) was used to test the 260/280 and 260/230 and obtain the concentrations of all samples as Nanodrop was proven effective to measure concentration without the use of Qubit (ThermoFisher) by previous tests. All isolated RNA was then frozen in -80° C.

Two-Step RT-qPCR

All RNA was diluted to 2 ng/uL then Master Mix was made according to the protocol TaqMan® Small RNA Assays (ThermoFisher). The reverse transcription (RT) reaction was prepared with total RNA following the single-stranded small RNA reaction procedures using the primers TaqMan® MicroRNA Assays 200a, 200b and 200c primers (Product #4440885, ThermoFisher) The ministar silverline centrifuge (VWR) and the analog vortex mixer (VWR) were used. The RT reactions were ran in the Biometra T-Professional Basic Gradient thermocycler as stated in the protocol TaqMan® Small RNA Assays (ThermoFisher) then stored in -20° C.

A qPCR Reaction Mix was prepared following the TaqMan® Small RNA Assay User Guide (ThermoFisher) with TaqMan™ Universal Master Mix II, no UNG (ThermoFisher) and the primers TaqMan™ MicroRNA Assays 200a, 200b and 200c (Product #4440885, ThermoFisher). The sequences for the primers and RNU48 along with their product details can be found in Table 2. Nuclease free water (VWR) was used as a non-template control (NTC). Samples, the reference control RNU48 and NTC were plated on MicroAmp Optical 96-well Reaction Plate (Applied Biosystems) in technical triplicates, the Applied Biosystems 7300 Real Time PCR Machine was used and the cycles performed according to the TaqMan® Small RNA Assay User Guide (ThermoFisher) protocol without the UNG Activation step.

Table 2. Primer sequences for each sample used in Two-step RT-qPCR reaction.

Primer	Primer Sequence (3' to 5')	Accession Number	Assay ID
hsa-miR-200a	UAACACUGUCUGGUAACGAUGU	MI0000737	000502
hsa-miR-200b	UAAUACUGCCUGGUAAUGAUGA	MI0000342	002251
hsa-miR-200c	UAAUACUGCCGGGUAAUGAUGGA	MI0000650	002300
RNU48	GATGACCCCAGGTAACTCTGAGTGTGTCGCTGATG CCATCACCGCAGCGCTCTGACC	NR_002745	001006

qPCR data was evaluated according to rules decided prior to analysis, it was decided that the difference between Cq values must be smaller than or equal to 0.5. Discard the results if all three values have a difference larger than 0.5, if two values are undetermined, and if one value is undetermined but the other two have a difference larger than 0.5. Take the average of the two best as the final Cq value if one value has a difference larger than 0.5, if one value is undetected but the other two values are 0.5 from each other, and if two values are within 0.5 but one value is not. Fold change was calculated according to Livak method and normalized expressions were

calculated using the comparative C_T method (ΔΔCt). Time point 1.1 that was taken after PMA and before LPS and 1.2 taken after LPS and before ATP were chosen as calibrators as they were not stimulated with ATP.

Results

Cell Culture

The pellets collected from cell culturing appeared to decrease in size as time went on following LPS and ATP stimulation. This was observed in both biological replicates one and two, whereas biological replicate three had consistently small pellets.

Isolation of miRNA

Following isolation of miRNA from the three replicates, the quantity and quality of the samples were determined using a Nanodrop spectrophometer (ThermoFisher). Values can be found in Table's 3-5 in Appendix 1. The concentrations of all three replicates varied but the quantity of RNA was high enough to continue the following steps. The 260/280 ratio is a measure of the purity and was around 2.0 for most samples which is expected, they were either just a bit lower or a bit higher. The 260/230 ratio was much lower than anticipated, all of them were less than 2.0.

Two-Step RT-qPCR

qPCR resulted in quantitative results that were analyzed with relative quantification, using the Livak Method. The raw qPCR data as well as averages of Cq values can be found in Appendix 2.The calibrator is normally the untreated sample, in this case time point 1.1 that was taken after PMA and before LPS and 1.2 taken after LPS and before ATP were chosen as they were not stimulated with ATP. Resulting in the normalized expression ratios.

After obtaining the Cq values from the qPCR reaction, the $2^{-\Delta\Delta CT}$ was calculated using the Livak Method and normalized using sample 1.1 as a calibrator in Figure 2A and using sample 1.2 as a calibrator in Figure 2B. This resulted in the normalized expression ratios shown. Time point 1.6 of 200a was discarded as an outlier during the analysis of Cq values based on the stated rules.

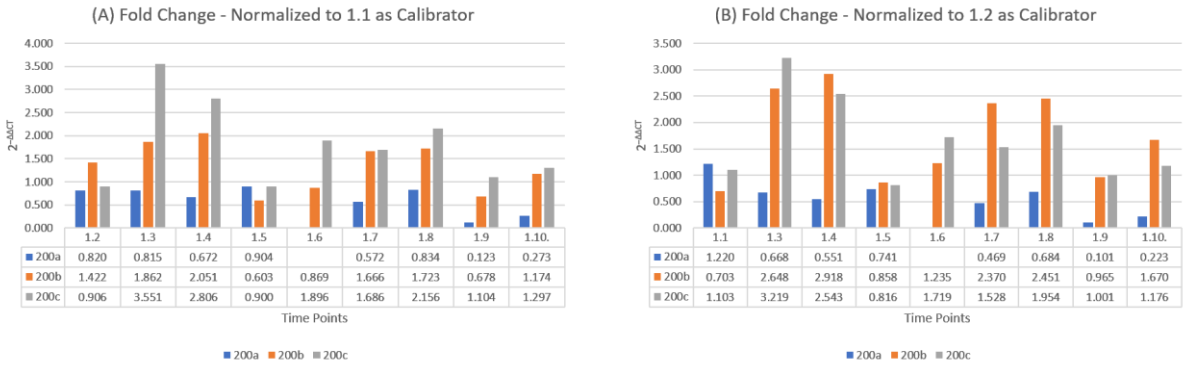

Figure 2. Fold Change of miRNA-200a, -200b, and -200c (A) Normalized expression ratio displayed as fold change, calculated with Livak method using the ΔCq value from sample 1.1 as a calibrator. (B) Normalized expression ratio displayed as fold change, calculated with Livak method using the ΔCq value from sample 1.2 as a calibrator. 200a time point 1.6 was removed as an outlier.

Log 2-Fold Change was calculated to better portray the qPCR results, the ΔCq values are illustrated as symmetric results. Depicting negative and positive values as either up or downregulated in Figure 3.

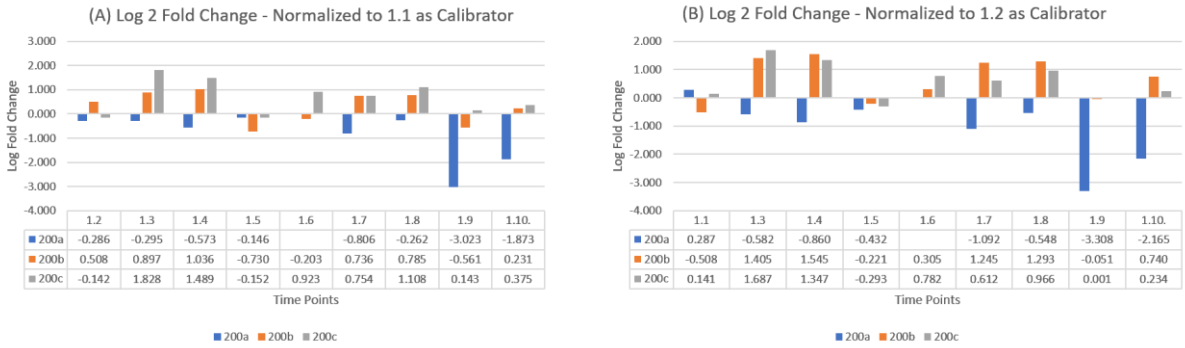

Figure 3. Log 2-Fold Change of miRNA-200a, -200b, and -200c (A) Log 2-Fold Change Normalized to the ΔCq value from sample 1.1 as a Calibrator. (B) Log 2-Fold Change Normalized to the ΔCq value from sample 1.2 as a Calibrator. 200a time point 1.6 was removed as an outlier.

Discussion

The aim of this study was to determine if miRNA expression changes once the inflammasome is activated. If the miRNA-200 family can be a regulator of the inflammasome, they would control whether it becomes activated and causes inflammation. Therefore, they could potentially stop inflammation and diseases.

Cell Culture

Three replicates of cultured THP1-ASC-GFP cells (Invivogen) were grown to a total of 50 million cells then differentiated with PMA as it was found to be one of the most effective differentiation agents for THP-1 cells (Chanput, Mes, & Wichers 2014). Following stimulation with LPS and ATP the ten samples collected for each replicate were pelleted and appeared to decrease in size as the time series continued. This gradual decrease was observed in both biological replicates one and two, whereas biological replicate three had pellets that were consistently small. It has been found in previous research that long exposure to and different amount of ATP in THP-1 cells and other cells causes decreased cell proliferation and apoptosis (Ciccarelli et al., 1994; Yoon, Lee, Kim, & Kim, 2006; Puchalowicz et al., 2020). This could be the reason for the decreased cell pellet size as the time series continued and thus increased ATP exposure in those samples.

Isolation of Total RNA

Following isolation of total RNA, Nanodrop was used to determine the quantity of total RNA and the purity of those samples. It was decided not to use Qubit, as previous testing prior to isolating the samples found that Nanodrop was sufficient in determining the concentration of the samples. Though it was decided that Nanodrop was effective, it is not always reliable in determining sample concentration as DNA, other RNA particles and contaminants are detected by absorbance at 260 nm. Therefore, it is unknown whether the Nanodrop truly measured the RNA concentration. The Qubit should have also been used to confirm the concentrations as it is more accurate. Garcia-Ellis et al. (2017) found that Qubit® Assay was the best method for quantifying miRNA compared to Infinite® 200 PRO Nanoquant, Thermo Scientific™ Nanodrop 2000, and Agilent 2100 Bioanalyzer. The Qubit's higher specificity for small RNA molecules allows it to measure the concentration of RNA more accurately, even at very low ranges.

The Nanodrop results seen in Appendix 1 indicate probable contamination from phenol used during isolation due to the high fluctuations in purity and exceptionally low results for the 260/230 readings. The 260/230 ratio should be around 2.0 and all samples had readings well below that (Desjardins & Conklin, 2010). The 260/280 readings for RNA are normally about 2.0 as well, lower readings would indicate DNA or protein contamination (Desjardins & Conklin, 2010). Most readings, especially those in replicate one which was the only one fully analyzed in RT-qPCR, had a purity around 2.0. The other two replicates had more variation, with most differing by slightly lower purities and only a few that were higher.

Two-Step RT-qPCR

Quantitative polymerase chain reaction (qPCR) is a fast, specific, and sensitive method to quantitively monitor the changes in nucleic acids, in this case miRNA 200 family expression levels. One large disadvantage with qPCR is that it can only be used for targeting known genes making it limited to analyzing known sequences. A major advantage is the ability to collect data in real time due to fluorescent labeling measuring the amplification of the PCR product through the increase in fluorescence during each PCR cycle, reflecting the amount of DNA amplicons at that time. This is an advantage over traditional endpoint PCR which analyses DNA amplification at the end of PCR (Smith & Osborn, 2008). Two-step RT-qPCR was chosen over one-step RT-qPCR as in eukaryotes one-step was found to be less sensitive as there are two enzymes in the same reaction and optimization of the reactions is impossible. Therefore, two-step was carried out so reverse transcription and qPCR reactions could be done separately. (Smith & Osborn, 2008). However, due to the two steps, an extra open-tube step, there is more pipetting, and increased hands-on time, leading to a greater risk of pipetting errors and contamination than one-step RT-qPCR. Though SYBR green assay can be more affordable and easier to use, TaqMan probes were chosen here as they are more reproducible, sensitive, specific, higher level of quantitation of gene expression, and can have predesigned assays. TaqMan use a fluorogenic probe ensuring that the fluorescence detected is only from the amplified target (Smith & Osborn, 2008; Tajadini, Panjehpour, & Javanmard, 2014).

NTCs were used as a control to detect contamination of the qPCR assay possibly from degraded nucleotide molecules, proteins, contaminants from the isolation process or presence of primer-dimer amplification (Bustin et al., 2009). RNU48 was used as a reliable endogenous control as it has good abundance and expression is stable making it good for normalization in miRNA qPCR analysis (Torres, Torres, Wdowiak, Paszkowski & Maciejewski, 2013). A reference gene should have a constant expression level on all samples and not be affected by changes in conditions during the experiment, unfortunately this was not validated prior to the experiment. The MIQE Guidelines state that it is not acceptable to use only one reference gene unless the scientist is able to confirm the expression does not change under the experimental conditions (Bustin et al., 2009). Several reference genes should have been tested in the experiment then analyzed with software such as NormFinder, GeNorm and BestKeeper to identify the most stable genes (Andersen, Jensen, & Ørntoft., 2004; Pfaffl, Tichopad, Prgomet, & Neuvians, 2004; Vandesompele et al., 2002). Then those most stable genes should have been used in the experiment and further normalized as it is more efficient to have multiple reference genes.

A no-RT control was not included in this experiment, this would have indicated if DNA contamination were present. Since it was not included, it us unknown whether there was contamination from DNA which could cause non-specific amplification and inaccurate results. DNA contamination could have also been detected with Qubit, had it been used, the DNA concentration could have been compared with the RNA concentration of the samples.

Following Two-step RT-qPCR, the Cq values obtained were then evaluated according to the previously decided rules mentioned in the materials and methods. As a result, time point 1.6 for miRNA-200a was removed as an outlier but the other data was evaluated using the Livak Method to calculate the $2^{-\Delta\Delta CT}$ of each time point. The calibrator is normally the untreated sample, in this case time point 1.1 (after PMA and before LPS addition) and time point 1.2 (after LPS and before ATP) were chosen as they were not stimulated with ATP. This resulted in the normalized expression ratios presented in fold changes seen in Figure 2. The Livak Method assumes that the amplification efficiencies of the target and reference gene are close to 100% and similar to each other within 5%, this should have been calculated prior to evaluation of the data using this method (Bio-Rad Laboratories, 2006; Bustin et al., 2009; Livak & Schmittgen, 2001). If this was tested, then optimizations could have been made to the assay if the amplification efficiency results were not similar to one another. Another method for relative quantification could have been used as well, the Pfaffl Method is used if the amplification efficiencies of the target and reference were not the same but each gene has the same efficiency in the test and calibrator samples (Bio-Rad Laboratories, 2006). As a result of not measuring the amplification efficiency prior to evaluation, the relative quantification results may not be accurate.

Normally statistics are used to determine a cutoff limit for up/downregulation but as the other two replicates could not be evaluated with qPCR, statistics could not be applied to the data. Since the other replicates could not be used in this study the results are instead treated as indicative results of a pilot study. Thus, a cutoff was not applied otherwise upregulated results would have a scale of one to infinity while downregulated results would have a scale of zero to one. Instead, Log 2-fold change was calculated to show true change and more visually accurate results that are easier to interpret, as presented in Figure 3. Overall, miRNA-200b and miRNA-200c have mostly positive values across all time points while miRNA-200a has almost all negative values. This would suggest that miR-200b and miR-200c show the potential of being upregulated and miR-200a has the potential of being downregulated.

While no distinct conclusions were drawn in this pilot study, there is a possibility the miR-200 family could regulate the NLRP3 inflammasome as there are several other microRNA's that have been found to be post-transcriptional regulators. Bauernfeind et al. (2012) following similar methods to this pilot study found that miR-223 negatively controls the NLRP3 inflammasome by binding to a conserved binding site within the 3' UTR, reducing inflammasome activity. Zhou et al., (2016) found that miR-7 negatively regulates NLRP3 inflammasome activation and researched the effects of that suppression in Parkinson's disease.

A study by Wendlandt, Graff, Gioannini, McCaffrey & Wilson (2012) researching targets of TLR4 signaling pathways and regulators of NF-κB, focused on the miR-200 family, and had similarities as well as a connection to this experiment. In NLRP3 inflammasome activation, following the priming step where the PAMP LPS binds to the pathogen recognition receptor TLR4, the NF-κB pathway is activated and initiates the expression of NLRP3, pro-caspase-1 and pro-IL-1β. In this study, HEK293 and THP-1 cells were used, THP-1 cells were differentiated with PMA and stimulated with LPS, similar to this experiment. RT-qPCR was done for miR-200b and miR-200c, with RNU48 as an internal control, and fold change was calculated using the comparative Ct method as was done in this experiment. Wendlandt et al. (2012) found that miR-200b and miR-200c influenced NF-κB reporter activity and MyD88 along with some signaling pathway proteins were targets for miR-200b and miR-200c, they were also able to suppress tested chemokines expression once THP-1 cells were stimulated with LPS (Wendlandt et al., 2012). Therefore, suggesting that miR-200b and miR-200c can affect NLRP3 inflammasome activation and the expression of cytokines. Though no concrete conclusion about the miR-200 family's expression level changes and possible regulation of the NLRP3 inflammasome could be made in this pilot

study due to the inability to analyze all biological replicates, this study by Wendlandt et al. (2012) proposes interesting connections between the miR-200 family and the NLRP3 inflammasome activation pathways.

The double-negative regulatory feedback loop between the miRNA-200 family and ZEB1 and ZEB2 have their role in tumor invasion and metastasis in various cancers. However, there is also a connection to the immune response with ZEB1 and ZEB2 expression in $CD8^+$ T cells. The miR-200 family regulates the differentiation of effector and memory $CD8^+$ T cells during an immune response by targeting the expression of ZEB1 and ZEB2. ZEB1 is a necessary regulator for the survival and homeostasis of memory $CD8^+$ T cells and inflammatory responses while ZEB2 is involved in the development of terminal effector $CD8^+$ T cells (Guan et al., 2019). Members of the miR-200 family inhibit ZEB2 expression and TGF-β activates ZEB1 but inhibits ZEB2 in $CD8^+$ T cells, allowing memory cells to form and prepare for future infections. Overexpression of the family members repressed terminal effector cells and induced memory cell development, while low expression resulted in the loss of memory $CD8^+$ T cells (Guan et al., 2019). The NLRP3 inflammasome's role in inflammatory responses correlates as it induces inflammation during an infection with the release of IL-1β and IL-18. IL-18 together with IL-12 activates naïve $CD8^+$ T cells and natural killer cells and stimulates production of interferon gamma in T-helper cells (IFNγ). The microRNA family's role in these various processes and the functions of the NLRP3 inflammasome suggest that members of the miR-200 family possibly also play a role in the NLRP3 inflammasome and the effects of inflammatory responses and possibly diseases, even cancer.

The next steps after this pilot study would be to further research the miRNA-200 family's response to NLRP3 inflammasome activation. The experiment should be repeated and fully completed. All three biological replicates being analyzed would provide more definitive conclusions about the changes in miRNA-200 family expression levels in stimulated THP-1 cells before and after the NLRP3 inflammasome is activated. Only three out of the five members of the miR-200 family were studied in this, miR-141 and miR-429 should be included in future research. Should members of the family be found to be up or downregulated, then continued research into those members should be pursued. Knowledge about regulation targets of inflammasomes brings the potential to prevent the inflammasome from activating and causing inflammation, therefore prohibiting inflammation and in the future, hopefully treating the causes of inflammatory diseases and even cancers.

Conclusion

The aim of this study was to observe how miRNA-200 family expression levels change in stimulated THP-1 cells as the NLRP3 inflammasome is activated. This became a pilot study as all biological replicates could not be analyzed, miR-200 family is showing a potential response to the activation of the NLRP3 inflammasome and they should be investigated further. Further studies will lead to learning if the miRNA-200 family can be regulators of the NLRP3 inflammasome, able to prevent inflammasome activation and potentially stop inflammation Expression of the miR-200 family have been found to be deregulated in numerous cancer studies. The miRNA-200 family are considered promising biomarkers and may serve as novel drug targets for the therapy of multiple types of cancer and inflammatory diseases.

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Appendix 1: Quantity and Quality for each replicate from Nanodrop

Table 3. Replicate 1 Nanodrop Results

Sample	ng/uL	260/280	260/230
1.1	20.1	2.00	0.56
1.2	21.7	2.00	1.48
1.3	6.2	2.17	1.66
1.4	26.5	2.16	1.86
1.5	22.8	2.00	1.72
1.6	25.5	2.00	0.55
1.7	15.4	1.95	0.39
1.8	13.1	1.95	0.14
1.9	7.9	2.04	0.43
1.10	20.1	1.99	0.57

Table 4. Replicate 2 Nanodrop Results

Sample	ng/uL	260/280	260/230
2.1	16.7	1.85	0.81
2.2	21.0	2.02	1.07
2.3	10.8	1.92	0.75
2.4	4.3	1.38	0.30
2.5	12.6	1.78	0.90
2.6	23.2	2.08	1.83
2.7	14.6	2.01	0.76
2.8	16.9	2.04	0.89
2.9	4.4	2.14	0.84
2.10	9.5	2.01	0.41

Table 5. Replicate 3 Nanodrop Results

Sample	ng/uL	260/280	260/230
3.1	5.6	2.26	0.62
3.2	5.5	3.57	0.65
3.3	10.0	1.95	0.20
3.4	7.5	2.03	0.56
3.5	5.8	2.13	0.18
3.6	23.2	1.7	0.14
3.7	11.1	1.87	0.25
3.8	13.9	1.84	0.68
3.9	30.8	1.94	1.06
3.10	36.8	1.94	1.04

Appendix 2: Cq Values from Raw qPCR data of miRNA-200 family and reference control RNU48

Table 6. Average of Cq values from qPCR for each miRNA-200 family and RNU48 reference control on each plate analyzed.

Sample	200a	200b	200c	Ref for Plate 1	Ref for Plate 2
1.1	34.825	33.02	33.415	22.18333333	23.63333333
1.2	35.175	32.575	33.45	22.24666666666667	23.52666667
1.3	34.19	33.57666666666667	34.75666666666667	21.84333333333333	23.14666666666667
1.4	32.205	32.01	33.09	20.13666667	21.82
1.5	34.58	32.19	33.17666666666667	22.08333333	23.54666666666667
1.6	Thrown	33.14	34.855	22.50666666666667	24.15
1.7	33.46	33.19666666666667	33.58	21.62333333	23.045
1.8	34.205	33.44666667	34.07666667	21.825	23.18666667
1.9	32.295	32.95333333	34.035	22.67666666666667	24.11
1.10	32.22333333	32.525	33.14666666666667	21.45666667	22.99

Table 7. Raw data from qPCR for miRNA-200a.

Sample Name	Cq	Std Dev Cq
1.1 a	33.50	0.767
1.1 a	34.91	0.767
1.1 a	34.74	0.767
1.2 a	34.19	0.604
1.2 a	34.97	0.604
1.2 a	35.38	0.604
1.3 a	34.01	0.558
1.3 a	34.37	0.558
1.3 a	33.28	0.558
1.4 a	32.14	0.447
1.4 a	32.97	0.447
1.4 a	32.27	0.447
1.5 a	35.12	0.319
1.5 a	34.52	0.319
1.5 a	34.64	0.319
1.6 a	35.18	0.581
1.6 a	Undetermined	Undetermined
1.6 a	36.00	0.581
1.7 a	33.54	0.532
1.7 a	33.38	0.532
1.7 a	32.55	0.532
1.8 a	33.72	0.305
1.8 a	34.09	0.305
1.8 a	34.32	0.305
1.9 a	32.31	0.422
1.9 a	33.02	0.422
1.9 a	32.28	0.422
1.10 a	32.27	0.038
1.10 a	32.21	0.038

1.10 a	32.19	0.038

Table 8. Raw data from qPCR for miRNA-200b.

Sample Name	Cq Values	Std Dev Cq
1.1 b	33.05	0.307
1.1 b	32.99	0.307
1.1 b	32.49	0.307
1.2 b	33.17	0.343
1.2 b	32.59	0.343
1.2 b	32.56	0.343
1.3 b	33.62	0.042
1.3 b	33.57	0.042
1.3 b	33.54	0.042
1.4 b	31.87	0.351
1.4 b	32.15	0.351
1.4 b	32.57	0.351
1.5 b	32.14	0.053
1.5 b	32.19	0.053
1.5 b	32.24	0.053
1.6 b	33.21	0.47
1.6 b	33.07	0.47
1.6 b	33.95	0.47
1.7 b	33.47	0.251
1.7 b	33.14	0.251
1.7 b	32.98	0.251
1.8 b	33.28	0.152
1.8 b	33.48	0.152
1.8 b	33.58	0.152
1.9 b	32.98	0.209
1.9 b	32.73	0.209
1.9 b	33.15	0.209
1.10 b	33.18	0.387
1.10 b	32.61	0.387
1.10 b	32.44	0.387

Table 9. Raw data from qPCR for reference control RNU48 on plate 1.

Sample Name	Cq Values	Std Dev Cq
1.1 R	22.19	0.006
1.1 R	22.18	0.006
1.1 R	22.18	0.006
1.2 R	22.23	0.033
1.2 R	22.28	0.033
1.2 R	22.23	0.033
1.3 R	21.77	0.064

1.3 R	21.86	0.064
1.3 R	21.90	0.064
1.4 R	20.06	0.066
1.4 R	20.17	0.066
1.4 R	20.18	0.066
1.5 R	22.04	0.045
1.5 R	22.13	0.045
1.5 R	22.08	0.045
1.6 R	22.55	0.046
1.6 R	22.46	0.046
1.6 R	22.51	0.046
1.7 R	21.68	0.142
1.7 R	21.46	0.142
1.7 R	21.73	0.142
1.8 R	23.01	0.69
1.8 R	21.92	0.69
1.8 R	21.73	0.69
1.9 R	22.65	0.023
1.9 R	22.68	0.023
1.9 R	22.70	0.023
1.10 R	21.47	0.041
1.10 R	21.41	0.041
1.10 R	21.49	0.041

Table 10. Raw data from qPCR for miRNA-200c.

Sample Name	Cq Values	Std Dev Cq
1.1 c	34.16	0.437
1.1 c	33.35	0.437
1.1 c	33.48	0.437
1.2 c	34.22	0.444
1.2 c	33.51	0.444
1.2 c	33.39	0.444
1.3 c	34.93	0.281
1.3 c	34.91	0.281
1.3 c	34.43	0.281
1.4 c	33.21	0.108
1.4 c	33.07	0.108
1.4 c	32.99	0.108
1.5 c	33.24	0.11
1.5 c	33.24	0.11
1.5 c	33.05	0.11
1.6 c	34.33	0.339
1.6 c	34.71	0.437
1.6 c	35	0.437
1.7 c	34.43	0.437

1.7 c	33.55	0.444
1.7 c	33.61	0.444
1.8 c	34.1	0.444
1.8 c	34.31	0.281
1.8 c	33.82	0.281
1.9 c	34.05	0.281
1.9 c	33.45	0.108
1.9 c	34.02	0.108
1.10 c	33.28	0.108
1.10 c	32.95	0.11
1.10 c	33.21	0.11

Table 11. Raw data from qPCR for reference control RNU48 on plate 2.

Sample Name	Cq Values	Std Dev Cq
1.1 R	23.59	0.037
1.1 R	23.66	0.037
1.1 R	23.65	0.037
1.2 R	23.58	0.055
1.2 R	23.47	0.055
1.2 R	23.53	0.055
1.3 R	23.17	0.059
1.3 R	23.08	0.059
1.3 R	23.19	0.059
1.4 R	22.43	0.546
1.4 R	21.39	0.546
1.4 R	21.64	0.546
1.5 R	23.51	0.081
1.5 R	23.64	0.081
1.5 R	23.49	0.081
1.6 R	24.25	0.472
1.6 R	24.05	0.472
1.6 R	24.95	0.472
1.7 R	24.23	0.69
1.7 R	22.97	0.69
1.7 R	23.12	0.69
1.8 R	23.32	0.12
1.8 R	23.09	0.12
1.8 R	23.15	0.12
1.9 R	24.1	0.087
1.9 R	24.2	0.087
1.9 R	24.03	0.087
1.10 R	22.89	0.118
1.10 R	23.12	0.118
1.10 R	22.96	0.118