UNIVERSITY OF SKÖVDE

GSK-3 and ROS (reactive oxygen species) inhibition modulate Vimentin expression

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Student: Ragad Hassano
a19ragha@student.his.se

Supervisor: Mikael Ejdebäck
mikael.ejdeback@his.se

Co-supervisor: Matthew Herring
matthew.heering@his.se

Examiner: Patric Nilsson
patric.nilsson@his.se

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Abstract

When exposed to pathogenic stress, cellular processes and survival are dependent on cytoskeletal proteins for structure and organisation of the cell to adapt and maintain homeostasis during inflammation. Vimentin is type III cytoskeletal protein, with an extensive cytoplasmic meshwork, across the cell and regulate the cell structure and cellular space and expressed strongly under tumorigenic events. GSK-3, a regulatory component of inflammation expressed in abundance of cell together with reactive oxygen species (ROS), a group of key complex signalling molecules that are oxygen metabolites which are partially reduced, with robust oxidising abilities, are believed to influence inflammasome formation and specifically vimentin expression upon inflammation. This project investigated the potential modulation vimentin mRNA expression utilising the two signal NLRP3 inflammasome activation theory, by inhibiting GSK-3 and ROS in signal I and or signal II in LPS and nigericin stimulated THP-1 cells, compared to non-inhibited LPS and nigericin THP-1 cells. Inhibition of GSK-3 in signal II downregulated vimentin expression, reflecting repressed phosphorylation of GSK-3 hence also the components required for vimentin; whilst upregulation of vimentin in signal I, reflects possible alternative pathways phosphorylating vimentin components. Overall upregulation of vimentin upon inhibiting ROS in both signal I and II, further proved that inflammasome activation is independent of ROS in the priming step. More research is required integrating vimentin activity and either GSK-3 or ROS, as the potential of these prominent inflammatory markers and their major regulatory presence across an abundance of cell may contribute to the future of drug development for inflammatory diseases.

List of abbreviations

ALRs	Absent in melanoma 2-like Receptors
ASC	apoptosis-associated speck-like protein containing a CARD
DAMPs	Damage Associated Molecular Patterns
EMT	epithelial mesenchymal transition
GSK-3	Glycogen Synthase Kinase-3
IF	Intermediate Filaments
IFN	Interferon
IL-	Interleukin-
LPS	Lipopolysaccharide
LRR	Leucine Riche Repeats
NF-dB	Nuclear Factor Kappa
NLRP3	NOD-like receptor
PAMP	Pathogen Associated Molecular Pattern
PMA	Phorbol Myristate Acetate
PRR	Pattern recognition Receptor
ROS	Reactive Oxygen Species
THP-1	Human leukaemia monocytic cell line
TLR	Toll-Like Receptor

Introduction

The innate immune system and mechanisms of inflammasome activation

Inflammation is the response of the immune system to injury and infection. It is a physiological response to potential pathogens or cell damage and activates macrophages to develop an immune response to the invading pathogen (Coant et al.,2011). Within cell tissues of dendritic cells, fibroblasts, macrophages, mast cells and leukocytes cell reside innate immune cells which recognize cell damage and invading pathogens with pattern recognition receptors (PRRs) (Newton & Dixit, 2012). Upon microbial infection, the cell release exogenous signals in the form of carbohydrates, lipoproteins and microbial nucleic acids called Pathogen associated molecular patterns (PAMPs), whereas the response of injured cells to endogenous stress is by releasing damage associated molecular patterns (DAMPs) (Tang et al., 2012). PAMPs and DAMPs are mainly recognized by membrane bound family group toll like receptors (TLR) group of PRRs, a type of transmembrane proteins containing leucine rich repats (LRRs), triggers the innate immune response and activates PPRs which induces a specific inflammatory pathway related to the infection or injury obtained, and the upregulation of pro-inflammatory cytokines forming the inflammasome complex (Kelley et al., 2019). The inflammasome complex, a multimeric protein complex, increases the abundance of inflammatory cell mediators such as cytokines and chemokines, leading to the elimination of microbes, by the release of toxic metabolites or phagocytosis and allowing tissues to repair (Nallar & Kalvakolanu, 2014).

The inflammasome complex, is constituted of different unique sensor molecules such as: absent in melanoma 2- like receptors (ALRs), nucleotide binding domain like receptors (NLRs) and Pyrin, which upon construction activate caspase-1(Tang et al., 2012). In addition, inflammasomes have an adaptor molecule apoptosis-associated speck-like protein (ASC) containing a CARD, consisting of a pyrin domain, which interacts with inflammasomes sensor molecules, and a caspase activation and recruitment domain, which initiates activation of capsase-1 by autocatalytic cleavage (Tang et al., 2012).

NLRP3 inflammasome

The NOD-, LRR- and pyrin domain containing 3 (NLRP3) inflammasome is the most well-studied inflammasome and is activated in response to pathogens. The construction of NLRP3 follows two distinctive signalling pathways, priming and activation (Swanson et al., 2019; Lamkanfi & Dixit., 2014).

In signal I, also named the priming step, different PAMPs as mentioned above, triggers the Toll-like receptors (TLR) signalling and activates the NF-kB pathway leading to the upregulation of NLRP3 and pro-IL-1β expression (Sanson et al., 2019). Signal II, also named the activation step, is initiated by endogenous signalling of cells, DAMPs, and it is when the multi-protein inflammasome complex is assembled leading pro-caspase-1 to cleave (Zhao & Zhao., 2020). The active caspase-1 molecule, activates pro-inflammatory cytokines including pro-IL-1β, induced by the activation of transcription factor nuclear factor-κB (NF- κB), and pro-IL-18, which its expression increases upon cellular activation (Latz et al., 2013). NLRP3 inflammasome is activated by varying stimuli including, ionic flux, mitochondrial dysfunction, lysosomal damage, and the production of reactive oxygen species (ROS) (Latz et al., 2013; Sharma & Kanneganti., 2016).

Intermediate filament Vimentin

When exposed to pathogenic stress, cellular processes and survival are dependent on cytoskeletal proteins, which facilitates the cellular structure and organisation for the cell to adapt and maintain biochemical processes required for homeostasis during inflammation (Tur-Garcia et al., 2021). Cytoskeletal proteins are classified into three main types, microtubules, microfilaments, and

intermediate filaments (IF), each are part their own network dedicated to executing specific task within the cell (Tur-Garcia et al., 202; Su et al., 2019). Third class cytoskeletal proteins are the intermediate filaments (IF), which possess the ability to crosslink to type one and two cytoskeletal proteins (Danielsson et al., 2018). They have an extensive cytoplasmic meshwork, which encompass throughput the cell and regulate the cell structure as well the cellular space and are constituted in response to mechanical stress, and therefore are considered future biomarker for many inflammatory diseases (Danielsson et al., 2018). They are classed into different cell type specific classes which includes epithelial cell types contains class I and II cytokeratin, mesenchymal cells contain vimentin of class III, neurofilaments contains class IV, and type V lamin found in nucleus of cells and type VI nestin which are embryonic neurons (Ong et al., 2020).

From the six IFs mentioned, vimentin is the predominantly expressed and highly conserved protein type in normal mesenchymal cells in CNS and in muscle cells (Lois-Bermejo et al., 2022). Vimentin consists of a highly stable coiled-coil polypeptide consisting of α-helical region edged by non α-helical N-terminal head and C-terminal end regulated by the phosphorylation, with distinct and complex phosphorylation pattern and signalling kinases involved (Cogli et al., 2013; Lois-Bermejo et al., 2022). Since vimentin is expressed in a variety of cells and it influences many cell and tissue functions and its involvement in the pathogenesis of varying inflammatory diseases, marks it a crucial component to investigate in regards of inflammation. A key study by dos Santos (2015), in which they state that NLRP3 inflammasome activation is regulated by vimentin, by investigating acute lunge injury in mice with knocked out vimentin function compared to wild type mice and observed that levels of IL-1β and active caspase-1 reduced, suggesting vimentin as a key regulator of the NLRP3 inflammasome (Gimena dos Santos et al., 2015). However, the underlaying mechanism of vimentin and how it interacts with different inflammatory signalling pathway regulators and inflammasomes formation is poorly understood in a molecular and clinical level and requires extensive research.

Glycogen synthase kinase 3

Glycogen synthase kinase-3 (GSK-3), a systolic serine/ threonine protein kinase, is the greatest expressed pro-inflammatory kinase in cells (Beuel & Jope, 2009). Fundamentally recognised for the regulation of the glycogen metabolism, GSK-3 is found and expressed in the mitochondria, nucleus, and other subcellular compartments, which highlights the extent of involvement and mechanism GSK-3 possess in human and animal tissues (Beuel & Jope, 2009). GSK-3 is divided into GSK-3α and GSK-3β, two functionally distinctive isomers of GSK-3, present in different cellular compartments (Tsai et al., 2011). The enzymes' ability to phosphorylate an abundance of substrates depends on its substrate specific action of regulation and the explicit signalling of GSK-3 (Yoshino & Ishioka, 2015). During inflammation, GSK-3 is exposed to stimuli such as lipopolysaccharide (LPS) from bacterial cell wall, which stimulates Toll-like receptor 4 (TLR4), and causes the activation of the enzyme, and its substrate pre-phosphorylate before entering the inflammatory pathway (Yoshino & Ishioka, 2015).

The ubiquity of the GSK-3 enzyme to other kinases and its involvement in all cellular signalling and an array of crucial cellular functions, marks it a rather interesting enzyme to investigate. Furthermore, GSK-3 multiple regulatory mechanism of inflammation, inducing pro-inflammatory mediators and expression across all cells, uniquely defines it as the main marker for understanding and developing therapies targeting inflammatory diseases. A recent study through a proteomic investigation followed by Western blot and fluorescence microscopy found that in glioblastoma cell lines, upon inhibiting GSK-3 with different inhibitors such as lithium chloride (LiCl) and 6-bromo-indirubin-3-oxime (BIO), significantly downregulated the expression of vimentin, and they are the first to propose further insights of the relationship between GSK-3 and vimentin dynamics (Michal et al., 2019). However, more studies regarding the mechanisms which

vimentin and GSK-3 interact are required to understand the extent of either of their functions in inflammation.

Reactive Oxygen Species (ROS)

Reactive oxygen species (ROS) are the product of biochemical reactions in cells and organelles (Hayyan et al., 2016). They are considered central for the progression of an abundance of inflammatory diseases. A group of key complex signalling molecules that are oxygen metabolites which are partially reduced, with robust oxidising abilities (Mittal et al., 2014). The ROS products have been generally regarded as toxic by-product of metabolism, which damages DNA due to them oxidising lipid cellular components and proteins (El-Kenawi & Ruffell., 2017). The oxidative stress in the cell is believed to contribute to the pathogenesis of an abundance of human diseases such as lung diseases and sepsis (Zhao et al., 2020). However, current research suggest the ROS embarks a bigger role in the cell than just being the metabolism by-products, and that they have active role in cell signalling and regulation (El-Kenawi & Ruffell., 2017; Feng et al., 2018; Zhao et al., 2020). Current research reported that upon stimulation with LPS, which activates the TLR4, the intracellular levels of ROS increased, which further highlights the relevance of ROS in the priming step of the NLRP3 inflammasome activation, yet the exact mechanisms of how ROS mediates the activation remains largely unknown (Feng et al., 2018).

The biochemical interactions of vimentin with either GSK-3 or ROS upon inflammation is not widely discussed, and important mechanisms upon altering GSK-3 and ROS function regarding vimentin expression remains unknown, as current research focuses on understanding the underlying mechanisms of respective component. The strong presence of either components upon inflammation and tumorigenic events mentioned above and the lack of studies investigating these interactions calls out for the need to analyse and discover the extent of these interactions and significance or if they are independent of each other. Acquiring further understanding of these interaction aids the overall goal towards potential drug development and diagnosis of different cancers and inflammatory diseases, as mentioned, both vimentin and GSK-3 are future biomarkers, and different ROS products are also strongly linked to inflammation, yet their interactions with vimentin is not extensively investigated.

Research question

The main question which arises is: how is vimentin expression modulated upon inhibiting GSK-3 or ROS in signal I compared to signal II in LPS and nigericin stimulated THP-1 ASC-GFP-macrophages?

Aim and objectives

The aim of this project is to investigate the modulation of mRNA expression level of vimentin upon inhibiting GSK-3 or ROS in pre- LPS priming (signal I) or post- LPS priming (signal II), followed by activation of the NLRP3 inflammasome complex with nigericin, in comparison to non-inhibited LPS and nigericin stimulated THP-1 cells. Moreover, the relationship between vimentin modulation and inflammasome formation was investigated in two different aspects. First, live cell imaging of all conditions compared to LPS and nigericin stimulated cells was performed. The second part focuses on studying the interplay of vimentin expression and the NLRP3 activation and inflammasome formation in cellular level, by ASC- speck formation in live cell imaging and staining of cells to measure the intensity of vimentin.

Materials and methods

Cell culture

THP-1 ASC-GFP cells derived from THP-1 human monocytic cells (Invivogen), were grown in RPMI 1640 media with 0.3 g/L L-glutamine (Sigma Aldrich), 10% Heat inactivated premium Grade Foetal Bovine Serum (Biowest), 1mM sodium pyruvate (Sigma Aldrich), 0.45% Glucose solution (Sigma Aldrich), 10 mM HEPES (Sigma Aldrich) and 1x Penicillin-streptomycin (Sigma Aldrich). Cells were kept at 5 x 10^5– 1.5 x 10^6 cells/mL and media was changed every other day. 100 µg/mL Zeocin (Invivogen) was added to growth media every other cell passage to maintain the selective pressure of the cells. The cells were maintained at 37 °C, 5% CO_2.

Cells were differentiated to macrophages with 100 ng/mL Phorbol Myristate Acetate (PMA) at density of 3 x 10^5 cells/mL and incubated at 37 °C, 5% CO_2 for 24h. Cells for mRNA quantification of vimentin were differentiated in t-25 flasks, and for live cell imagining in 24-well plates. Cells for staining and fixation were differentiated in 4-well glass slides. Cells were washed twice with 1x PBS (Sigma Aldrich) and incubated for 48h in fresh growth media. All incubation steps were at 37 °C and 5% CO_2 incubator.

Stimulation of cells

Cells for mRNA quantification of vimentin, live cell imaging and staining and fixation were treated in five different conditions. All cells were stimulated with 500 ng/mL LPS for 4h and10 µM Nigericin for 45min. First condition and the control sample are LPS and nigericin stimulated THP-1 macrophages. Second and third condition is inhibiting ROS products and GSK-3 in the first signal. ROS products are inhibited with 10 µM DPI- diphenylene iodonium chloride (Sigma Aldrich) for 1h for sample two followed by LPS and Nigericin, whilst in sample three GSK-3 is inhibited with 10 µM SB-216763 inhibitor followed by LPS and Nigericin. Fourth and fifth condition is inhibition of GSK-3 and ROS in signal II. Cells of these conditions were primed with LPS followed by inhibition of ROS with 10 µM DPI for condition four, or inhibition of GSK-3 with 10 µM SB-216763 for condition five for 1h, followed by activation with nigericin. Stimulation of cells for imaging were performed in minimal lighting contact to prevent photo-bleaching.

Following stimulation, supernatant was collected before scraping of cells, and stored at -18°C, until use. For RNA extraction, cells were washed twice with ice cold 1x PBS, pelleted and stored at -82°C until extracted.

Fixation and staining of cells

Fixation and staining were performed immediately after stimulation. All incubation steps were performed on a rocking table in the dark and at room temperature.

Cells were rinsed twice with 37°C 1x PBS followed by fixation with 37°C, 4% Paraformaldehyde (PFA; Merck) in 1x PBS and incubated for 10 minutes. Cells were washed twice with 1x PBS for 5 min, followed by washing with de-ionized water twice for 5 minutes. To permebilise the cells, cells were incubated for 10-minute with 1% FBS (Biowest) in PBS with 0.4% Triton X-100 (Merck), followed by incubation with 5% FBS (Biowest) in PBST (Fisher BioReagents) for 30 min to block non-specific binding. To prevent non-specific binding, cells were incubated with Vimentin primary antibody Rb 154207 (ABCam) for 1.5h, followed by washing the cells twice with PBST for 10 minutes. Finally, cells were stained with DAPI (Sigma Aldrich) and incubated for 10 min, followed by washing them with de-ionized water for 10 min and they are directly imaged.

Imaging

All cell imagining of vimentin samples was performed using the EVOS M7000 Imaging System (Thermofisher). Imaging was performed at 20x magnification and included Trans, GFP, TX red and

DAPI channels. Trans channel imaged cells without any fluorescence and was used to determine which areas of the slide/ wells of the samples to image in the other channels. GFP channel, imaged the ASC-specks formed in the cells. TX red channel, imaged vimentin fluorescent of the cells. DAPI channel imaged cell nucleus stained with DAPI.

For live cell imaging, cells in each condition were counted in triplicates as well as ASC-specks formed. Cells were investigated in the GFP-channel, and ASC-specks were examined by looking at the trans channel to make sure they are true specks. True specks will not emit in the trans channel and only in the GFP channel, which minimises the error of counting non inflammasome specks formed. Specks formed were quantified as a percentage of the cells counted in each replicate.

Criterions for cell counting for live cell imaging and stained cells included: manual counting of cells, which excluded cells that were clumped together and had no distinguished cell shape separating the cells. Stained cells were counted using the DAPI channel to make sure true nuclei were counted Moreover, the specks counted had to be attached to single cell and did not include clumps of cells that had a speck attached to them. Specks attached to cells were analysed in the GFP-channel, and to confirm, specks were analysed in the trans channel as true specks fluorescent in the GFP-channel.

Vimentin intensity measurement of cells with an inflammasome compared to cells without an inflammasome in the same image, was conducted in Fiji:Image J. Cells were again examined in the DAPI channel as well as the TX channel for vimentin and measured the average pixel intensity using the free hand selection for each cell and subtracted it against the back-ground pixel intensity of the image.

RNA extraction and reverse transcription

RNA extraction was conducted using the RNeasy Mini kit (Qiagen). RNA concentration and purity was measured using the DS11 spectrophotometer (DeNovix). Following RNA extraction, reverse transcription to obtain cDNA was preformed using the High-Capacity RNA to cDNA Reverse Transcription kit (Thermofisher).

Quantification of mRNA expression

qPCR was conducted using the SYBR Select Master Mix kit (Applied Biosystems) and AriaMX Real-Time PCR System (Agilent).

Reference genes

For vimentin, the mRNA expression stability of reference genes ACTB, HRPT1, RPLP, TBP, TUBB and YWHAZ from the SYBR Human Endogenous Control Reference Gene Panel (TATAA Biocenter) and raw Cq data obtained were evaluated to determine appropriate reference gene were evaluated using RefFinder which includes different algorithms to determine the most stable expressed reference gene among all reference genes mentioned. GeNorm determined RPLP and TBP as the most appropriate reference gene, and they were both used in this experiment. Target genes were normalised against the geometric mean of RPLP and TBP and evaluated relative to differentiated LPS and nigericin stimulated THP-1 cells. All data analysis were conducted using the software GenEX (2010).

Vimentin primer design and validation

The choice of appropriate primer pair for the gene of interest was conducting by preforming a 7-fold dilution of 100ng unstimulated THP-1 macrophages and the efficiency was determined by plotting the logarithmic value of sample dilution factor in AriaMX. The primer pair chosen has a 98% efficiency and was designed to cover a junction. Forward: 5′-AAGACACTATTGGCCGCCTG-3′, Reverse: 5′-GGCAGAGAAATCCTGCTCTC-3.

Statistical analysis

All statistical test preformed using IBM SPSS Statistics version 28, by One-way ANOVA followed by post-hoc Tukey's test, for mRNA expression levels and mean percentage of cells containing and inflammasome. Independent samples t-test was used for measuring the mean pixel intensity of vimentin. Significant difference is displayed by $p<0.05$ (*), $p<0.01$ (**) and $p<0.0001$ (***).

Results

Selection of a stable reference gene

qPCR is used to quantify gene expression levels, therefore a stable refence gene is required for normalising the gene of interest to ensure true expression changes of the experiment. For this project, seven primers from the SYBR Human Endogenous Control Reference Gene Panel (TATAA Biocenter), including RPLP, TBP, TUBB, UBC, HRPT1, β-Actin and YWHAZ were evaluated (Figure 1). Using the raw qPCR, data, fluctuating Cq data trends could be observed among the different treated samples, yet no large variation (SD≥0.3) could be noticed among the technical replicates for all primers. Following these observations, GeNorm was used to identify the most stable and suitable refence genes for the experiment. According to GeNorm, RPLP and TBP from the SYBR Human Endogenous Control Reference Gene Panel (TATAA Biocenter) combined are deemed as the most stable and suitable reference genes to normalise against the vimentin mRNA expression levels, with the lowest M-value of 0.474 RPLP and TBP combined, whilst YWHAZ had the highest M-value of 3.703 as the least stable as displayed in Figure 1. Moreover, within RefFinder algorithms BestKeeper, Delta CT and Normfinder all suggested RPLP as the most stable reference gene followed by TBP as second best.

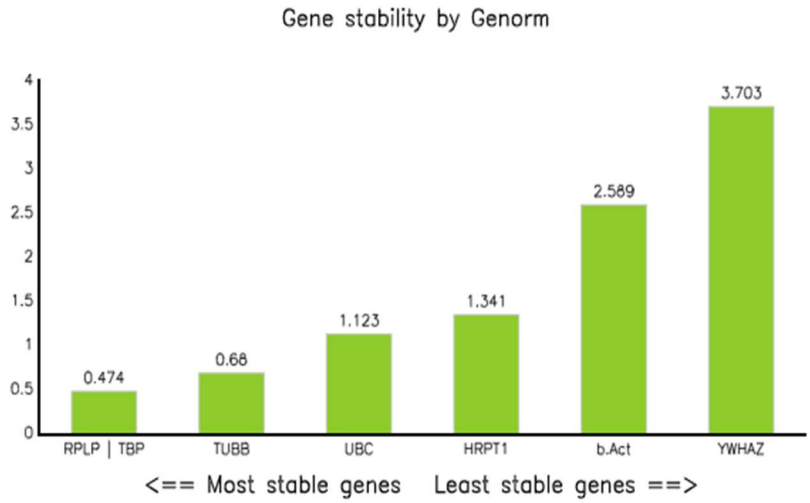

Figure 1. GeNorm suggests RPLP and TBP combined most stable reference gene in GSK-3 or ROS inhibited LPS and nigericin stimulated THP-1 macrophages. The bar chart illustrates gene expression normalisation factor by GeNorm and the stability of seven reference genes RPLP, TBP, TUBB, UBC, HRPT1, β-Actin and YWHAZ from the SYBR Human Endogenous Control Reference Gene Panel (TATAA Biocenter). GeNorm suggest the calculated geometric mean (0.474) of primers RPLP and TBP combined the most suitable and stably expressed reference genes.

Inhibition of GSK-3 or ROS modulated vimentin expression

Inhibition of GSK-3 in signal I and signal II

To investigate potential modulation in the mRNA level of vimentin upon inhibiting GSK-3, mRNA level of vimentin was quantified using qPCR to evaluate the effect of GSK-3 inhibition (SB-216763) in signal I or signal II in LPS and nigericin stimulated THP-1 macrophages (Figure 2). Inhibition of GSK-3 in signal I, meaning pre-priming with LPS for 1h, followed by 4h LPS and 45min activation with nigericin (Figure 2), vimentin mRNA expression level is significantly increased (mean log 2-fold change = 0.61, $p<0.001$) compared to LPS and nigericin stimulated THP-1 macrophages (Figure 2). In contrast, inhibition of GSK-3 in signal II, meaning post- priming with LPS 4h,

followed by the inhibitor for 1h and nigericin 45min, significantly downregulated vimentin expression (mean log 2-fold change= -0.44, $p<0.02$), compared to LPS and nigericin stimulated THP-1 cells as seen in Figure 2. Overall, GSK-3 inhibition in signal I or signal II significantly modulated mRNA expression of vimentin in LPS and nigericin stimulated THP-1 macrophages (Figure 2).

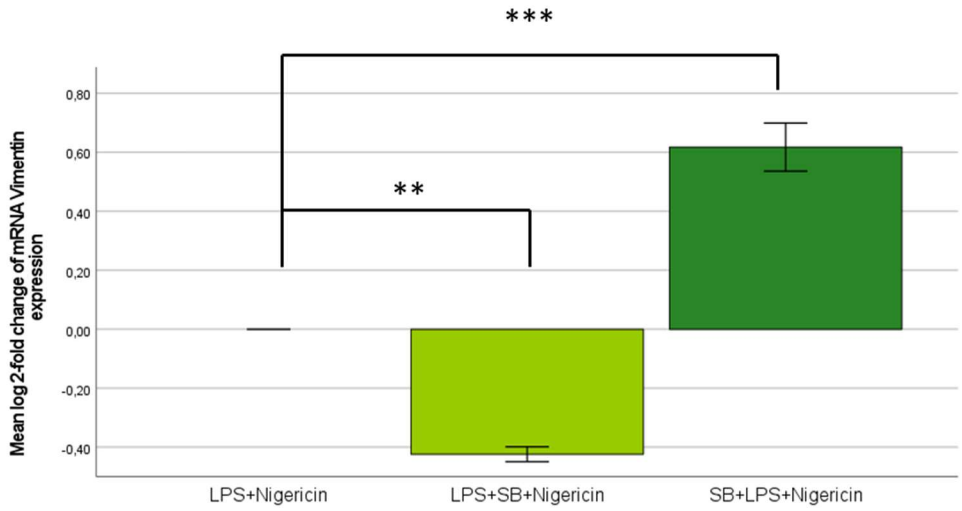

Figure 2. GSK-3 inhibition significantly increases vimentin mRNA expression level in signal I and decreases in signal II in LPS and nigericin stimulated THP-1 macrophages. The figure displays the mean log 2-fold change (ΔΔCt) of Vimentin mRNA expression levels were normalised against reference genes RPLP and TBP combined and calculated relative to LPS and nigericin stimulated THP-1 macrophages. Inhibition of GSK-3 (SB-216763, 10 μM) in signal I, meaning pre- LPS priming, or inhibition of GSK-3 (SB-216763, 10 μM) in signal II, meaning post-LPS priming, were compared to only LPS and nigericin stimulated THP-1 macrophages. The bars represent the mean values ± 1 SD. Statistical significance was determined by One-Way ANOVA ($F_{2,9} = 7.25$, $p<0.001$), followed by Tukey's post-hoc test (n=3 in each group). Asterisks denotes significant differences from the control LPS + Nigericin (***$p<0.001$ and **$p<0.01$).

Inhibition of ROS in signal I and signal II

This experiment quantified vimentin mRNA expression levels using qPCR to evaluate the effect of ROS inhibition (DPI) in signal I or signal II in LPS and nigericin stimulated THP-1 macrophages (Figure 3). Inhibition of ROS in signal I, meaning pre-priming with LPS for 1h, followed by 4h LPS and 45min activation with nigericin (Figure 3) or inhibition of ROS in signal II meaning post-LPS 4h, followed by the inhibitor for 1h and nigericin 45min vimentin expression is significantly ($p<0.01$) increased in both conditions compared to only LPS and nigericin stimulated macrophages (Figure 3). Furthermore, a 60% increase of vimentin expression is observed of the mean log2-fold change upon inhibiting ROS at signal I and II compared to inhibition in signal I (Figure 3).

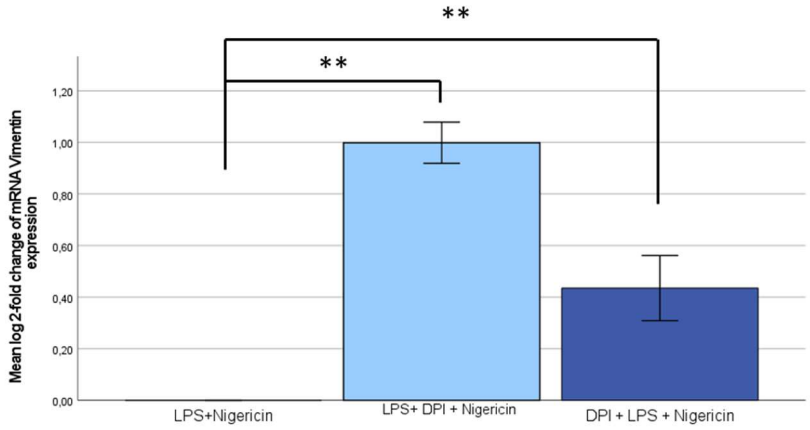

Figure 3. ROS inhibition (DPI, 10 μM) in signal I or signal II significantly increases vimentin mRNA expression levels in LPS and nigericin stimulated THP-1 macrophages. The figure displays the mean log 2-fold change (ΔΔCt) of Vimentin mRNA expression levels were normalised against reference genes RPLP and TBP combined and calculated relative to LPS and nigericin stimulated THP-1 macrophages. The bars represent the mean values ± 1 SD. Statistical significance was determined by One-Way ANOVA ($F_{2,9} = 12.16$, $p<0.001$), followed by Tukey's post-hoc test (n=3 in each group). Asterisks denotes significant differences from the control LPS + Nigericin (**$p<0.001$).

Inhibition of GSK-3 and ROS does not affect ASC-speck formation

Live-cell imaging was used to investigate the formation of ASC-speck upon inhibiting GSK-3 or ROS in either signal I or II in LPS and nigericin stimulated THP-1 cells (Figure 4). Figure 4A is 4h LPS treated cells followed by 45min of nigericin; 4B are GSK-3 inhibited cells in signal I, meaning pre-priming with LPS for 1h, followed by 4h LPS and 45min activation with nigericin. Figure 4C displays inhibition of ROS in signal I, meaning pre-priming with LPS for 1h, followed by 4h LPS and 45min activation with nigericin. Figure 4D displays inhibition of GSK-3 in signal II, meaning post- priming with LPS 4h, followed by the inhibitor for 1h and nigericin 45min. Lastly figure 4E displays inhibition of ROS in signal II meaning post-LPS 4h, followed by the inhibitor for 1h and nigericin 45min. Overall, specks are formed in all different treatments in the GFP channel (Figure 4).

Using live-cell images of all treated samples mentioned above were compared to the control treated sample of only LPS and nigericin stimulated cells (Figure 4A), were used to quantify percentage of cells containing an ASC-speck (Figure 5). As figure 5 displays, no significant difference of ASC-speck formed between the different stimulated samples ($p=0.161$). Overall, no significant ASC-specks abundance is formed upon inhibiting GSK-3 or ROS compared to only LPS and nigericin stimulated cells. Therefore, it's not possible to observe correlation of ASC-speck

formed and NLRP3 activation upon inhibition to the Vimentin mRNA expression level results in Figure 2 and 3.

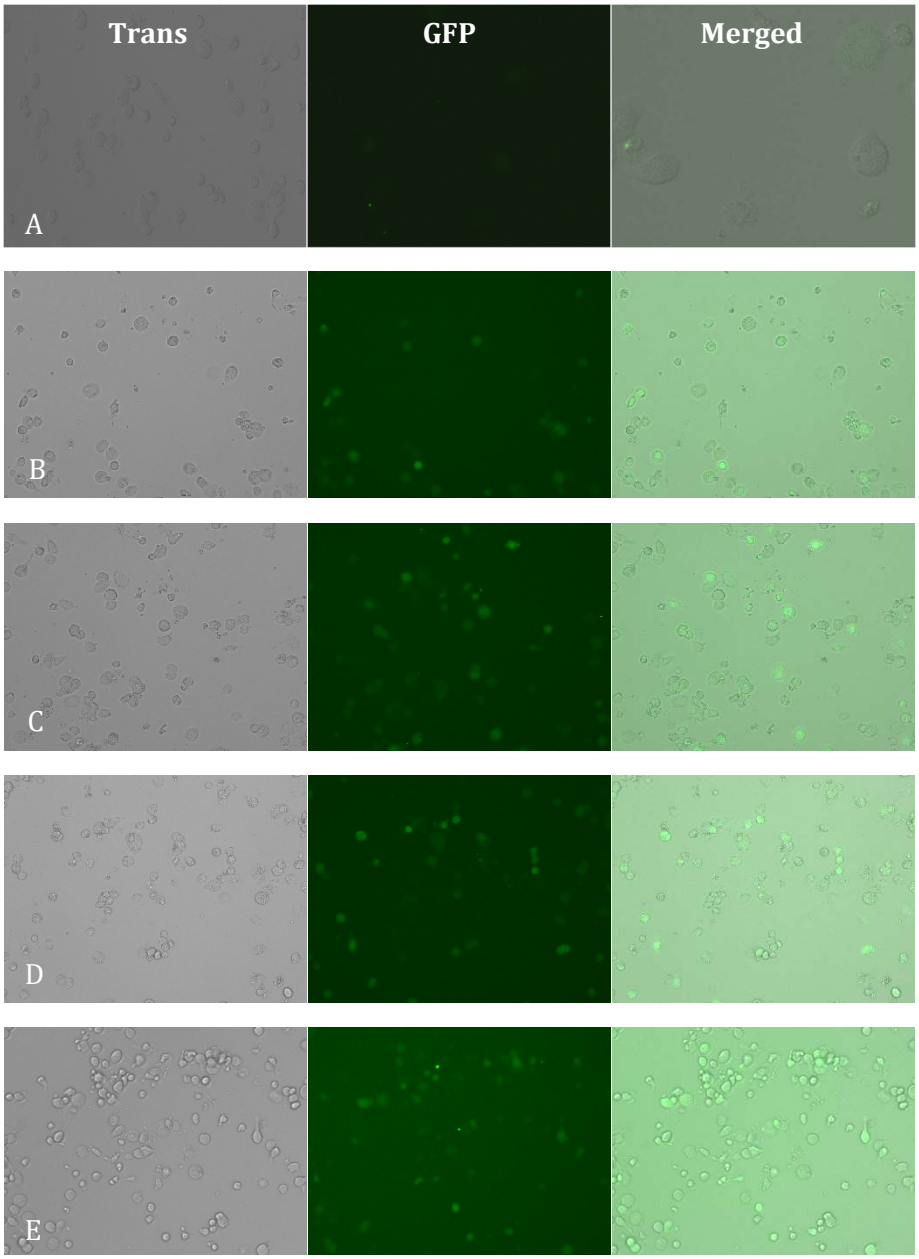

Figure 4. ASC-speck formation in live- cell imaging using fluorescence microscopy for five treated conditions. Top of the figure shows three different channels: transmission, GFP and merged images channel. THP-1 cells are stimulated according to: (A) LPS 4h and nigericin 45min; (B) inhibiting (SB-216763, 10 μM) of GSK-3 in signal I followed by LPS and nigericin activation; (C) inhibiting (DPI, 10 μM) ROS in signal I followed by LPS and nigericin; (D) inhibiting (SB-216763, 10 μM) GSK-3 in signal II meaning post- LPS stimulation; (E) inhibiting (DPI, 10 μM) ROS in signal II, meaning post-LPS stimulation followed by using fluorescence microscopy. Images are representative of 5 separate experiments.

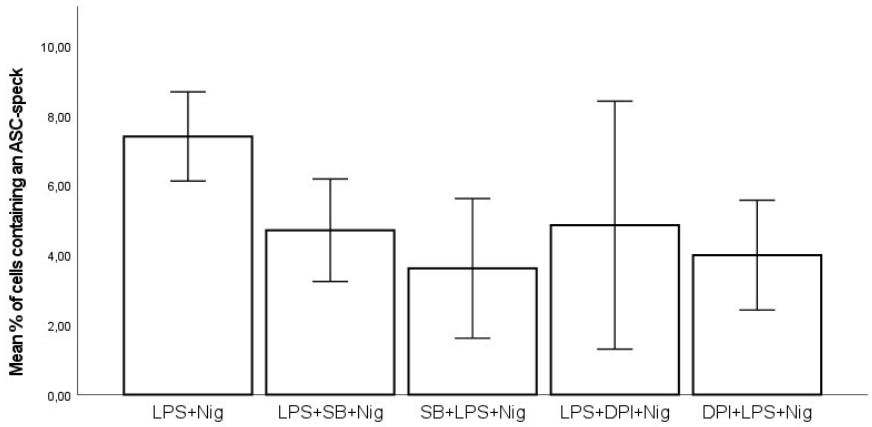

Figure 5. Mean percentage of cells containing an ASC-speck count in THP-1 macrophages for live cell imaging. From left to right, LPS 4h and nigericin 45min; inhibiting (SB-216763, 10 μM) GSK-3 in signal II meaning post- LPS stimulation; inhibiting (DPI, 10 μM) ROS in signal II, meaning post-LPS stimulation LPS and nigericin stimulated cells; inhibiting (SB-216763, 10 μM) of GSK-3 in signal I followed by LPS and nigericin activation; inhibiting (DPI, 10 μM) ROS in signal I followed by LPS and nigericin. The bars represent the mean values ± 1 SD. Statistical significance (n=3 in each group) was determined by One-Way ANOVA (p=0.161).

Average pixel Vimentin intensity of cells with an ASC-speck and cells without an ASC-speck

Upon inducing cellular stressors, vimentin activity is robust. This experiment investigated the average pixel intensity of cells with an ASC speck and cells without an ASC-speck in the same image of DAPI stained and fixated of 4h LPS stimulated and 45min nigericin activated THP-1 cells. Figure 6 displays fluorescence microscopy of LPS and nigericin stimulated cells in four channels: (6A) GFP channels showing an ASC-speck attached to a cell; (6B) DAPI channel showing cell stained with DAPI staining the cell nucleus; (6C) TX red channel showing stained cytoskeletal structure vimentin; and lastly (6D) a merged image of all the channels displaying a single cell with an ASC-speck attached to it. Moreover, Figure 7 displays mean pixel vimentin intensity of cells containing an ASC-speck compared to those without an ASC-speck in the same image of only LPS and nigercin stimulated THP-1 cells. Independent samples t-test was conducted showed no statistical significance in vimentin intensity (t_{62}= 7.27, p=0.917), between cells with speck and without speck.

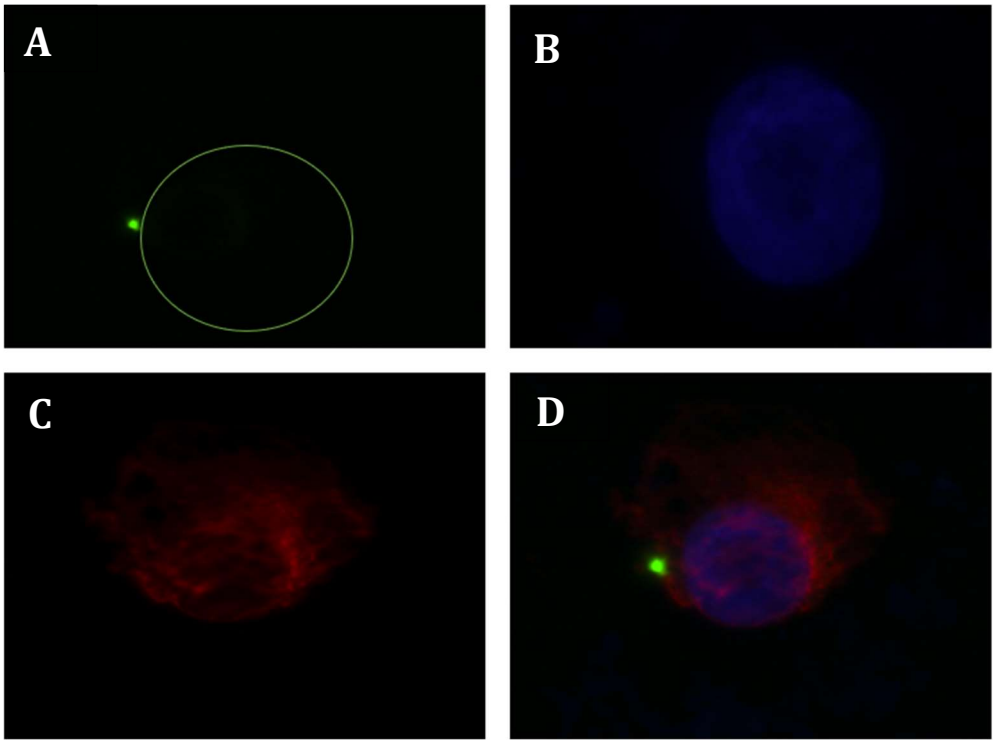

Figure 6. Fluorescence microscopy of THP-1 LPS and Nigericin stimulated cells, in three different channels and a merged imaged. (A) GFP channel shows ASC-speck attached to the cell, marked out, (B) DAPI channel shows cell nucleus, (C) TX red channel shows Vimentin around the cell nucleus and (D) Merged image of all channels.

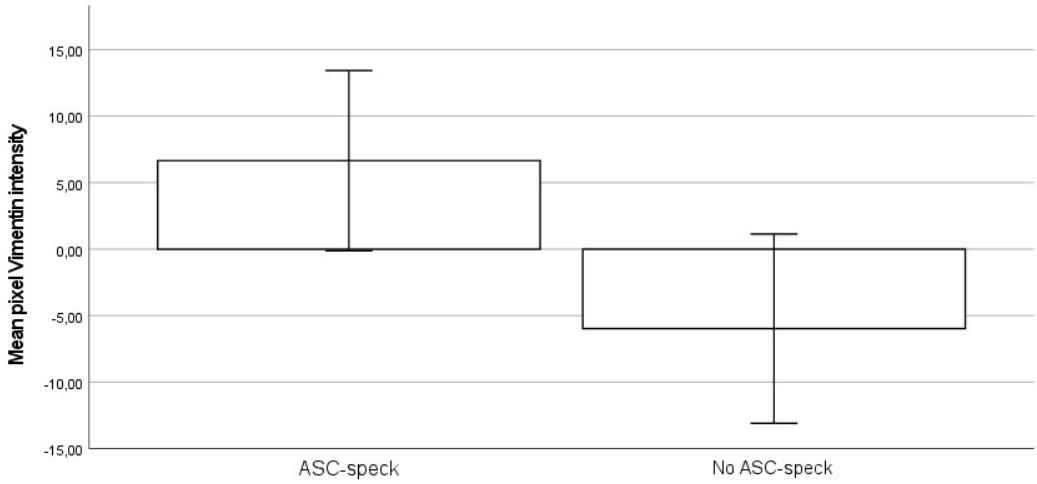

Figure 7. Mean pixel Vimentin intensity measure within the same image of stained THP-1 LPS and Nigericin stimulated macrophages. The bars represent the mean values ± 1 SD. An independent samples t-test was run to determine significant difference of Vimentin expression of cells with an ASC-speck and cells without an ASC-speck. Cells with an ASC-speck (6.65 ± 6.776) and cells without an ASC-speck (-5.97 ± 7.12) was not statistically significantly different (t_{62}= 7.27, p=0.917).

Discussion

Reference gene selection and stability

The choice for an appropriate reference gene for the reliability of qPCR analysis is crucial as they adjust the errors and technical variation introduced during extraction of RNA or/and upon reverse transcribing the RNA to cDNA, therefore, target genes are normalised against reference genes to reflect true significant change in the expression of the target gene (Manoli et al., 2012; Kozera & Rapacaz, 2013).

For this experiment, seven primers from the SYBR Human Endogenous Control Reference Gene Panel (TATAA Biocenter) investigated and evaluated using RefFinder (Figure 1). The requirements for a reference gene are stable expression level when quantified and normalised against samples, and that the reference gene expression cannot be affected by any abiotic or biological stress (Wang et al., 2019). Moreover, selection of a candidate reference gene was based on results of RefFinder, which includes different algorithms that allowed to compare different reference genes to normalise against the gene of interest (Zhou et al., 2018).

Prior to using RefFinder, the SD of raw cq values had to be within the range of 0.5 to ensure random error is minimised, followed by a complied cq expression of the replicates for each of the reference genes (Karlen et al., 2007). Moreover, another requirement for the reference gene is that the fold expression should not exceed >1 between the different samples, this is to further minimise unreliable results due to systemic variation (Schmittgen et al., 2000; Karlen et al., 2007). RefFinder algorithms NormFinder, BestKeeper and the comparative delta- Ct method suggested RPLP the most stable reference gene across all treated samples. However, upon evaluating the raw Cq values among the different treated samples, fluctuating Cq among GSK-3 inhibited cells compared to ROS inhibited cells (1- 3.57-fold changes) could be observed and therefore highly unlikely to be the most stable. Moreover, Cq values of TBP were also fluctuating among the GSK-3 inhibited compared to ROS inhibited samples (1- 2.38-fold changes). Therefore, it would be most appropriate to utilise more than one reference genes to normalise the target gene against.

Based on the m-value (0.475) GeNorm suggested RPLP and TBP the most stable to normalise the gene of interest against (Figure 1). GeNorm utilises the geometric mean of expression (m-value) to determine reference gen stability, in which m-value<0.5 indicates high expression stability (Wang et al., 2019). By setting limits and evaluating each of the reference genes across the different treatments, as well as the different algorithms ensured the choice of RPLP and TBP would yield true expression of vimentin following normalisation of the data.

Inhibition of GSK-3 or ROS modulated Vimentin expression

Within healthy cells, vimentin is stably expressed in low concentration and change is mediate due to cell stressors, such as endoplasmic, mitochondrial, and oxidative stresses. However, upon the rise of cancerogenic events and inflammation, vimentin expression is upregulated. The discovery of potential molecular targets for cancer therapy is a crucial step towards understanding the complex nature of the rising of tumorigenic events and how vital biochemical pathways of the cell adapt to these changes. Recent research found vimentin, important component for cell structure and regulation of cell function, recently been adapted as a biomarker for epithelial mesenchymal transition (EMT) (Chen et al., 2021). Due to the wide distribution of vimentin across an abundance of cells and their cell regulatory function marks it further as an excellent candidate molecular target for cancer therapy.

GSK-3 inhibition modulates Vimentin mRNA expression levels

Vimentin activity is regulated by a complex phosphorylation pattern in combination with an array of kinases that regulates both the function of vimentin as well as its assembly, resulting in specific

sites and protein interactions depending on the state of the cell. (Satelli & Li, 2011). The main signalling pathway which regulates phosphorylation of vimentin are AKT/ PKB (protein B), STK33 (serine/threonine kinase 33) and NF-kB cell signalling pathways which alters the protein-protein interactions of vimentin with other cell regulatory kinases by degrading the vimentin polymers and ultimately diminishing its function (Ivask et al., 2007; Herrmann & Aebi et al., 2016; Tur-Gracia & Martinez., 2021). Regarding inhibition of GSK-3, current research exploring how and to what extent GSK-3 act as a regulator of vimentin activity upon inflammation regarding signalling pathways, migration, and development of the cells, emphasises the relationship between GSK-3 and vimentin and the need to further investigate how this affects inflammasome formation (Nowicki et al., 2019; Duda et al., 2020; Hajka et al., 2021).

This experiment investigated the complex nature and interaction of GSK-3 and vimentin by inhibiting the cells pre- (signal I) or post- (signal II) LPS priming, followed by activation with nigericin, compared to non- inhibited LPS primed and nigericin activated macrophages. Overall, inhibition of GSK-3 modulated the mRNA expression level of vimentin, with upregulation of vimentin in signal I and down regulation in signal II as displayed in Figure 2. As previously mentioned, upon priming, vimentin expression will increase, and the main signalling pathways that are activated interlinking GSK-3 and vimentin are: PI3K-PKB/AKT (phosphoinositide-3-kinase–protein kinase B/Akt) signalling networks which phosphorylate GSK-3 and NF-kB, increasing the GSK-3 activity in signal I and inducing pro-inflammatory cytokines, chemokines, and inflammatory mediators (Dan et al., 2008; Troutman et al., 2012; Guo et al., 2016; Stutz et al., 2017). This is a possible reason to why vimentin expression was downregulated in signal II (Figure 2), as repressing this activity will reduce the activation of GSK-3 and significantly reduce pro-inflammatory cytokines required for NLRP3 formation such as pro-IL-1β and pro-IL 18 (Dos Santos et al., 2015; Zhao et al., 2020). Therefore, this eventually is followed up by downregulation of vimentin since GSK-3 is not able to phosphorylate components required for the NF-kB signalling pathway, to which Vimentin is a major NF-kB target gene (Dan et al., 2008). Interestingly, upregulation of vimentin in signal I (Figure 2), do not align with the proposed PI3K-PKB/AKT pathway, as phosphorylation reduces the expression of vimentin, yet in this experiment vimentin expression is increased.

Current studies suggest the mechanism of GSK-3 ability to phosphorylate an abundance of substrates, requires extensive research to comprehend due to the complex nature of these mechanisms (Yoshino & Ishioka, 2015; Zheng et al., 2020). A paper by Zheng et al. (2020) propose that strong regulatory mechanism of GSK-3 is due to its regulatory substrate specific action, and the explicit signalling of GSK-3 allows the interchange ability of the kinases which phosphorylate GSK-3. However, more ongoing research is needed to investigate these specific signalling pathways and the post- translational modifications regulating GSK-3 (Yoshino & Ishioka, 2015). Pre-phosphorylation of the GSK-3 substrate requires a specific signal that primes the substrate for the activation of kinases, for GSK-3 to recognise the substrate (Zheng et al., 2020). Knock out genes of vimentin or/and GSK-3 to limit external cellular interactions can further proof these interactions. Observations can also be made by the effect of different GSK-3 inhibitors affect vimentin expression level. Moreover, protein level interactions can be observed and evaluated by different immunoassay techniques, including vimentin measurement at protein levels as well as secretion of major pro-inflammatory cytokines IL-1β and IL-18 (Beurel et al., 2015; Wang et al., 2018; Liu et al., 2017; Lang et al., 2018). Secretion of pro-inflammatory cytokines can be utilised to target specific pathways by measuring relevant cytokine distinct to a particular pathway.

ROS inhibition modulates vimentin mRNA expression levels

A major conflict in current research is to what extent does vimentin mediate inflammasome activation. Specifically, the relationship between ROS products, which are crucial for the activation

of the NLRP3 inflammasome, and vimentin which is known to modulate mitochondrial motility was needed to be investigated. In this experiment, upon inhibiting ROS pre- (signal I) or post- (signal II) LPS, followed by activation with nigericin, the vimentin mRNA expression level was upregulated in both inhibition of signal I and signal II, compared to non-inhibited LPS primed cells, with a 60% increase of vimentin expression in signal II compared to signal I (Figure 3).

In this experiment, upon inhibition of ROS after the priming of the cells, mRNA expression levels of vimentin increased significantly (Figure 3). This can be explained by the controversial paper by Bauerfeind et al (2011), in which they suggest that ROS inhibition will block the priming but not the activation of the NLRP3 inflammasome. They conclude that various ROS inhibitors, in particularly DPI, block the priming signals required for recruiting NLRP3 components by observing the caspase-1 activation, IL-8 and IL-1β protein levels upon adding DPI before or after LPS priming followed by Nigericin stimulation. Upon inhibiting the cells before priming, inhibited the upregulation of NLRP3, whilst adding DPI after LPS they observed no change on the caspase-1 activation and the pro-inflammatory cytokines, however the kinases which alters this interaction are not determined. Furthermore, the findings of the paper also suggests that vimentin deficient cells have more robust inflammasome signalling, which is partially fulfilled upon inhibiting ROS in signal II in this experiment, by the 60%-fold increase compared to inhibition at signal I (Figure 3).

The conical approach to inflammasome activation proposed ROS products to activate signalling pathways in signal II by the mitogen-activated protein kinases (MAPK), specifically MAP 3K-MAP 2k-MAPK pathway and NF-kB through the AKT1-IKK-KB pathway (Kovtun et al., 2000; McCubrey et al., 2006; Bulua et al., 2011; Choi et al., 2016; Swanson et al., 2019). Moreover, current studies suggest studies in particular NOX enzymes the source for ROS leading to the activation of the NLRP3, this is proven by the diminishing of the NLRP3 inflammasome upon blocking of NOXs and phagocyte oxidase (Mittal et al., 2014). It has been reported that cell surface expression of vimentin in macrophages is enhanced by PKC phosphorylation and PKC is also an activator of NADPH oxidase (Ryan et al., 2004; Mangat et al., 2006; Park et al., 2006). However, DPI is also an inhibitor of both NADPH oxidase and nitric oxide synthase.

Recent research has described upregulation of vimentin in mRNA level is possible by investigating the inhibition of ROS with ROS scavengers Mitoquinone (Q10), a coenzyme analogue that acts as a ROS scavenger, contains a lipophilic triphenyl phosphonium cation that causes the antioxidant to accumulate several hundredfold within mitochondria because of the high mitochondrial membrane potential, and inhibited varying cell lines pre-LPS priming (Jing et al., 2015; Mahesh et al., 2016; Håversen et al., 2018). Utilising different ROS product inhibitors, among them DPI, they compared the levels of IL-6 and IFN-β in DPI inhibited cell lines, among them THP-1 cells, and observed a significant reduction of the IL-6, concluding if the ROS products are inhibited, cytokine production is reduced in LPS induced cell lines. Therefore, investigating the transcription factors involved in inflammasome activation, due to ROS production upregulation of pro-inflammatory gene expression in various innate immune signalling pathways. As mentioned earlier the exact pathways of this inhibition is unknown and further investigation utilising quantification of pro-inflammatory cytokines, different activators, and knockouts genes for either vimentin or ROS products will lead to proof conclusion. Furthermore, the critical paper by dos Santos et al (2014), which largely demonstrated that vimentin modulates NLRP3 inflammasome activation, and together with previously mentioned findings, demonstrate that ROS is required for signal II of NLRP3 activation, and vimentin expression is upregulated.

Imaging and ASC-speck formation

As previously mentioned, activation of NLRP3 inflammasome upregulates the mRNA vimentin expression and cells containing an ASC-speck will have a robust vimentin expression (Gimena et

al., 2014; Dos Santos et al., 2015; Danielsson et al., 2018; Su et al., 2019). Further investigation of the relation between inhibition of ROS or GSK-3, vimentin and how they correlate to inflammasome formation was investigated by live cell imaging and stained cells ASC speck formation was utilised as readout for inflammasome activation.

Live cell imaging quantified the percentage of cells containing an ASC-speck upon inhibition of ROS and GSK-3 in signal I and II compared to only LPS and nigericin stimulated cells (Figure 4 and 5). Although mRNA levels showed significant difference of vimentin in between the different treatments (Figure 2 and 3), ASC-speck as a read out for inflammasome formation would've correlated, as upregulation of vimentin is followed by increased NLRP3 activity (Zhu et al., 2011; Lamkanfi et al., 2014; Danielsson et al., 2018; Lang et al., 2018; Su et al., 2019). This significant variance was not observed on the live cell imaged the cells. A possible explanation could be the introduction of errors by manually counting the cells, as cells clumped together with an ASC-speck and no clear distinguished cell morphology were excluded. The exclusion of cells might've significantly affected the statistical analyses. To reduce this error, Jensen (2013) suggests the elimination of cells should be conducted by comparing equally treated samples which were exposed to light and compare them with those not exposed to light. Furthermore, cells are also prone to photodamage, and exposing the cells to light from the microscopy excite the ASC-GFP, and upon reacting with oxygen free radicals are produced, which under an extended amount of time affects the morphology of the cells (Tosheva et al., 2019).

Moreover, LPS and nigericin stimulated THP-1 cells were investigated, as LPS alone can induce NLRP3 activation and ASC-speck are formed, to further explore vimentin and NLRP3 interactions upon stimulation. As shown in Figure 7, no statistical significance of the average vimentin pixel intensity is observed in cell with a speck and cells without a speck in the same image. As previously mentioned, upregulation of vimentin will further increase the NLRP3 activity hence a robust vimentin intensity (Dos Santo et al., 2015; Pan et al., 2021; Lois-Bermejo et al., 2022). The findings of experiment don't correlate to the other mentioned findings, which again could be an extension of the determining conditions of cell counting and morphology, as excluding cells alters the results and its significance (Jensen et al., 2013; Cadena-Herrera et al., 2015).

Conclusion

In conclusion, the results obtained in this project showed that vimentin mRNA expression level can be modulated upon inhibiting GSK-3 or ROS in either signal I and signal II in LPS and Nigericin stimulated THP-1 macrophages. Inhibition of GSK-3 in signal I upregulated vimentin, whilst upon inhibition in signal II downregulated vimentin expression as PI3K-PKB/AKT, phosphorylation component of both GSK-3 and NF-kB, is required to recruit pro-inflammatory mediators signalling as stressors within the cell and vimentin expression is therefore elevated in the cell. Inhibition of GSK-3 in signal II repressed this activity, resulting in the down regulation of vimentin. However, upregulation of vimentin in signal II is contradicts the suggested activity of vimentin upon inflammation, and alternative pathways may activate NF-kB pathway which vimentin is a main target gene for, resulting in higher vimentin expression although the GSK-3 activity is repressed. Further studies are required to understand the scope of the complex pathways involved in GSK-3 and vimentin activity upon inflammation. Moreover, inhibition of ROS in either signal I or II upregulated vimentin expression, upregulation of vimentin in signal I upon ROS inhibition is further proved to only block the priming but not the activation of NLRP3 inflammasome, as pro-inflammatory mediators are still released within the cells further elevating vimentin levels. This highlights the importance of understanding ROS and vimentin activity upon inflammation and which stages of inflammation are crucial to target for development of therapeutical methods and inhibitory drugs for various inflammatory diseases. Lastly, the findings of this project may provide further insights into the scope of cellular interaction of vimentin upon inflammation and direct research towards understanding target signalling pathways involved and inflammatory mediators released to further utilizing the extensive presence of vimentin across an abundance of cell for developing therapeutical strategies and future drug development for varying inflammatory diseases, overall improving public health.

Ethical considerations and impacts on society

A crucial ethical aspect to investigate in regards of this project is the utilisation of THP-1 cells lines. Within the field of molecular medicine, cell culture is crucial for investigation of cellular signalling and mechanisms and follows manipulation of these cell lines to mimic the behaviour of true animal cells. The THP-1 cells used stem from immortalised cell line of an individual with peripheral blood acute monocyte leukaemia, and these cells have since been modified and transformed into varying cell lines, due to the structural nature THP-1 exhibit to primary monocytes and macrophages allowing to experimentally explore immune-related diseases without further ethical issues. In this project, THP-1 cells were utilised as the have been proven to be a sufficient and cost-effective invitro model to study modulation of differentiated macrophages when exposed to cellular stressors. Additionally, no ethical considerations are required for the use of the commercially available cell lines (Unger et al., 2008). Therefore, no further experimental ethical consideration was made, as the cells have been extensively propagated and were prior to the experiment precultured. Regarding the environmental impact of this project the hazardous extent of kits and chemicals used in the project were assessed using KLARA, which further aided in the correct handling and disposal of chemicals used. Moreover, it is important to discuss the impact of the extensive use of THP-1 cells and their effect on progresses made with in immune-related diseases. This project aimed to enlighten and further validate the relation of vimentin during inflammatory events upon inhibiting GSK-3 or ROS to partake into larger in vivo research and towards decreasing the gap within this area of research and possibly gives rise to novo inhibitory drug development targeting varying inflammation related diseases by targeting these molecules, will lead to improvement and increase the life expectancy of individuals with these diseases, decreasing the hospitalisation of patients and allowing more resources towards improving the health care system.

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