Mutations E688K and G569R within the *NALP3* gene, associated with development of hereditary autoinflammatory disorders

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Abstract

The NALP3 protein plays an important role in activation of inflammasomes upon stimulation by different ligands. Different mutations in the NALP3 gene are believed to be associated with several inherited auto-inflammatory diseases such as familial cold inflammatory syndrome (FCAS), neonatal onset multisystem inflammatory disorder (NOMID) and muckle-wells syndrome (MWS). In order to express and eventually study the function of the two novel mutations G569R and E688K, they were supposed to be created in the cloned wild type NALP3 gene, sequenced and later expressed in a bacterial host for functional studies. Sequencing results was only obtained for mutation G569R that was re-cloned and expressed in bacterial cells. The expression of this recombinant target protein could not be verified by SDS-PAGE analyses, due to some cellular or mutational problem. Further work on this item should focus on to achieve a verified expression of the desired mutations in both bacterial and mammalian cells. Upon obtaining positive expression results, the recombinant mutants as well as wild type NALP3 proteins should be appointed to studies of function in bacterial and mammalian protein-protein interaction models.
## Abbreviations

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>ASC</td>
<td>Apoptosis-associated speck-like-protein</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BIR</td>
<td>Baculovirus inhibitor of apoptosis protein repeats</td>
</tr>
<tr>
<td>CARD</td>
<td>Caspase associated recruitment domains</td>
</tr>
<tr>
<td>CATERPILLER</td>
<td>caspase associated recruitment domains (CARD), transcription enhancer, R (purine)-binding, pyrin, lots of leucine repeats</td>
</tr>
<tr>
<td>OD value</td>
<td>Optical Density (measured experimentally to estimate the quantity of an oligonucleotide)</td>
</tr>
<tr>
<td>CIITA</td>
<td>MHC class II transcription activator</td>
</tr>
<tr>
<td>HET-E</td>
<td>Incompatibility locus protein from <em>Podospora anserina</em></td>
</tr>
<tr>
<td>FCAS</td>
<td>Familial cold inflammatory syndrome</td>
</tr>
<tr>
<td>FCU</td>
<td>Familial cold urticaria</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-β-D-thio-galactopyranoside</td>
</tr>
<tr>
<td>MWS</td>
<td>Muckle-wells syndrome</td>
</tr>
<tr>
<td>NACHT</td>
<td>NAIP, CIITA, HET-E and TP-1</td>
</tr>
<tr>
<td>NAIP</td>
<td>Neuronal apoptosis inhibitory protein</td>
</tr>
<tr>
<td>NALP3</td>
<td>Nacht domain, leucine-rich repeat-, and PYD-containing Protein 3</td>
</tr>
<tr>
<td>NLR</td>
<td>protein NOD-like receptor</td>
</tr>
<tr>
<td>NBD</td>
<td>Nucleotide-binding domain</td>
</tr>
<tr>
<td>NOD</td>
<td>Nucleotide-binding oligomerization domain</td>
</tr>
<tr>
<td>NOMID</td>
<td>Neonatal onset multisystem inflammatory disorder</td>
</tr>
<tr>
<td>LPS</td>
<td>Lippolysaccharide</td>
</tr>
<tr>
<td>LRR</td>
<td>Leucine-rich region</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TP1</td>
<td>Telomerase-associated protein</td>
</tr>
</tbody>
</table>
1 Introduction

The *NALP3/cryopyrin* gene, localized to chromosome 1q44, is member of a mammalian gene family known as CATERPILLER (caspase associated recruitment domains (CARD), transcription enhancer, R (purine)-binding, pyrin, lots of leucine repeats) (Duncan et al., 2007). This gene family has three structural domains; an intermediary NACHT (NAIP, CIITA, HET-E and TP-1) domain also known as conserved nucleotide-binding domain (NBD), a leucine-rich region (LRR) and an effector domain that can be a pyrin domain (PYD), baculovirus inhibitor of apoptosis protein repeat (BIR) domain or a caspase recruitment domain (CARD) (Benitez et al., 2008). The *NALP3* protein is implicated in the processing of interleukin-1β (IL-1β) by consolidating the multimeric complex of the inflammasome and it has similarities in structure with a subfamily of plant proteins that are responsible for disease resistance (Duncan et al., 2007). The actual protein also function as an ATPase, which catalyzes the conversion of adenosine triphosphate (ATP) to adenosine diphosphate (ADP), it also binds ATP/deoxyATP, which is important for its promotion of inflammatory signalling (Mariathasan and Monack, 2007).

The *NALP3* protein is a component of inflammasomes, which are cytoplasmic multiprotein complexes important in inflammatory reactions in response to infections. They activate different cytokines that play an important role in activation and recruitment of leukocytes to sites of infection. Appropriate production of these cytokines is beneficial for an efficient immune response. On the other hand, inappropriate production of especially interleukin-1β due to mutations in for example the *NALP3* gene is linked to cryopyrin-adherent periodic syndromes (CAPS) (Stehlik and Dorfleutner, 2007). The central effector protein of the inflammasome is caspase-1. This effector protein is activated when the inflammasome acts as a molecular scaffold for activation of the caspase. There are several types of inflammasomes that have been revealed recent years such as the *NALP1, NALP3* and *IPAF* inflammasomes identified and defined by the NLR protein that they compose (Fink et al., 2008).

It is generally believed that the activation of inflammasomes and thereby release of interleukin-1β requires a combinations of two signals, alternatively that it is enough with only one signal and that the other might establish a fail-safe mechanism, which secures that the activation of a strong inflammatory response only happens in presence of tissue injury and/or invasive pathogens (Mariathasan and Monack, 2007). The initiating stage in the pathway for activation of the first signal is followed after stimulation of one of the Toll-like receptors (TLR) by ligands such as lipopolysaccharide (LPS), lipopeptides Pam3CSK4, cytidine-phosphate-guanosine (CPG) DNA motifs, dsRNA, peptidoclycan, flagellin and imidazoquinolines. Next stage in the first signal activation is followed by stimulation of NF-κβ by the signal transduction pathway triggered by the ligand-TLR binding. Activation of NF-κβ, in turn activates the inflammasome (Akira et al, 2003). The second signal is a trigger induced by different types of agents such as maitotoxin (a marine toxin that comes from dinoflagellates), nigericin (a potassium ionophore) and pannexin-1(a membrane protein in mammalian cells). These agents can trigger
intracellular events such as membrane perturbation and potassium efflux leading to an activation of caspase-1 and interleukin-1β (IL-1β) secretion (Pelegrin and Surprenant, 2007). ATP is another trigger that can activate the inflammasome by activating the plasma membrane P2X receptor. When activated, this receptor is able to trigger potassium efflux by ionic perturbations and thereby activating the inflammasome (Brough et al, 2009).

It is specifically believed that the NALP3 inflammasome is activated by different ligands such as various pathogens (Listeria monocytogenes, Aeromonas hydrophilia and Staphylococcus aureus), gout crystals, bacterial RNA and toxins via the Toll-like receptor (TLR) as shown in Figure 1. These ligands are able to trigger a conformational change in the intracellular inactive NALP3 protein by an unknown mechanism. This leads to an exposure of the NACHT domain, which promotes an activation of the inactive NALP3 protein. Upon activation of the NALP3 protein, NALP3 oligomerizes. After oligomerization, the protein interacts via pyrin domains (PDSs) and apoptosis-associated speck-like-protein (ASC) (Mariathasan and Monack, 2007). The ASC contains also a caspase-recruitment domain (CARD) which interacts by homophilic interactions with the CARD domain of the pro-caspase-1 (Yamamoto et al, 2004). This interaction is believed to induce conformational alternations within pro-caspase-1 that leads to a proteolytic cleavage and activation of the caspase. Upon activation of caspase-1 pro-interleukin-1β (IL-1β) and pro-interleukin-18 (IL-1β) can be cleaved proteolytically to their bioactive forms interleukin-1β (IL-1β) and interleukin-18 (IL-18) and cause macrophage cell death (Mariathasan and Monack, 2007).

Figure 1. Shows how different ligands via the Toll-like receptor (TLR) such as various pathogens (Listeria monocytogenes, Aeromonas hydrophilia and Staphylococcus aureus), gout crystals, bacterial RNA and toxins promote an activation of inactive NALP3 protein. After oligomerization, the activated NALP3 protein interacts via pyrin domains (PYDs) and apoptosis-associated speck-like-protein (ASC). Conformational alternations within pro-caspase-1 lead to a proteolytic cleavage and activation of the caspase. The activated caspase-1 trigger proteolytical cleavage of pro-interleukin-1β (IL-1β) and pro-interleukin-18 (IL-18) to their bioactive forms interleukin-1β (IL-1β)
and interleukin-18 (IL-18) and cause macrophage cell death (adapted from Mariathasan and Monack, 2007).

Mutations in the NALP3 gene are thought to have strong genetic link with human diseases and most of the known mutations that are related to these diseases resides in the nucleotide-binding domain (NBD) (Zhengmao et al., 2008). It is believed that the mutations within the exon 3 reduces self-association, macromolecular complex formation, caspase-1 activation, cell-death, interleukin-1β (IL-1β) production, association with the inflammasome component ASC and ATP binding. The mutated variant of the gene is especially associated with inherited autosomal dominant diseases such as neonatal onset multisystem inflammatory disorder (NOMID), familial cold urticaria (FCU) and muckle-wells syndrome (MWS) (Duncan et al., 2007). The uncommon autosomal dominant disease muckle-wells syndrome (MWS) is characterized by periodic arthritis, general signs of inflammation, recurrent urticaria, sensorineural deafness and secondary amyloidosis (AA type). Other individual symptoms are painful joints, episodic fever and chills. The disease might also cause metabolism anomalies, nephritic syndrome and diverse extents of renal disorder (Grateau, 2005). Familial cold urticaria (FCU), also called the familial cold inflammatory syndrome (FCAS), is also an inherited inflammatory disease but very rarely occurring (at a rate less than 1:1,000,000) and mainly observed in families from Europe and North America. It is characterized by frequent repeated periods of fever/chills, fatigue, joint pain, and rash following exposure to cold. The disease debuts primarily among infants (< six months) and last throughout life. The life expectancy among afflicted patients is mainly normal (Hoffman, 2005). The neonatal onset multisystem inflammatory disorder (NOMID) is a rare congenital hereditary disorder characterized by urticarial eruptions that are presents at birth, bony overgrowth, sensory neural hearing loss, chronic aseptic meningitis and chronic papilledema due to an increased intracranial pressure. The NOMID is extremely rare occurring disease and the rate of mortality before adulthood is about 20% (Goldbach-Mansky et al., 2006).

The novel mutation G569R is thought to be genetically, associated with development of muckle-wells syndrome (MWS). It is also believed that this gene play a role as a modifier gene in determining the phenotype of the MWS. There are some indications that the actual gene is inherited with a low penetrance, that is according to an analysis study of pedigree of twelve families with MSW and FCU. This analysis study revealed that an asymptomatic individual (female) carrying a dominant predisposition MWS mutation (G569R) not necessary have to bee affected by the disease, but the risk to get an affected offspring is still as high as for other mutations associated with MWS (Dodé et al., 2002).

The mutation E688K is believed to be associated with the neonatal onset multisystem inflammatory disorder (NOMID). The actual mutation is specifically associated with perinatal events among individuals suffering from NOMID such as neonatal extended icterus that requires more than 24h of phototherapy, fetal distress and small for pregnant age with neonatal birth weight two kilogram at 40 weeks (Caroli et al., 2006).
The aim of this project was to create the mutations G569R and E688K within the wild type NALP3 gene and to study their expression and function compared to wild type. Upon construction of these mutant NALP3 genes, they were mutated, sequenced, re-cloned and expressed in bacterial cells (E.coli). Finally, the expression of the actual mutant NALP3 proteins was checked using SDS-PAGE to verify whether the recombinant plasmids, have been successfully transformed into bacteria cells (E.coli). The actual mutant NALP3 proteins were further supposed to be used in protein-protein interaction studies, to study their function in mammalian and bacterial cells.

2 Materials and methods

2.1 Site-Directed Mutagenesis

The specific primers for mutagenesis reaction were designed by using a design QuickChange® Primer Design Program (Stratagene, 2008). The primer sequences G/C content, melting (Tm) and annealing (TA) temperatures for each mutagenic primer used are shown in the Table 1.

Table 1. Mutagenic primers

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence</th>
<th>Tm (°C)</th>
<th>TA (°C)</th>
<th>G/C content</th>
</tr>
</thead>
<tbody>
<tr>
<td>E688K_sense</td>
<td>5’ CCATAACATGCCCAGGAGAGAGGAGGA 3’</td>
<td>70</td>
<td>65</td>
<td>51.5</td>
</tr>
<tr>
<td></td>
<td>3’</td>
<td>47</td>
<td>47</td>
<td>1</td>
</tr>
<tr>
<td>E688K_antisense</td>
<td>5’ TCCTCTCCCTCTGCTGCTTGGTGGGCTGTTATGG 3’</td>
<td>70</td>
<td>65</td>
<td>51.5</td>
</tr>
<tr>
<td></td>
<td>3’</td>
<td>47</td>
<td>47</td>
<td>1</td>
</tr>
<tr>
<td>G569R_sense</td>
<td>5’ GAAAACTATGGGAAATTCAAGGATTTTATGGTTGTTGTAAGTCTTTTTCGAATT 3’</td>
<td>75</td>
<td>70</td>
<td>31.2</td>
</tr>
<tr>
<td></td>
<td>3’</td>
<td>65</td>
<td>65</td>
<td>5</td>
</tr>
<tr>
<td>G569R_antisense</td>
<td>5’ ACGTACAAACAAATCAGAATACCTCTTTTCTCTTCTGGGTTTTC 3’</td>
<td>75</td>
<td>70</td>
<td>31.2</td>
</tr>
<tr>
<td></td>
<td>3’</td>
<td>65</td>
<td>65</td>
<td>5</td>
</tr>
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</table>

The mutagenesis synthesis reactions were performed according to the QuickChange® II Site-Directed Mutagenesis Kit (Stratagene, 2004) where 25 ng/µl of wild type NALP3 gene was used as template (Appendix A). The PCR reaction was run for 16 cycles according to Appendix B. After Thermal Cycling 10 U of Dpn I restriction enzyme (10U/µl) was added to each amplification reaction. One micro liter of the Dpn I - treated DNA from each sample reaction was transferred to a 50 µl aliquot of the XL1-Blue supercompetent cells (E. coli). After adding 0.5 ml of LB-medium to each transformation reaction, they were incubated for one hour at 37 °C with shaking at 225-250 rpm. Cells were then spread on LB-ampicillin (the concentration of the ampicillin used was (100 µg/µl) agar plates. The plated transformation reactions were then incubated overnight at 37 °C for > 16 hours.
After overnight incubation, single colony was picked and used for plasmid preparation according to the QIAprep® Miniprep Handbook (Qiagen, 2006). The obtained plasmid DNA and its concentration were measured using the ND-1000 Spectrophotometer from NanoDrop Technologies, Inc, Germany.

2.2 Sequencing

The obtained plasmid DNA samples (E688K and G569R) were subjected to PCR sequencing using BigDye Terminator cycle sequencing kit (Applied Biosystems, 2002). The concentration of the sequencing primers were 1µg/µl and the nucleotide sequence for the left primer 1816 used for sample E688K was 5’ CTGGAGCTGCTGAAATGGAT 3’ and the nucleotide sequence for right primer 1818 used for sample G569R was 5’ CAGCCTGATTTGCTGAGAGA 3’. The melting and annealing temperatures (Tm and Ta) for primers 1816 and 1818 were Tm =60.36 ºC, Ta = 50.00 ºC, Tm=59.27 ºC and Ta=50.00 ºC., respectively. PCR sequencing reaction (Appendix C) was run for 30 cycles and its running conditions were set according to Appendix D. Purification of the extended PCR plasmid DNA sample products were performed by using DyeEx 2.0 Spin Kit (Qiagen, 2002). After purification of the extended PCR plasmid DNA sample products, they were analyzed on an ABI Prism 310 Genetic Analyzer machine from Applied Biosystems, USA.

2.3 Re-cloning

The DNA sequencing sample G569R was amplified for re-cloning using standard PCR procedure according to the protocol provided by FastStart High Fidelity PCR system (Roch Applied Science, 2008). The concentrations of the downstream primer 1179 with sequence 5’ GGCAGCCTTCAGTCTGATTC 3’ and the upstream primer 1818 with sequence 5’ CAGCCTGATTTGCTGAGAGA 3’ were 0.4 µM, the PCR amplification was carried out for 36 cycles, and its profile was set as shown in Appendix E. To remove dNTPs completely from the amplified PCR reaction it was purified using the QIAprep Spin Miniprep Kit (Qiagen, 2006). All further re-cloning steps were performed according to the protocols in the EK/LIC Cloning Kits (Novagen, 2008). Upon PCR amplification, the amplified sample G569R product was subjected to agarose gel electrophoresis to confirm a positive amplification. Then a purification of the PCR sample followed and its concentration was measured by using the ND-1000 Spectrophotometer from NanoDrop Technologies, Inc, Germany. After nanodrop measurement of it was subjected to T4 DNA Polymerase treatment to generate compatible overhangs to the plasmid DNA sample. The concentration of the T4 DNA Polymerase used was 0.2 U/µl instead of 2.5 U/µl, however the amount of enzyme was the same and the concentration of the PCR product used was 0.2 pmol. Then the T4 DNA polymerase treated PCR product was annealed to the pTriEx-6 3C/LIC vector (Appendix F). Upon annealing of insert into the 3C/LIC vector one micro liter of the annealing reaction was used in the transformation reaction for initial cloning into 50µl NovaBlue GigaSingles™ Competent Cells.
according to manufacturers instructions. To select transformants, the cells were
spread on a LB-medium plate containing ampicillin (100 µg/µl) and incubated
over night at 37 ºC. Colonies were picked and screened for correct insert by
colony PCR. The sequence of the upstream primer (vector-specific) pTriExFP
5’ CTGGTTATTGTGCTGTCTCATCA 3’. The sequence of the downstream
157 primer (insert specific) was 5’ CCACATGGTCTGCCTTCTCT 3’. After
PCR master reaction mix setup, in which the NovaTaq DNA polymerase was
included with a concentration of 1.25 U a colony PCR with sample G569R was
run for 35 cycles and the PCR setting conditions are as shown in Appendix G.
The obtained PCR product was then analyzed on a 1% agarose gel. The sample
G569R bands obtained from agarose gel electrophoresis were assumed to be
correct size which might confirm that the colonies contain the correct inserts.

2.4 Protein Expression in E.coli

The isolation and transformation of plasmid DNA to and induction in the
expression host was carried out according to pTriEX™ System Manual
(Novagen, 2006). A colony was picked for pre-culturing from a transformation
plate, and placed in a culture tube containing 3 ml of LB-medium containing
(100 µg/µl) ampicillin and 3 % of glucose, for incubation overnight at 37 ºC
and shaking at 250 rpm. After overnight shaking the OD600nm value was
measured to 0.86. The pre culture was then transferred to a flask with 50 mL
LB-medium containing the same ingredients as described above and all of the
pre culture was added. After shaking the prepared pre culture at 250 rpm for
two hours in a heat cabinet at 37 ºC, the protein induction was performed as
described below. The OD value was measured for each sample and collected at
zero hours, after one hour and after two hours. When the first sample was
collected at zero hour and its OD value was measured, 500 µl of Isopropyl-β-
D-thio-galactopyranoside (IPTG (100mM)) was added to the pre-culture
mixture to start the protein induction process.

To verify the expression of recombinant NALP3 protein the sodium dodecyl
sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used. The
recombinant NovaBlue competent cells were first harvested and the desired
protein (recombinant NALP3 protein) was isolated by using Complete Lysis-B
(2X) Manual (Roche Applied Sciences, 2006). Then the glass cassette, casting
stand and the electrophoresis module were assembled and the SDS-PAGE was
run according to Mini-PROTEAN® 3 Cell Instruction Manual (Bio-Rad
Laboratories, Inc, 2006). The recipes followed for SDS-PAGE preparation and
running are shown in Appendix H. The molecular weight standard (MW
Standard) used in the sample setup is from Kaleidoscope Prestained Standards
(Bio-Rad Laboratories, 2000) (Appendix I). After obtaining the SDS-PAGE
gel with the actual samples separated, the staining with coomassie blue was
performed to make the MW standard and sample bands more visible, the dye
used for staining was Coomassie Brilliant Blue R-250 (Sigma, B-O149).

3. Results
The sequencing sequence result of the *NALP3* gene containing the G569R mutation can be seen in Appendix J. Figure 2 below shows a protein database search results using blastx (NCBI) of a translated nucleotide query of the *NALP3* gene sequence containing the G569R mutation at position 569. The blastx alignment results confirm that the creation of mutation G569R within the *NALP3* wild type gene was successful.

![BLASTX Alignment Results](image)

Figure 2. The blast alignment results above show that the creation of mutation G569R within the *NALP3* wild type gene has been successful. The amino acid glycine (G) underlined and yellow coloured has been changed to amino acid (R) underlined and blue coloured.

The PCR amplification of the “G2b1” sample corresponding to (G569R) was analyzed with gel electrophoresis to confirm the accuracy of the G2b1 DNA sample amplification as shown in Figure 3. The accuracy of the amplification is confirmed by comparing the band length of the actual sample, which should be 3kb and are supposed to be in line with the 3kb band length on the 2 kb ladder, which is shown in the Figure 3.
Figure 3. The first lane from left of the gel image represents the 2kb ladder and lane two represents the PCR amplified G2b1 sample.
Figure 4 represents a gel electrophoresis image of a PCR colony that was performed for recombinant protein verification. Lane 1 (L1) represents the 2 kb ladder used and lane 3 (L3), 4 (L4) and 5 (L5) shows the PCR amplified sample products, g2b1a, b and c composing the G569R mutation.

<table>
<thead>
<tr>
<th>Sample (mL)</th>
<th>Time (h)</th>
<th>ODI-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5</td>
<td>0</td>
<td>0.58</td>
</tr>
<tr>
<td>1.5</td>
<td>1</td>
<td>1.08</td>
</tr>
<tr>
<td>1.5</td>
<td>2</td>
<td>1.54</td>
</tr>
</tbody>
</table>
Figure 5 shows the resulting SDS-PAGE image of the G569R samples, which was run for SDS-PAGE.

![Figure 5. Represent a SDS-PAGE electrophoresis gel image. In the middle of the gel picture, the molecular weight standard ladder (MW) and its different band lengths are presented. To the left of the molecular weight standard ladder, the soluble protein G5569R samples zero, one and two hours (SP 0, 1 and 2) and their band lengths are shown. To the right of the MW, the insoluble protein G569R samples zero, one and two hours (IP O, 1 and 2) and their band lengths are shown. The MW magenta coloured band position on the gel represents the molecular weight of 112, 231 Daltons for protein beta-galactosidase. The MW blue coloured band on the gel represents the molecular weight of 6, 668 Daltons for protein Aprotinin.](image)

4. Discussion

It is believed that a single or a combination of different mutations might play a more or less fundamental role for development of hereditary autoimmune diseases. The mechanisms of function of these mutations are mostly unknown, although researches have expressed many of these mutations in different experiments (Church et al., 2008). The two novel mutations E688K and G569R that were studied in this thesis work, have showed an altered function that might play some role in development of the diseases chronic infantile neurological cutaneous and articular (CINCA) and familial cold-induced autoinflammatory syndrome (FACS) respectively (Caroli et al., 2006; Dodé et al., 2002).

The mutation E688K is of missense type and located in the exon three of the NALP3 gene. It has an alternation by substitution of base guanine (G) to alanine (A) at the DNA sequencing position 2062, located at the first site of the codon that code for the amino acid sequence position 688. This substitution of G to C causes a codon change from GAG coding for amino acid glutamic acid
to AAG (GAG→AAG), which codes for amino acid lysine. Mutation G569R on the other hand is of missense type located in the exon three, which encodes the nucleotide-binding domain (NBD) of the NALP3 gene. It has a base substitution where the base guanine (G) has altered to cytosine (C) at the DNA sequencing position 1705, located at the first site of the codon that code for the amino acid sequence position 569. This alternation of G to C causes a codon change from GGG coding for amino acid glycine to CGG (GGG→CGG), which codes for amino acid arginine (Touitou, 2009).

The first phase of this work started with a construction of the mutations E688K and G569R on a wild type NALP3 gene template by mutagenesis followed by sequencing. Only one of the mutations was created, namely G569R mutation and it was aligned by blast software (NCBI, 2009) based on a final sequencing of sample G569R mutated DNA (Appendix K). The alignment that was performed showed a good result with a 98 % homology, no gaps and the expected point mutation at amino acid sequence position 569, which was altered from glycine to arginine (Figure 2). The other mutation E688K was not successfully created. Reasons for that can be many but some troubleshooting was performed to obtain a successful insert even for this mutation. One possible reason might be the transformation part in which no single colonies could be obtained at the first attempts. When four micro liters of Dpn I digested, PCR product of E688K sample was used instead of one micro liter, a few single colonies were obtained on the agar plate indicating a poor amplification, possibly because of ineffective mutagenesis primers. Some of the colonies were later used for further experiment and might have been of low quality due to bad transformation level in the XL1-Blue super competent cells (E. coli) or maybe to insufficient DNA template was used due to a pipetting error in this specific case. To exclude this possibility the amount of DNA in the sequencing reactions were increased up to four-fold the normal amount used. Even another bacterial strain was used “XL Gold Super Competent Cells” to achieve better plasmid DNA concentration and purity. It was also believed that the amount of the primer was insufficient; therefore the amount of the primer was increased. Finally the most likely reasons for bad sequencing results were contamination errors such as proteins, RNA, residual salts and detergents during the plasmid DNA purification and later preparation of DNA templates before PCR cycle sequencing. To exclude these eventualities the purification step that was performed before the preparation of PCR cycling sequencing, two different kits were used DyeEx 2.0 Spin Kit (Qiagen, 2002) and Mini-M™ Plasmid DNA Extraction System (Viogene, 2009). During the preparation for PCR sequencing the DNA template was in one case purified using “DyeEX spin kit” and in the other case using “Isopropanol precipitation” which has good salting out properties. Despite of the actions mentioned above the sequencing signal for sample E688K obtained was to low and yielded a lot of undefined bases “N” to produce a positive sequencing result.

Upon obtaining a positive sequencing result for the G569R mutant the work could proceed for re-cloning. The mutated NALP3 Gene containing the mutation G569R was subjected for PCR amplification and then analyzed on an agarose electrophoresis (Figure 3) to verify a positive clone production. Lane two shows a band at approximately 3kb which proves that the DNA product
has been amplified and it also corresponds to the right size of the NALP3 gene. It was further assumed that the T4 DNA Polymerase treated Ek/LIC insert had been successfully annealed into the pTriEx-6 3C/LIC vector after a re-cloning colony was screened by colony PCR and analyzed on a 1 % agarose electrophoresis gel (Figure 4). The samples in lane 3, 4 and 5 shows bands at around 400 kb which approximately corresponds to the expected base pair size between the pTriEx upstream and the 157 downstream primers that was annealed to the sequence on exon 2 containing the “HRV 3C/LIC Cloning Site” of the pTriEX™ vector. The chosen colony for the G569R sample that was were, further used for protein expression within a bacteria E. coli host and analyzed on a SDS-PAGE gel to verify the mutant NALP3 gene expression (Figure 5). A similar experiment especially concerning the mutagenesis part (where a nucleotide binding was demonstrated), was performed by Duncan et al, 2007 on disease-associated mutant cryopyrin that was expressed in Hi5 insect cells infected by baculovirus and later analysed by immunoblotting with anti-FLAG antibody. The mentioned experiment done by Duncan and colleagues showed that disease-associated mutant cryopyrin is ATP-dependent and are thought to play an important role for association with ASC and thereby controlling the inflammasome assembly.

The sample preparation before SDS-PAGE gel casting with the optical density (OD) value measurement for each sample is shown in Table 2. The SDS-PAGE gel results shows no clear expression bands at all for the mutant NALP3 gene within the insoluble protein G569R samples zero, one and two hours (ISP O, 1 and 2) where they are most likely supposed to be present and up-regulated. The reason for assuming this is that inclusion bodies contain misfolded proteins (mostly insoluble proteins) as a result of expression of recombinant proteins in bacteria. The expected bands for the actual mutant NALP3 protein, which has a molecular weight (MW) of approximately 114 kD, should have been in line with the magenta coloured band on the MW ladder. The magenta coloured band corresponds to the beta-galactosidase protein on the MW ladder, which has a molecular weight of around 112 kD. In Figure 5, there is no clear separation of the bands on the MW ladder and neither for the insoluble or soluble protein bands. The magenta coloured band position is not distinguishable and the insoluble protein samples were badly separated the bands are too unclear, so it was not enough verification to conclude that the desired mutated NALP3 protein was expressed. To confirm the negative results from the protein expression part the SDS-PAGE gel electrophoresis should have been run once more and for a longer time to achieve a better and clearer band separation and make it easier to ascertain the obtained results. Whether the SDS-PAGE gel electrophoresis gel shows a negative or positive result a double verification of obtained results were planned to be performed but could not be done because of time limit.
5. Conclusion

The expected results for each sample G569R and E688K are the same, namely to construct the mutations within the wild type \textit{NALP3} gene and then re-clone them into a different hosts both bacteria and mammalian cells. Upon a successful mutant \textit{NALP3} protein expression within these cells (both the mutant and wild type \textit{NALP3} protein) they were supposed to be subjected to, further studies of function of these proteins by protein-protein interaction models in mammalian and bacterial cells. For future work, it is very important to achieve and verify a positive recombinant protein expression by different techniques such as mentioned above before continuing with function analyses.

6. Acknowledgement

I wish to acknowledge and express my sincere gratitude to my supervisor Prof. Mikael Ejdebäck for giving me an opportunity to carry out my thesis work and for his admirable guidance throughout the project. Further I would like to thank all teachers in molecular biology at Skövde University for their counselling during the period at the lab. Finally I would also like to thank my family members and friends for their backing up which gave me the strength to accomplish my final project.

7. References


Coomassie Brilliant Blue R-250 (Sigma, B-O149).


Kaleidoscope Prestained Standards (Bio-Rad Laboratories, 2000).


QuickChange® II Site-Directed Mutagenesis Kit (Stratagene, 2004).


8. Appendices

Appendix A

Wild type *Nalp3* gene nucleotide sequence

```
atgaagatgg caagcacccg ctgcaagctg gccaggtacc tggaggacct ggaggatgtg
gacttgaaga aatttaagat gcacttagag gactatcctc cccagaaggg ctgcatcccc
tctcccgaggg gtcagacaga gaaggcagac catgtggatc tagccacgct aatgatcgac
tttcaatgggg aggagaaggc gtgggccatg gccgtgtgga tcttcgctgc gatcaacagg
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ggcagggatt gggaaaacaa tcctggccag gaagatgatg ttggactggg cgtcggggac actctaccaa gacaggtttg actatctgtt ctatatccac
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tcagcagcct ccagcagcct ccagcagcct ccagcagcct ccagcagcct ccagcagcct ccagcagcct ccagcagcct ccagcagcct ccagcagcct
Appendix B

The Thermal Cycling Parameters for the Mutagenesis Reaction

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<td>15</td>
<td>95º C</td>
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<td></td>
<td>55º C</td>
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Appendix C

PCR cycle sequencing reaction mixtures

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<td>Template</td>
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<td>Primer</td>
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Appendix D

PCR cycle sequencing setup

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Appendix E

Standard PCR setup profile

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Appendix F

Colony PCR setup profile

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<td></td>
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Appendix G

The vector expression map
Appendix H

Recipes for SDS-PAGE preparation and running

Separating 15 % polyacrylamide gel, one 1.5 mm gel

3.65 ml deionised water
3.75 ml 40 % acrylamide: bisacrylamide
(37.5:1) NB Very toxic!
2.5 ml 1.5 M Tris-HCL pH 8.8
0.1 ml 10 % SDS

Mix the solution by inverting the closed tube. Do not shake the tube. Add 50 µl fresh 10 % APS and 5 µl TEMED to the solution immediately before gel casting. Mix by inverting the closed tube a few times. Do not shake the tube.

Stacking 4 % polyacrylamide gel, one 1.5 mm gel

3.2 ml deionised water
0.5 ml 40 % acrylamide: bisacrylamide
(37.5:1) NB Very toxic!
1.25 0.5 M Tris-HCL pH 6.8
0.05 ml 10 % SDS

Mix the solution by inverting the closed tube. Do not shake the tube. Add 25 µl fresh 10 % APS and 5 µl TEMED to the solution immediately before gel casting. Mix by inverting the closed tube a few times. Do not shake the tube.

Reducing sample buffer

For making 0.5 ml buffer, mix…
475 µl Laemmli sample buffer
25 µl β-mercaptoethanol
Use gloves and work in fume hood!

Transfer buffer

25 mM Tris, 192 mM glycine, 20 % methanol, pH 8.3
3.0 g Tris
14.4 g glycine
200 ml methanol
Water to 1 liter.

Keep in the cold room at 4 ºC.
Appendix I

The molecular weight standard (MW) ladder from Bio-Rad Laboratories.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Colour</th>
<th>MW (Daltons) on Bis-Tris gel</th>
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<tbody>
<tr>
<td>Myosin</td>
<td>Blue</td>
<td>196,621</td>
</tr>
<tr>
<td>Beta-galactosidase</td>
<td>Magenta</td>
<td>112,231</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>Green</td>
<td>59,761</td>
</tr>
<tr>
<td>Carbonic anhydrase</td>
<td>Violet</td>
<td>26,601</td>
</tr>
<tr>
<td>Soybean trypsin</td>
<td>Orange</td>
<td>22,020</td>
</tr>
<tr>
<td>inhibitor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lysozyme</td>
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</tr>
<tr>
<td>Aprotinin</td>
<td>Blue</td>
<td>6,668</td>
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</table>
Appendix J

Sequencing result from G569R mutant

TTNCTGCAACTTTATTTCTCCTCCCAAATTAGAGGTTCCCTCCTGTTTACCAGGCCAAAGA
GAAACTGCAACACAAATCAAATACCTCTCTTTCTCAATTGATCCAGGCCAAGGAAACGTACAACAAAAATCAAATACCTCTTTTCGAATTTGCCATAGTTTTCCAGAAGGA
CTGTCAGTGTTGCTGGCTGGGAAGCTTCAAACGACTCCCTGGAACGTTCGTCCTTCCTTCCTTTTCCTCTTCCAGCAGGTAGTACATGGCGGGCAAAGAACTCCTGGAAAGTCATGTGGATG
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GACACATCCGCCTTTGCAAGCAGTTCCATGATTTCCGAGGGTCGGACTCCTCTCAAACAGGATTCTTC
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