
Interleukin-1 α Induction in Human Macrophages

Master's Thesis

Medicine with Industrial Specialization

Translational Medicine

Group 1005

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AALBORG UNIVERSITY
STUDENT REPORT

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Department of Health Science and Technology

Medicine with Industrial Specialization

Master's Thesis

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Abstract

Inflammation is a complex biological process in response to foreign substances, and inflammatory cytokines are known to have an essential role in the initial part of the immune response. However, many inflammatory diseases are related to malfunctions in the inflammatory processes, thus investigations of cytokine responses are of great interest. This study aims to characterize the interleukin-1 α (IL-1 α) response in blood macrophages from healthy individuals by stimulation with lipopolysaccharides (LPS). Mononuclear cells were extracted from ten healthy donors and the kinetics of IL-1 α was examined by stimulating the cells with LPS for a varying amount of time. RNA was extracted from the cells and cDNA synthesis was conducted prior to quantitative PCR, in order to investigate the transcription of IL-1 α . The protein synthesis was examined by use of flow cytometry. Additionally, cDNA was used for DNA sequencing of the IL-1 α gene to identify any eventual single nucleotide polymorphisms (SNPs). The transcription revealed great inter-individual variability, though in general a rapid increase in mRNA expression was observed within 2-4 hours following LPS stimulation. The response remained highly elevated throughout the 20 h investigated. No significant difference ($P>0.05$) was observed in the amount of mRNA when comparing the different times that the cells were stimulated in. This indicates that a regression does not occur within the 20 h. The protein synthesis of IL-1 α revealed a slow but steady enhancement over time. This was indicated by a statistically significant increase in the fluorescence intensity of the macrophages between 0 and 4h, 4 and 8 h, and 8 and 20 h ($P<0.000$). DNA sequencing of the IL-1 α gene demonstrated a known SNP in six of the ten subjects. No association was found between the presence of the SNP and the IL-1 α response. This study revealed that an IL-1 α response to LPS stimulation contains both individual variations and general characterizations, and it forms the basis of further study of IL-1 α in health and disease.

1. Introduction

1.1 Spondyloarthritis

Spondyloarthritis (SpA) is a term covering a group of inflammatory articular diseases consisting of ankylosing spondylitis (AS), reactive arthritis (ReA), psoriasis arthritis, arthritis associated with idiopathic inflammatory bowel disease and undifferentiated SpA^{1,2}. These disorders exhibit common clinical features including inflammatory back pain, asymmetric peripheral arthritis predominantly in the lower extremities, and they are often connected with sacroiliitis, spondylitis, enthesitis and uveitis^{1,2}. The majority of the subtypes are thought to have a genetic background with a strong relation to the major histocompatibility complex (MHC) class I molecule, human leukocyte antigen B27 (HLA-B27)¹⁻³. Environmental factors also contribute to the development of SpA, especially in ReA where bacterial infections are associated with the onset of disease^{4,5}. The similar characteristics in SpA make it difficult to distinguish between the subtypes, along with the fact that more than one disorder may emerge consecutively in the same individual¹.

1.1.1 Ankylosing Spondylitis

AS is the most frequent, best defined and most studied subtype^{4,6,7}. It primarily affects young people before the age of 30 and males are more exposed than women, which is different from other immunological diseases⁸. The clinical features of AS include back pain due to sacroiliitis, inflammation in other parts of the axial skeleton, peripheral arthritis, enthesitis and uveitis^{1,2}. Besides back pain,

typical symptoms are spinal stiffness and decreased mobility due to inflammation and structural damage. Structural bone damage often includes formation of syndesmophytes caused by osteoproliferation and the development of osteoporosis⁹. The pathogenesis of AS is still unknown, but genetic factors are believed to have an essential role. AS is the type of SpA which possesses the greatest association with HLA-B27, since about 90-95% of AS patient are HLA-B27 positive. However, the majority of HLA-B27 positive individuals are healthy and AS strikes only about 5 % of the HLA-B27 positive population². Thus, other factors than HLA-B27 must be essential for the development of the disease. This is supported by twin studies indicating that HLA-B27 accounts for 20-30 % of the genetic susceptibility to AS¹⁰. It is still uncertain which genes outside the MHC cluster that predisposes to AS, but studies indicate that endoplasmatic reticulum aminopeptidase 1 (ERAP1), tumor necrosis factor (TNF) α , interleukin-23 receptor (IL-23R) and interleukin-1 (IL-1) might be involved^{3,11-13}.

The function of HLA-B27 in the pathogenesis of disease is still uncertain and several theories have been studied over time. Currently three dominant hypotheses exist^{14,15}. The arthritogenic-peptide theory proposes that HLA-B27 binds a specific arthritogenic peptide which is recognized by CD8+ T lymphocytes and consequently causes an inflammatory response. Two other antigen-independent theories are based on misfolding of the HLA-B27 molecule during its formation in ER^{14,16}. Misfolding of HLA-B27 is believed to create a free heavy chain molecule without the β_2 microglobulin. Two different scenarios are hypothesized to be a consequence of this misfolding. One theory is that the free heavy chain molecules accumulate in the endoplasmic reticulum (ER) resulting in a proinflammatory stress response. The other theory is that the free heavy chains molecules form homodimers which, when located on the cell surface, activate inflammatory NK cells and T lymphocytes¹⁶.

1.1.2 Reactive Arthritis

ReA is one of the SpAs which are less affected by the genetic factor HLA-B27, though 60-80 % of ReA patient are HLA-B27 positive it is not believed to influence the onset of disease, but is rather affecting the severity^{5,17}. The disease mostly affects young people in the age 20-40, and men are at greater risk than women like in AS. ReA is an autoimmune disease which develops following an infection especially in the gastrointestinal or urogenital track. Bacteria associated with ReA are *Campylobacter*, *Salmonella* and *Shrigella* in the gastrointestinal tract, and primarily *Clamydia trachomatis* in the urogenital tract^{4,5}. The triggering antigen is lipopolysaccharide (LPS) found on the surface of the aerobic gram-negative bacteria⁴. The disease is often visualized one to three weeks after the infection where the antigen is no longer to be found, thus the disease is believed to be a cause of an over-stimulated autoimmune response⁴. Imbalance in the production of proinflammatory cytokines in mononuclear cells have been observed in the disease. However, the exact biological reaction which causes the disease remains unclear^{4,5}. The disease develops in 1-15 % of the infected individuals and is mainly characterized by inflammation of large joints, conjunctivitis and enteritis or urethritis^{4,5}.

1.1.3 Classification

Several classification systems for SpA have been establish and modified over the years^{1,2}. AS is the subtype that has been most clearly defined according to the modifications of the New York classification criteria for AS from 1984¹⁸. This classification is primarily based on the presence of sacroiliitis on x-ray. These criteria have been widely used in clinical studies of AS, however they lack sensitivity in diagnosing early disease¹⁷. In the beginning of the nineties the European Spondyloarthropathy Study Group and the Amor classification criteria were formed with the focus of covering all types of SpA, including the less specific forms^{19,20}. These criteria included both axial and peripheral manifestations, and the presence of sacroiliitis was less predominant, allowing earlier

diagnosis. The most recent set of criteria is the Assessment of SpondyloArthritis international Society (ASAS) axial and peripheral SpA classification criteria^{17,21}, see figure 1. These criteria divide the patients into a group of axial SpA and a group of peripheral SpA¹⁷. Patients under the age of 45 years with back pain for at least three months are candidates for a diagnosis of axial SpA^{17,21}. Patients with axial SpA can be classified as having either radiographic or non-radiographic axial SpA. Radiographic axial SpA is diagnosed in patients with sacroiliitis on imaging along with at least one other SpA feature, while non-radiographic axial SpA is diagnosed in HLA-B27 positive patients with at least two other SpA features^{17,21}, see figure 1. A diagnosis of a radiographic axial SpA corresponds to the diagnosis of AS²¹. In patients with only peripheral manifestations, peripheral SpA can be diagnosed in the presence of arthritis, enthesitis or dactylitis together with at least one or two SpA features (shown in figure 1)¹⁷. This new classification system includes the use of magnetic resonance imaging (MRI), which makes it possible to diagnose patients in earlier stages of the disease²².

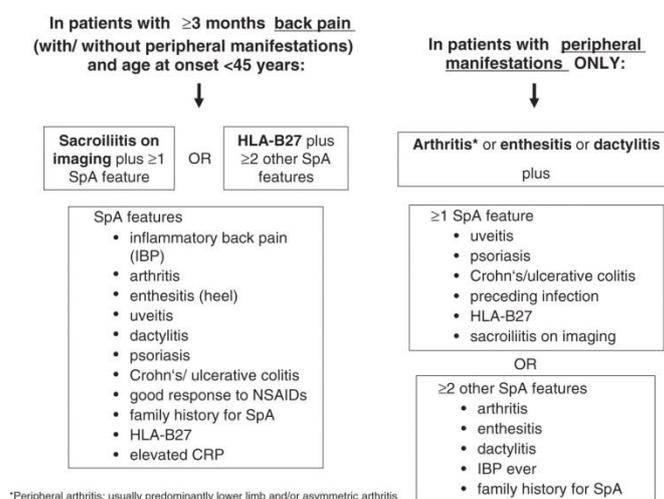


Figure 1: Overview of the combined use of the Assessment of SpondyloArthritis international Society (ASAS) criteria for axial spondyloarthritis (SpA) and the ASAS criteria for peripheral SpA. At the left is seen the criteria for axial SpA, with or without peripheral manifestations. At the right are the criteria for peripheral SpA, they are used when only peripheral manifestations are present. Source: ¹⁷.

1.1.4 Treatment

The treatment of SpA is based on a combination of pharmacologic and non-pharmacologic therapies, with the focus of relieving pain, increasing mobility and preventing inflammation^{2,23}. Non-pharmacologic therapies include physiotherapy, exercise and education. In severe cases joint replacement and spinal surgery may be necessary^{2,23}. The standard pharmacological treatment for axial SpA is the use of non-steroidal anti-inflammatory drugs (NSAIDs), which reduces pain and stiffness¹³. In peripheral arthritis disease-modifying antirheumatic drugs (DMARDs), especially sulfasalazine, are predominantly used². DMARDs have not demonstrated any effect on axial SpA. If the initial treatment is ineffective then TNF inhibitors can be used according to the updated ASAS recommendations⁹.

1.2 Lipopolysaccharide as an Inducer of the Innate Immune System

LPS is a molecule known to induce a strong inflammatory response in humans. Thus, it is widely used as pathogen when studying the innate immune system in vitro²⁴. LPS is found on the surface of the outer membrane of gram-negative bacteria. The molecule consists of three parts: an O-antigen oligosaccharide chain, a core carbohydrate and a lipid A which anchors in the outer membrane²⁴⁻²⁶.

The structure of LPS varies significantly amongst bacteria species which may influence the immune response²⁴. Thus, it is important to consider from which bacteria LPS is extracted. In the present study LPS from *Escherichia coli* (*E. coli*) was used, since it is known to be a strong inducer of immune response signaling^{24,25,27}. The lipid A of *E. coli* LPS is composed of six fatty acyl chains on a glucosamine backbone and forms a hydrophobic part. This hydrophobic lipid A is the most essential part of LPS for binding to the immune receptors^{25,27}. When LPS have entered the human body, it is bound by LPS binding protein (LBP)^{25,26}. LBP presents and binds LPS to the protein CD14, which exists in a soluble form or bound to the cell membrane of e.g. macrophages^{26,28}. CD14 then transfers LPS to the toll-like receptor (TLR) 4 and its co-receptor myeloid differentiation factor 2 (MD-2) which induces a signal cascade, as seen in figure 2²⁶.

1.2.1 Toll-like Receptor 4 Signaling

TLR4 consists of an extracellular part with leucine-rich repeat (LRR) domains, a transmembrane part and an intracellular part with a Toll/IL-1R (TIR) domain^{25,27}. The TIR domain was named on the basis that the intracellular domain of the TLR family is highly homologues with that of the IL-1 receptor (IL-1R) family^{29,30}. Thus, the two receptor families constitute a TLR/IL-1R superfamily. TLR4 forms a complex with MD-2, since it is unable to bind LPS on its own^{25,27}. MD-2 contains the ligand binding site but is incapable of transmitting a signal without TLR4, since it has no link to the cell membrane. MD-2 consists of two β -sheets that forms an open pocked where the ligand interacts^{25,27}. When a ligand is presented to the TLR4-MD-2 receptor complex a dimerization of two receptor complexes occurs, as seen in figure 2. The joined intracellular TIR domains are responsible for the following signal transduction^{25,26}. Two different signal pathways exist in response to TLR4 activation: the myeloid differentiation primary response gene 88 (MyD88)-dependent and MyD88-independent pathway. The MyD88-dependent pathway mediates activation of proinflammatory cytokine genes, while the MyD88-independent pathway is responsible for the production of Type I interferons, as seen in figure 2^{25,26}.

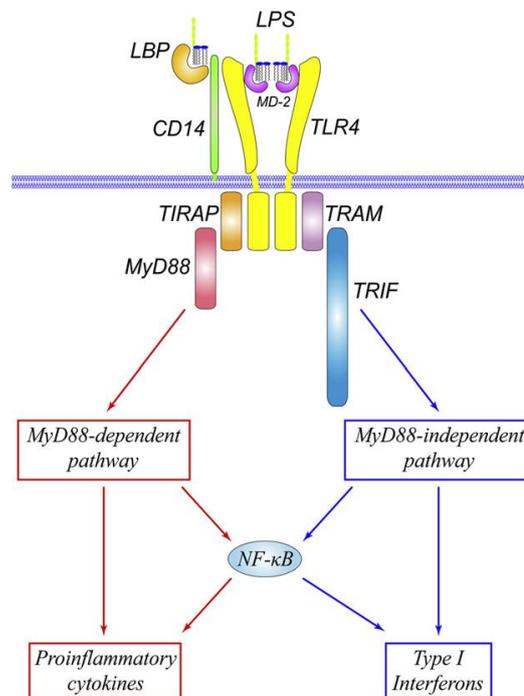


Figure 2: Overview of the LPS-TLR4-MD-2 signaling pathways. LPS is bound by LBP which presents LPS to TLR4. Two signaling pathway are activated, the MyD88- dependent which results in transcription of proinflammatory cytokines. And the MyD88-independent pathway which causes transcription of Type 1 interferones. Picture Source:²⁶

1.2.2 The MyD88-dependet pathway

In the MyD88-dependet pathway the TIR domains recruit the adaptor proteins TIR-domain-containing adaptor protein (TIRAP) and MyD88^{25,29}. Both proteins contain a TIR domain which binds them to the TLR4. Additionally, MyD88 contain a death domain (DD) which is responsible for further recruitment of the DD-containing IL-1 receptor-associated kinase (IRAK) 4 and IRAK1^{26,29}. IRAK4 activates IRAK1 causing phosphorylation of IRAK1 and subsequent recruitment of TNF receptor-associated factor 6 (TRAF6). TRAF6 along with IRAK1 separates from the receptor complex to form a new complex with transforming-growth-factor- β -activated kinase 1 (TAK1), TAK1-binding protein (TAB) 2 and TAB3^{26,29}. IRAK1 is degraded and the remaining complex connects with ubiquitin-conjugating enzyme 13 (UBC13) and ubiquitin-conjugationg enzyme E2 variant 1 isoform A (UEVIA). This leads to activation of TAK1 which further activates IKK (I κ B kinase) and mitogen-activated protein kinase (MAPK) pathways^{26,29,31}. In the IKK pathway, TAK1 interacts with IKK α , IKK β and IKK γ causing phosphorylation and degradation of I κ B proteins with subsequent release of the transcription factor nuclear factor κ -light-chain-enhancer of activated B cells (NF- κ B). NF- κ B then translocate to the cell nucleus where it promotes transcription of genes encoding proinflammatory cytokines^{26,29,32}. In the MAPK pathway, MAPK activates the transcription factor activator protein-1 (AP-1), which also influences the expression of proinflammatory cytokines ²⁶.

1.2.3 The MyD88-independenth pathway

In the MyD88-independent pathway, TLR4 recruits TIR-domain-containing adaptor-inducing interferon- β (TRIF) through the presence of TRIF-related adaptor molecule (TRAM)^{26,29}. TRIF then recruits TRAF3 which activates the transcription factor interferon regulatory factor 3 (IRF3). This causes transcription of Type I interferons^{26,29}. However, TRIF also activates MAPK and IKK pathways through the activation of receptor-interacting protein 1 (RIP1), which helps the transcription process of IRF3^{26,29}.

1.3 IL-1 α

IL-1 cytokines are essential in the control of proinflammatory reactions in response to pathogens such as LPS³³. Today there are 11 members of the IL-1 family, of which three of the earliest discovered and most studied is IL-1 α , IL-1 β , and the IL-1 receptor antagonist (IL-1Ra)³⁴. The function of IL-1 β in the inflammatory response wave been widely studied, while little is known about the role of IL-1 α in the innate immune response³⁵.

IL-1 α is found in several cell types including endothelial cells, epithelial cells, fibroblasts and macrophages³³. It is synthesized as a precursor protein named pro-IL-1 α with a molecular weight of 31 kD^{36,37}. Translation of the protein takes place in association with microtubules in the cytosol, this is unlike most proteins which are translated in the endoplasmic reticulum³⁷. Pro-IL-1 α is an intracellular protein, which is not recorded in the human circulation or any organs, unless if it is released from dying cells^{33,37}. Following translation pro-IL-1 α stays in the cytosol were it can translocate to the nucleus or it can be myristoylated and translocate to the cell membrane³⁷, see figure 3. The nuclear translocation is possible since the N-terminal part of the protein contains a nuclear localization sequence (NLS)³⁸. Its nuclear functions are many including activating transcription of pro-inflammatory cytokines^{27,33}. Membrane bound pro-IL-1 α is likewise involved in the inflammatory process by causing autocrine signaling through juxtacrine interactions ^{37,39,40}. Thus, IL-1 α is highly active in its precursor form. However, pro-IL-1 α can also be cleaved into two parts by the cysteine protease calpain (see figure 3), but this primarily occurs at abnormal conditions^{37,38}. The 16-kD N-

terminal part, termed IL-1 α propiece, remains intracellular were it due to its NLS can translocate to the nucleus and induce transcription like its precursor^{37,38}. The 17-kD C-terminal part forms the mature IL-1 α which is released into the extracellular compartment were it causes paracrine signaling^{37,38}.

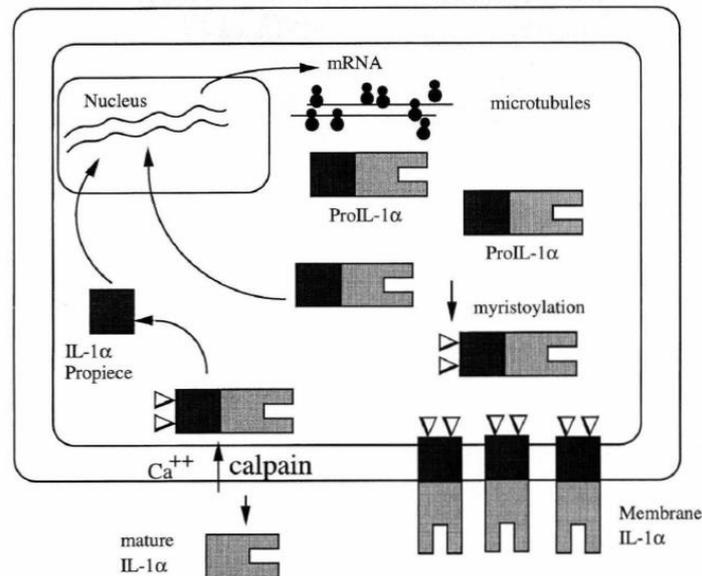


Figure 3: Production of IL-1 α in macrophages. Transcription of mRNA to pro- IL-1 α takes place on microtubules in the cytol. Here it can translocate to the nucleus or it can be myristoylated and anchor the cell membrane. In both ways pro-IL-1 α is involved in inflammatory responses. Pro- IL-1 α can also be cleaved by calpain into the intracellular IL-1 α propiece and the mature IL-1 α which is released form the cell Picture source:³⁷

1.4 IL-1 Receptor Signaling

IL-1 α and IL-1 β share similar biologic effect by signaling through the Type 1 IL-1 receptor (IL-1RI)^{41,42}. The IL-1RI is a member of the IL-1R family which holds 9 members³⁶. The majorities consist of an extracellular part of three immunoglobulin (Ig)-like domains and an intracellular TIR domain ^{29,30,41}. The TIR domain is essential for signal transduction why IL-1R and TLR utilize similar signaling pathways, resulting in enhanced gene expression of inflammatory and immunologic cytokines ^{29,37,41,42}. In order to cause signaling, IL-1RI is dependent on interaction with the IL-1 receptor accessory protein (IL-1RAcP)^{29,41,42}. IL-1 α binds to the two outer Ig-like domains of IL-1RI and is then linked to IL-1RAcP to form a complex^{41,43}. Both IL-1RI and IL-1RAcP contain a TIR-domain that when joined triggers a signal cascade starting by recruiting the adaptor protein MyD88^{30,36,41}. This signaling pathway is very similar to the MyD88-dependet pathway described in section 1.2.2^{26,29}. The downstream signaling leads to association of TRAF6 with TAK1 and MAPK kinase kinase 3 (MEKK3)^{26,29,33,41}. This causes activation of three different pathways; the NF- κ B, the c-jun N-terminal kinase (JNK) and the p38 MAPK pathways³³. The NF- κ B pathway was described previously in section 1.2.2. The JNF pathway leads to activation of MKK4 and 7, which further activates JNF. JNF then causes phosphorylation of AP-1, especially c-Jun and activating transcription factor 2 (ATF-2)³³. The p38 MAPK pathways is activated by MKK3, 4 and 6, which causes stabilization of IL-1 induced mRNA by p38 MAPK ³³. Furthermore, nuclear p38 MAPK may also activate ATF-2. Together these pathways results in the transcription of several proinflammatory cytokines such as IL-6, IL-8 as well as IL-1 α and IL-1 β itself ³³.

1.5 IL-1 Inhibitors

The biological response of IL-1 is regulated by several inhibitors which work in different manners^{30,40,42}. The Type 2 IL-1 receptor (IL-1RII) binds both IL-1 α and IL-1 β but due to its lack of a TIR domain this receptor is unable to cause a signal^{41,42}. This is in spite the fact that IL-1RII, when activated, also recruits the TIR-domain-containing IL-1RAcP, se figure 4 (1). Thus, IL-1RII acts as a decoy receptor suppressing the IL-1 system^{30,42}. Single Ig IL-1-related receptor (SIGIRR) is another receptor of the IL-1R family which possesses an inhibitory effect on IL-1^{30,36}. The receptor contains only one extracellular IgG-like domain and the intracellular TIR domain is dysfunctional due to the lack of two amino acids^{30,40}. The altered TIR domain in SIGIRR causes reduced activation of the intracellular NF- κ B pathway, thus limiting IL-1 signaling, se figure 4 (2)⁴⁰. IL-1Ra, also known as the drug Anakinra, is a naturally occurring antagonist and is competitive to IL-1 α and IL-1 β in binding to IL-1RI^{40,41}. When IL-1Ra binds to IL-1RI no signaling develops, thus it can be used for blocking IL-1 activity as seen in figure 4 (3). IL-1Ra is approved for the treatment of inflammatory diseases ^{40,44,45}.

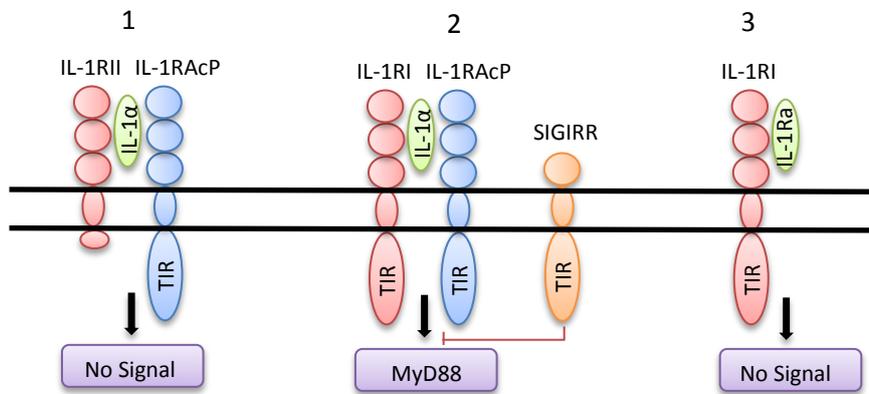


Figure 4: Inhibitory factors of IL-1 signaling. 1: IL-1 binds to IL-1RII with high affinity, but IL-1RII lacks a TIR domain, thus no signaling occurs. 2: SIGIRR is an IL-1R with an altered TIR domain which interferes with the MYD88-patway and reduces the signal. 3: IL-1Ra is a receptor antagonist which by binding the IL-1RI prevents IL-1 in binding it and causing signaling.

2. Aim

As mentioned above, the role of IL-1 α in the immune response is not fully understood. Therefore, the aim of this study was to induce and characterize an IL-1 α response in human macrophages of healthy subjects. This was done by stimulating mononuclear cells with LPS in a varying amount of time. The kinetics of IL-1 α was investigated by quantitative polymerase chain reaction (qPCR) and flow cytometry. A second aim was to investigate if the IL-1 α gene contained any SNPs in a healthy Caucasian population. The hypothesis was that the expression of IL-1 α differed depending on the amount of time that the cells were stimulated. Further, it was hypothesized that no expression of IL-1 α was present in the non-stimulated control cells.

3. Methods

Blood samples were collected from ten healthy donors of Caucasian origin (males: n=6, females: n=4) with the age of 21-29. The study was conducted in relation to a trial approved by the Ethics Committee of Region Midtjylland, case number 1-10-72-291-12. Written informed consent was obtained from all donors.

3.1 Experimental Design

Human mononuclear cells were isolated from blood samples of ten healthy subjects. Immunofluorescence staining was conducted on cells from one donor in order to investigate the expression of IL-1 α propiece. The cells were analyzed following 12 hours of incubation, were half the cells were stimulated with LPS and half the cells were used as controls. Cells from all ten subjects were used for analyzing the kinetics of IL-1 α over time. Again half the cells were stimulated with LPS and half the cells were used as controls. The setup was performed with cells from each donor which was stimulated with LPS for 0, 2, 4, 6, 8 and 20 hours, respectively. One part of the cells was used for cDNA synthesis for two purposes; Firstly, to investigate the quantitative mRNA expression of IL-1 α by use of qPCR and secondly to demonstrate any variation in the IL-1 α gene by DNA sequencing. Another part of the cells was used to study the protein expression of IL-1 α propiece over time by use of flow cytometry.

3.2 Isolation of Mononuclear Cells and LPS Stimulation

Blood from the donors was collected by s-monovette® (SARSTEDT) and diluted 1:1 in 0.9 % NaCl containing 4 u/mL heparin (LEO). Then mononuclear cells were isolated using the density gradient Lymphoprep™ (Fresenius Kabi AS) by centrifugation for 30 min at 20 °C, 300g. Three different procedures were tested for cell isolation; 50 mL tubes (VWR International), 50 mL accuspin™ tubes (Sigma Aldrich), and 15 mL tubes (VWR International). The 15 mL tubes produced the thickest and most distinct layer of buffy coat, making it optimal to collect. Thus, these were used throughout the study. The buffy coats were transferred to a new tube and washed with RPMI 1640 medium (Lonza) containing 1.98 mM L-glutamin (Merck) and 0.012mg/mL hexamycin/gentamicin (Sandoz). Following 5 min of centrifugation the pellet was resuspended in wash media containing 16.5 % fetal calf serum (Biochrom AG). The cells were counted using a Bürker-Türk counting chamber, and 200 μ L suspension was distributed to the wells of two Nunc™ MicroWell™ Plates (Thermo Fischer Scientific) with a density of 1-1.5 million cells per well. The cells in one plate were stimulated with 5 μ g/mL lipopolysaccharides (LPS) from *E. coli* (Sigma-Aldrich), while the other plate remained non-stimulated as a control. The cells were placed in a 5 % CO₂ incubator at 37 °C for 2, 4, 6, 8 and 20 hours, respectively. Additionally, an immediate sample was made as a non-stimulated control at 0 hours. For immunofluorescence staining, LPS-stimulated and non-stimulated cells from one donor were seeded in a 16-well permanoks LabTek® Chamber Slide™ (Thermo Fisher Scientific).

3.3 Immunofluorescence Staining

The cells in the permanoks chamber were fixed with 3.7 % formalin in phosphate buffered saline (PBS) for 20 min at 4 °C. The cells were washed twice in PBS and then permeabilized with PBS containing 0.2 % triton X-100 for 7 min at room temperature. The cells were washed twice and then incubated at 37 °C for 15 min with blocking buffer consisting of PBS with 0.05 % bovine serum albumin (BSA). Primary antibody, MAb1.3.2 mouse-anti-human against IL-1 α propiece (Loke Diagnostics), was diluted 1:10 in blocking buffer and added to each well (antibodies are presented in

table 1). The cells were incubated for 30 min at 37 °C and subsequently washed three times in PBS. Secondary antibody, fluorescein isothiocyanate (FITC) conjugated goat-anti-mouse (Jackson ImmunoResearch Laboratories Inc.) diluted 1:100 in blocking buffer, was added and incubated for 30 min at 37 °C. Following two washes, nuclear counterstaining was conducted with 1µM To-Pro-3 iodide (Invitrogen). The cells were then incubated for 10 min at room temperature. Lastly, the cells were washed twice in PBS and the wells were removed from the slide. Antifade (Loke Diagnostics) and coverslip were added before examination of the cells with a fluorescence microscope (Leica DM5500). Pictures were taken with a Q imaging retiga 2000R camera.

Additionally, immunofluorescence staining was conducted with FITC conjugated MAb1.3.2 mouse anti-human against IL-1α propiece for validation of the antibody prior to flow cytometry. The procedure was identical with the above mentioned, except that MAb1.3.2 Propiece IL-1α FITC was used as primary antibody with a concentration of 2µg/mL (see table 1). Furthermore, no secondary antibody was necessary since the primary antibody was FITC conjugated.

Primary antibody	Secondary antibody
MAb1.3.2 mouse-anti-human IL-1α propiece	Goat-anti-mouse FITC
MAb1.3.2 mouse-anti-human IL-1α propiece FITC	None

Table 1: Overview of primary and secondary antibodies used for immunofluorescence staining

3.4 RNA Extraction

Lysis of the cells and subsequent RNA extraction was performed using a GeneJET RNA Purification Kit (Thermo Scientific) according to the manufacturer’s protocol. The cells were washed in PBS and transferred to microcentrifuge tubes. Cell lysis was performed by adding 400 µL lysisbuffer containing 2 % β-mercaptoethanol (AppliChem). Subsequently, the lysate was mixed with 240 µL 99 % ethanol and transferred to purification columns. The cells were washed once in wash buffer 1 and twice in wash buffer 2 according to the manufacturer’s protocol. Lastly, the RNA was eluted from the columns with nuclease-free water. The quantity of RNA was assessed by a Nanophotometer (IMPLEN). Lastly, the RNA solution was stored at -80 °C.

3.5 DNase Treatment

In order to prepare DNA-free RNA for cDNA synthesis, DNase treatment was conducted. Depending on the RNA concentration, a volume of maximal 8 µL was transferred to a microcentrifuge tube. A concentration of approximately 140ng RNA per tube was striped for. When the volume of RNA solution was less than 8 µL, nuclease-free water was added to a total volume of 8 µL. To each tube 1 µL RNase-free DNase I and 1 µL 10X reaction buffer with MgCl₂ was added and incubated for 30 min at 37 °C. Following, 1 µL EDTA was added and incubated for 10 min at 65 °C.

3.6 cDNA Synthesis

Synthesis of cDNA was performed using a Maxima H Minus First Strand cDNA Synthesis Kit (Thermo Scientific). Each reaction had a total volume of 20 µL consisting of 11 µL DNase treated RNA solution and a 9 µL mastermix of the following:

Mastermix	Volume (μL)
Random Hexamer primer (0.2 $\mu\text{g}/\mu\text{L}$)	0.25
Oligo(dT) ₁₈ primer (0.5 $\mu\text{g}/\mu\text{L}$)	0.25
dNTP Mix	1
5 x RT Buffer	4
Maximum H Minus Enzyme Mix	1
Nuclease-free water	2.5
In total	9

The tubes were placed in a Thermal Cycler (BIO-RAD T100™) with a three-step temperature curve: 25 °C for 10 min, 55 °C for 15 min and 85 °C for 5 min. The cDNA was stored at -25 °C.

3.7 Design of Primers for qPCR

Primers for *IL1A*, *IL1B*, *B2M* and *CD68* were designed using Primer-BLAST. All primer pairs were designed with the attempt to match the same melting point, and to avoid known SNPs in the primer sequence. Additionally, a primer length of 18-22 base pair was strived for. Optimal conditions of a primer include a length at 18-22 base pairs, a melting temperature about 52-58°C, a GC content of 40-60% and presence of G or C the final bases of the 3' end⁴⁶.

3.8 Quantitative Polymerase Chain Reaction

Primers for *IL1A*, *IL1B*, *B2M* and *CD68* (DNA Technology) were used for qPCR, see table 2 for primer information. Initially, all primers were tested in order to obtain their efficiency by a five-fold serial dilution of cDNA from one subject. The cDNA from cells stimulated with LPS for 4 hours was used, and diluted with nuclease-free water. For each reaction a master mix with a total volume of 20 μL was made, containing 12.5 μL Maxima SYBR Green (including 10nM ROX), 8.5 μL nuclease-free water, 1.5 μL forward primer (5 μM) and 1.5 μL reverse primer (5 μM). The master mix was mixed with 5 μL diluted cDNA. For each gene a negative control was made of mastermix with nuclease-free water instead of cDNA.

Gene	Product size (bp)	Type	Primer	T _m (°C)
IL1A	160	Forward	GGCGTTTGAGTCAGCAAAGAAG	60.60
		Reverse	CATGGAGTGGGCCATAGCTT	59.82
IL1B	176	Forward	CCACCTCCAGGGACAGGATA	60.03
		Reverse	TGGGATCTACACTCTCCAGC	58.22
β 2M	105	Forward	AGATGAGTATGCCTGCCGTG	59.89
		Reverse	GCGGCATCTTCAAACCTCCA	60.96
CD68	203	Forward	GGTTGAGCAACTGGTGCAGA	60.82
		Reverse	TGGTTGTTCCAGTGCTCTCT	58.87

Table 2: Information about the primers used for qPCR, including primer sequence, product size and optimal temperature (T_m) conditions.

The qPCR was carried out in a Perkin-Elmer Thermo Cycler with the following program setup:

1 Cycle	40 Cycles	1 Cycle
95 °C in 10min	95 °C in 15 sec	95 °C in 1 min
	53 °C in 30 sec	55 °C in 30 sec
	72 °C in 30 sec	95 °C in 30 sec

Primers with an efficiency of 90-110 % are accepted for obtaining data with good quality. Thus, only primers for *IL1A* and *B2M* were used for further study, see section 4.3 in results. The procedure used

to test the quantitative expression of IL-1 α and β_2 -microglobulin was similar to the above-mentioned, with the exception that a 10-time dilution of cDNA was used as template.

Efficiency curves and Ct-values were extracted by use of the software Mx-Pro (2007). Result with a Ct value more than 30 in the β_2 -microglobulin sample was excluded from the analysis since this indicated an inadequate amount of cDNA. All Ct-values were normalized to the housekeeping gene β_2 -microglobulin and fold change of IL-1 α expression was calculated using the Pfaffl equation:

$$Ratio = \frac{E_{target}^{\Delta Ct_{target} (control-treated)}}{E_{ref}^{\Delta Ct_{ref} (control-treated)}}$$

3.9 Preparation of Cells for Flow Cytometry

Cells were transferred from the microwell plates to 5mL polypropylene round-button tubes (BD Falcon™), with approximately 3 million cells per tube. The cells were washed in PBS and fixed with 1 % formaldehyde for 20 min at room temperature. The cells were centrifuged for 10 min at 20°C, 250g and stored in PBS over night at 5°C. Permabilization and blocking buffer was made consisting of PBS with 0.3 % BSA, 0.05 % sodium azid (Merck) and 0.2 % Triton X-100 (Sigma). The buffer was filtrated through a 0.2 μ m filter before use. The cells were centrifuged for 10 min, then permabilization and blocking buffer was added and incubated for 10 min at room temperature. The cells were centrifuged for 10 min and washed twice in PBS, with centrifugation for 5 min at 20 °C, 300 g between each wash. Then 2 μ g/mL FITC conjugated MAb1.3.2 mouse-anti-human antibody against IL-1 α propiece (Loke Diagnostic) diluted in permabilization and blocking buffer was added to each tube along with 0.5 μ g/mL APC anti-human CD68 antibody (Dako) (antibodies are presented in table 3). The tubes were wrapped in aluminum foil and incubated for 30 min at room temperature. Following incubation, the cells were centrifuged for 5 min and washed three times in PBS, with 5 min centrifugation between each wash. The cells were kept in PBS for flow cytometry, still covered with aluminum foil. Flow data were extracted by use of a BD FACSCanto™ Flow Cytometer (BD Bioscience, San Jose, USA) and BD FACSDiva software. In each sample 10,000 events were recorded, gated by setting side scatter (SSC) at 350 and forward scatter (FSC) at 44. Finally, flow data was analyzed using FlowJo version 10.

Additionally, isotope controls were performed in order to investigate if any non-specific binding of the antibodies against IL-1 α and CD68 existed. IgG1 was used as isotope control for antibody against IL-1 α , while IgG2b was used as isotype control for antibody against CD68. Preparation of the cells was conducted with identical protocol as above, with exception of the antibodies. FITC conjugated MAb1.3.2 mouse-anti-human against IL-1 α propiece was double stained with 0.5 μ g/mL APC mouse-IgG2b, κ Isotype Control (BioLegend®), while APC anti-human CD68 was double stained with 0.5 μ g/mL FITC mouse IgG1 κ Isotype Control (BD Pharmingen™) (antibodies are presented in table 3).

Antibodies for Flow Cytometry	Isotype Control
MAb1.3.2 mouse-anti-human IL-1 α propiece FITC	FITC mouse IgG1 κ Isotype Control
APC anti-human CD68	APC mouse-IgG2b, κ Isotype Control

Table 3: Overview of the antibodies and their respective isotype controls used in flow cytometry

3.10 Polymerase Chain Reaction

Five microliter cDNA was used as template cDNA for PCR. The cDNA was added to a master mix containing 5 μ L 10xDreamtaq buffer (containing 20mM MgCl₂), 5 μ L dNTP mix, 1 μ L dreamtaq

polymerase and 34 μL nuclease-free water. Additionally, 2.5 μL forward primer (5 μM) and 2.5 μL reverse primer (5 μM) were added to each tube, see table 4 for additional primer information.

Cytokine name	Type	Primer
IL-1 α	Forward	GCTGCCAGCCAGAGAGGG
	Reverse	CATGGTACATATGAACTGTCAACACTGCAC

Table 4: Primer sequence of the primers used for PCR

The PCR tubes were placed in a thermal cycler and PCR were carried out with the following program setup:

1 Cycle	40 Cycles	1 Cycle	Hold
95 °C in 30 sec	95 °C in 30 sec 58 °C in 30 sec 72 °C in 60 sec	72 °C in 7 min	4 °C hold

3.11 Gel Electrophoresis

Following PCR, the products were analyzed by gel electrophoresis. 10 μL PCR product with 2 μL 6x DNA loading die (Thermo Scientific) was added in each well of a 1 % agarose gel (TBE-buffer containing 1 % agarose NA (GE Healthcare Bio-Science AB) and 0.3 $\mu\text{g}/\text{mL}$ ethidium bromide). GeneRuler 1kb DNA ladder (Thermo Scientific) was used as a reference. The gel electrophoresis was carried out at 100V for 30 min. The gel was visualized using a MiniBIS Pro (DNR Bio-Imaging Systems) and the software GelCapture.

3.12 PCR Purification

Prior to DNA sequencing the PCR products were purified by use of a GeneJET PCR Purification Kit (Thermo Scientific) according to the manufacturer's protocol. The PCR product was mixed with binding buffer and isopropanol in a concentration of 1:1:1. The solution was transferred to a purification column and washed with washing buffer according to the manufacturer's protocol. Lastly, the PCR product was eluted from the column with elution buffer and stored at -25 °C.

3.13 DNA Sequencing

PCR product from cells stimulated with LPS for 4 hours was used for sequencing the IL-1 α gene, since gel electrophoresis showed a strong band at this time. For each subject two PCR samples were sent to sequencing, one containing 2.5 $\mu\text{M}/\mu\text{L}$ forward primer and one containing 2.5 $\mu\text{M}/\mu\text{L}$ reverse primer. Samples for all subjects were sent to GATC Biotech AG (Konstanz, Germany) for DNA sequencing. Results from sequencing were displayed through CLC Genomic Workbench 6.5.1.

3.14 Statistical analysis

Data were presented as the mean \pm standard deviation of the mean (mean \pm SD). One way repeated measures analysis of variance (ANOVA) was used for statistical analysis. Bonferroni was used as post hoc test. When the data was not normally distributed, Friedman's test was performed. The significance level were set at $P < 0.05$. All statistical analysis was performed using SPSS software version 22.

4. Results

4.1 IL-1 α Propiece Identification by Immunofluorescence Staining

To confirm that macrophages stimulated with LPS causes IL-1 α production, immunofluorescence staining was performed. Mononuclear cells were isolated by use of lymphoprep™ and seeded in a permanox chamber (see figure 5A). The cells were incubated for macrophages to attach to the surface. Thus, when the cells were washed, a selection of primarily macrophages remained in the wells for immunofluorescence staining, as seen in figure 5B. Following 12 hours of incubation with LPS the cells were stained with primary antibody MAb 1.3.2 against IL-1 α propiece and secondary antibody FITC conjugated goat-anti-mouse. Microscopy showed that IL-1 α propiece was expressed primarily in the nucleus of LPS-stimulated macrophages, indicated by the green fluorescence color seen in figure 6A. This is contrary to the non-stimulated cells which did not express IL-1 α propiece, see figure 6B.

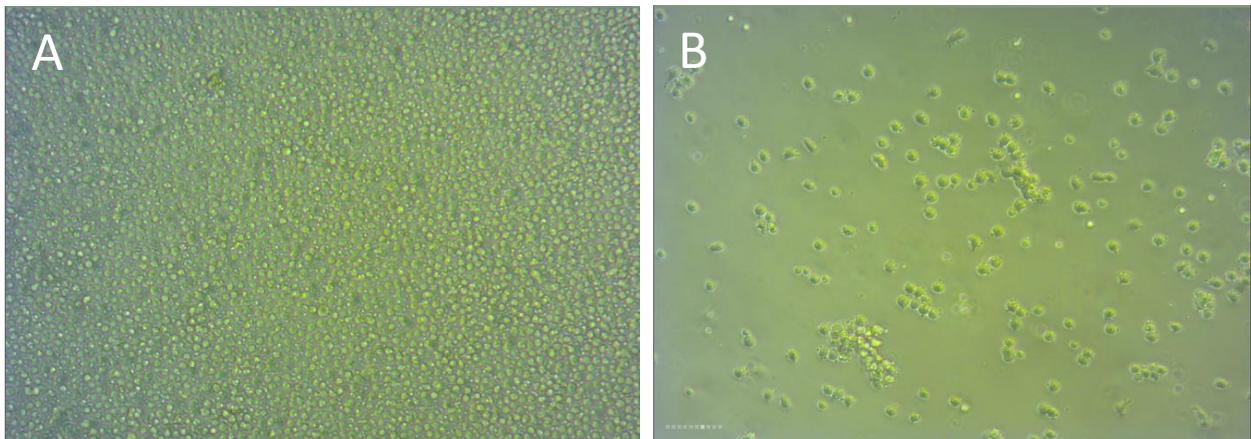


Figure 5: Mononuclear cells in a permanox chamber before and after 4 hours of incubation with subsequent washing. A: Mononuclear cells immediate after transfer to the well. B: Mononuclear cells following 4 hours of incubation and subsequent washing. Macrophages are attached to the surface, while other cells are washed away. Microscopy was performed with an inverted microscope (Carl Zeiss Primo Vert) with a Primo Plan ACHROMAT 20xobjective.

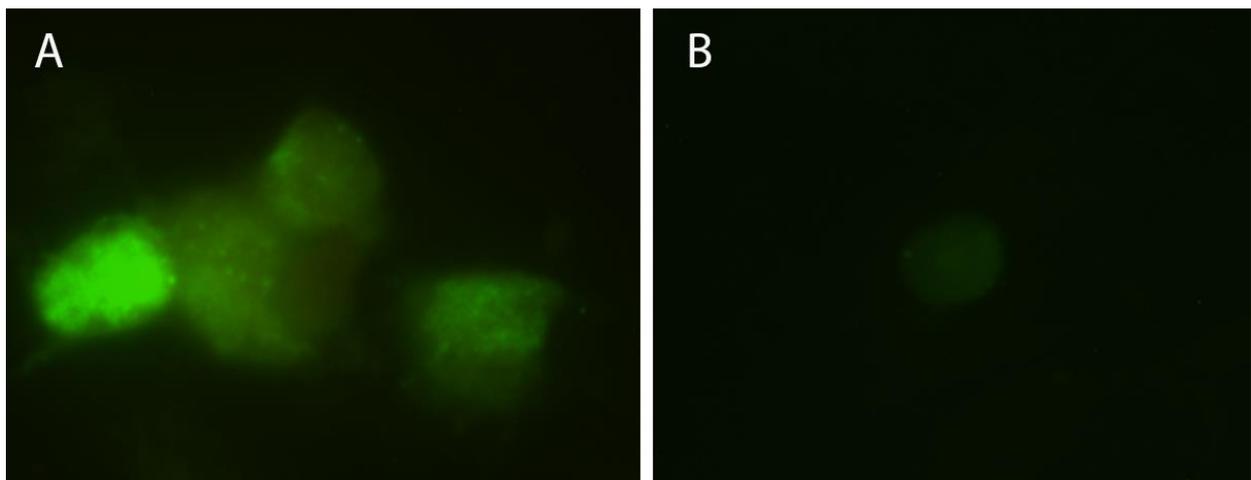


Figure 6: Immunofluorescence microscopy of macrophages stained with MAb 1.3.2 against IL-1 α propiece and FITC conjugated goat-anti-mouse following 12 hours of incubation with or without LPS. Green fluorescence color indicates expression of IL-1 α propiece. A: Macrophages stimulated with LPS, demonstrates that IL-1 α propiece is present following stimulation. B: Non-stimulated macrophages do not express IL-1 α propiece. Microscopy was performed with a Leica DM5500 fluorescence microscope with a 100xobjective.

4.2 Validation of RNA Ekstraction and cDNA Synthesis

RNA was extracted from mononuclear cells with the purpose of synthesizing cDNA to perform qPCR as well as PCR for DNA sequencing. During the process of making cDNA several validation steps were performed to make sure that the samples contained sufficient product. Firstly, RNA was extracted and the quantity was assessed by a nanophotometer. Additionally, gel electrophoresis was performed on two random samples to confirm that RNA was present after freezing for several days. As seen in figure 7A, RNA was observed in the samples demonstrated by the two ribosomal RNA bands in each sample (the two boom lines). The high molecular weight band in the top indicates that the samples were contaminated with genomic DNA. This confirms why DNase treatment was necessary prior to cDNA synthesis. Following DNase treatment, DNA was destroyed which was confirmed by lack of the upper band in the RNA gel illustrated in figure 7B. Figure 7B also confirmed that RNA was present after DNase treatment and thus cDNA synthesis could be performed. The final validation step prior to PCR and qPCR assessment was gel electrophoresis of cDNA. Electrophoresis of cDNA forms a smear in the gel as illustrated in the middle of figure 7A. Thus, cDNA was present.

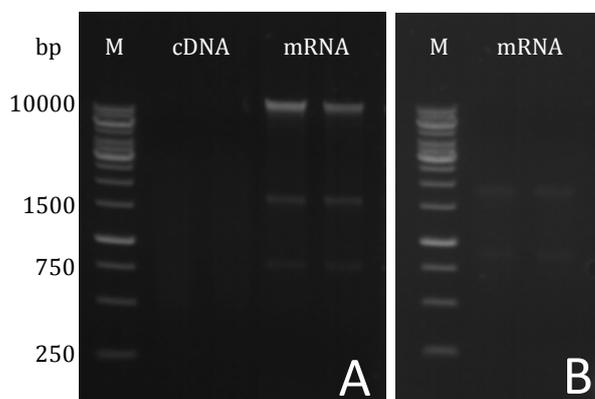


Figure 7: Two gels with RNA and cDNA from mononuclear cells of two different subjects. RNA was extracted from the cells (A), treated with DNase (B) and subsequently cDNA synthesis was done (A). The column at the left in both A and B is the DNA marker (M) which is a 1 kb ladder. A: The two columns at the right show RNA following extraction with two ribosomal RNA bands. The high molecular weight band in the top indicates presence of DNA in the RNA samples. The two columns in the middle demonstrate a smear from DNase-free cDNA. B: RNA following DNase treatment. DNA was no longer present in the sample, indicated by the disappearance of the upper band as seen in gel A. The images were carried out with a MiniBIS Pro.

4.3 Efficiency Curves for qPCR

A five-fold serial dilution of cDNA from LPS-induced macrophages was performed to test the primers amplification efficacy for qPCR. Primers for *IL-1 α* , *IL-1 β* , β_2 -microglobulin and *CD68* genes were tested and their efficiency curves are presented in figure 8-11. The efficiency of primers for *IL1A* and *B2M* was 98.8 % and 101.4 %, respectively, while the Pearson Correlation Coefficients (R^2) was 0.999 and 0.998, respectively (see figure 8-9). Primers for *IL1B* and *CD68* had an efficiency of 76.0 % and 133.4 %, respectively, and their linear fit were $R^2=0.882$ and $R^2=0.933$, respectively (figure 10-11). An efficiency of 90-110 % and a linear fit of $R^2\geq 0.985$ were considered acceptable, why only primers for *IL1A* and *B2M* were further used in the study.

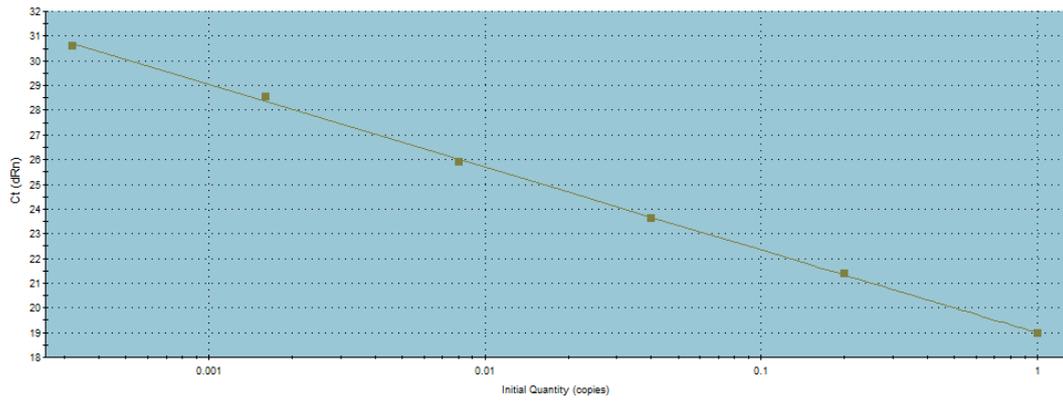


Figure 8: Efficiency curve of primers for *IL1A*, efficiency=98.8 %, $R^2=0.999$.

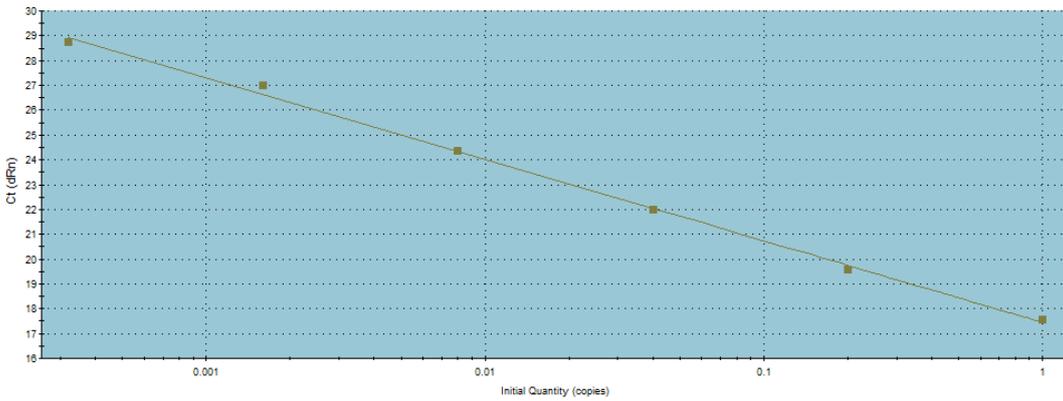


Figure 9: Efficiency curve of primers for *B2M*, efficiency=101.4 %, $R^2=0.998$.

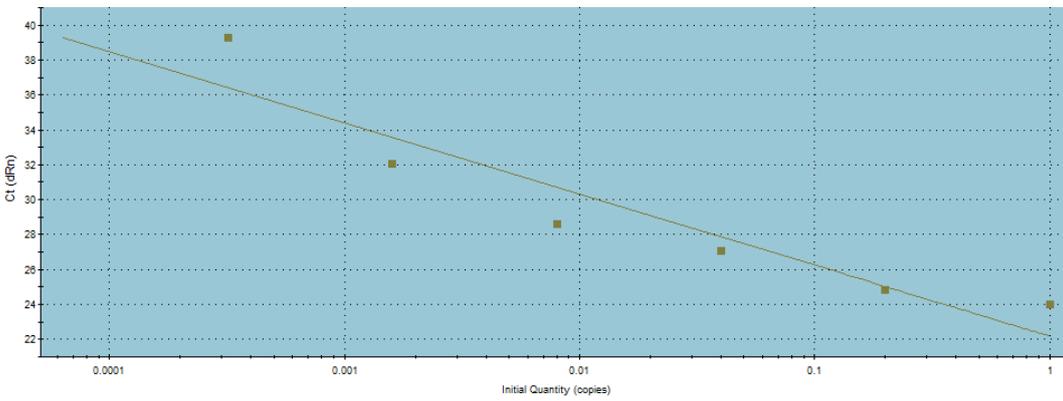


Figure 10: Efficiency curve of primers for *IL1B*, efficiency=76.0 %, $R^2=0.882$.

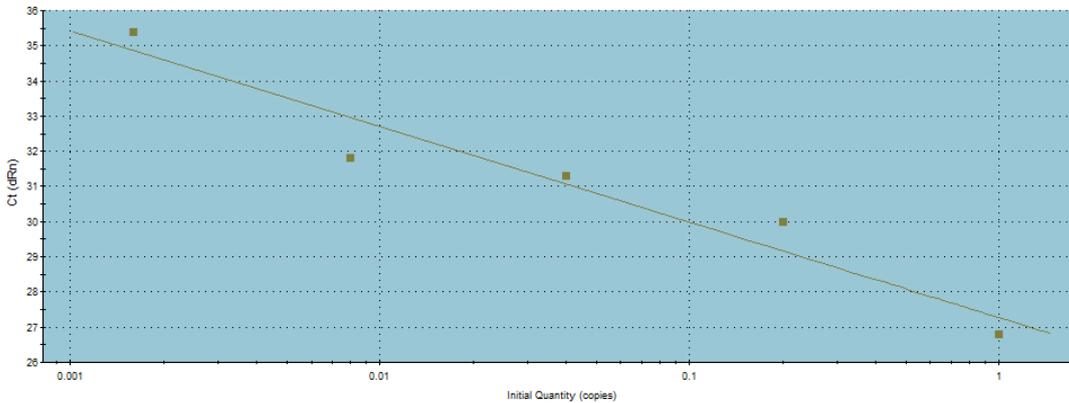
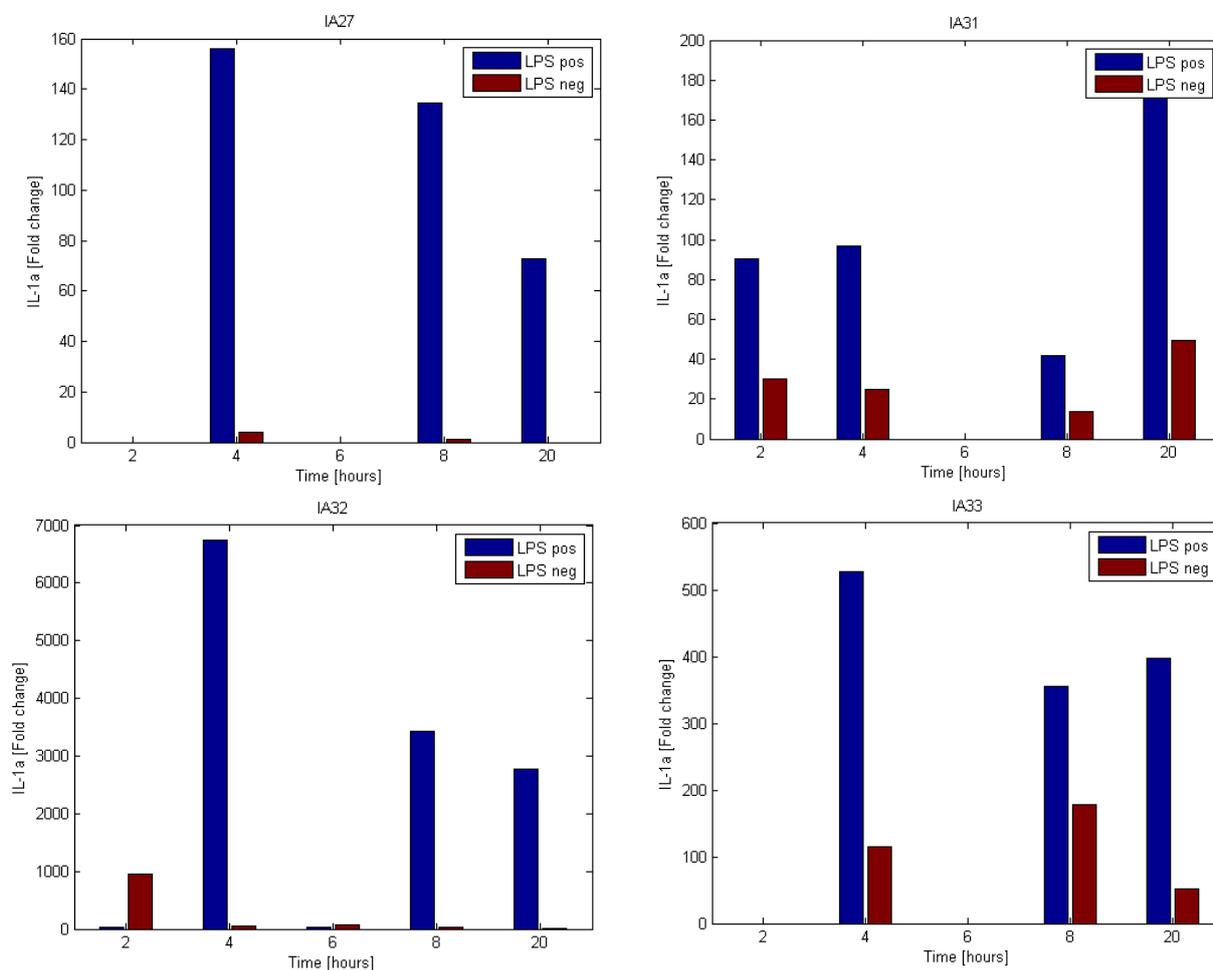


Figure 11: Efficiency curve of primers for *CD68*, efficiency=133.4 %, $R^2=0.933$.

4.4 IL-1 α mRNA Expression by qPCR

The kinetics of IL-1 α mRNA expression in blood macrophages from ten healthy subjects was examined by qPCR. The expression of IL-1 α was investigated by extracting mRNA from mononuclear cells which were stimulated with LPS for 2, 4, 6, 8 and 20 hours, respectively. Likewise, mRNA was extracted from non-stimulated control cells that were incubated in the same amount of time, including a sample incubated for 0 hours. The mRNA samples were used for cDNA synthesis and subsequently qPCR was performed. B₂-microglobulin was used as a reference gene in calculating the expression of IL-1 α by the Pfaffl equation which is found in section 3.8. The equation presented the IL-1 α expression as a fold change compared to the non-stimulated, non-incubated (0 hours) control sample. The results revealed high inter-individual variability in the up-regulation of IL-1 α mRNA, as seen in figure 12. However it was clear that LPS stimulation caused an enhanced transcription of IL-1 α within the first two to four hours. Moreover, the results indicated that LPS-stimulated cells caused a greater expression of IL-1 α mRNA compared to non-stimulated control cells. Though, because of the high inter-individual variability it was difficult to state any general assumptions regarding difference in the expression of IL-1 α mRNA over time. Non-stimulated control cells generally demonstrated low up-regulation of IL-1 α mRNA, with the exception of high expression in cells incubated for two hours in some individuals. The means of the mRNA expression was conducted in order to perform statistical analysis, and Friedman's test was used since the data were not normally distributed. No significant difference was observed between the stimulated cells ($P>0.05$) which probably was due to the high standard deviations as seen in figure 13. When comparing the stimulated cells with the non-stimulated controls at equal time point, a significant difference was seen at 4 and 20 h ($P=0.011$, $P=0.002$ and $P=0.002$). While no difference was demonstrated at 2, 6 and 8 h ($P=0.480$, $P=1.000$ and $P=0.058$).



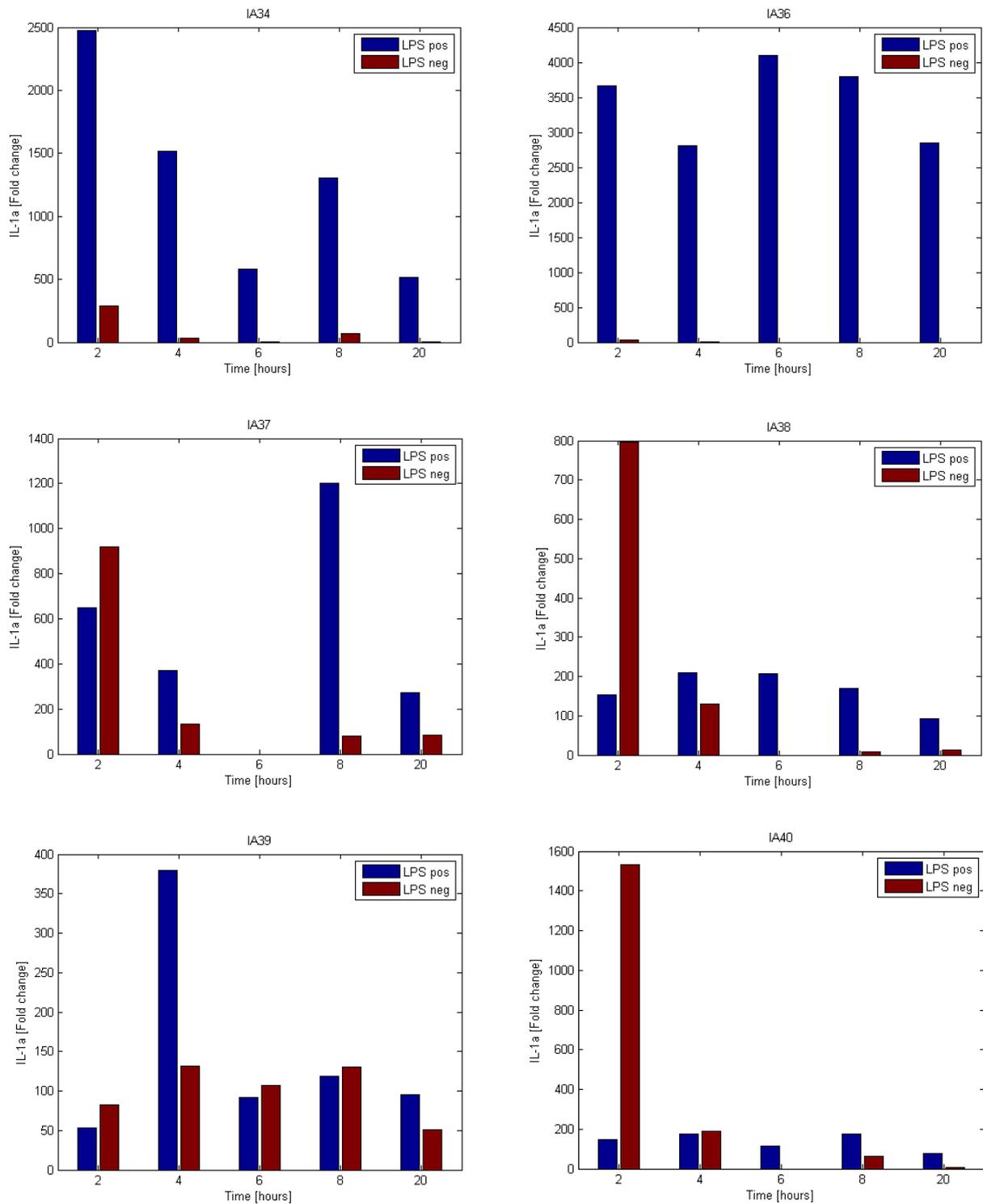


Figure 12: IL-1 α mRNA expression in LPS-stimulated (blue bars) and non-stimulated (red bars) macrophages from ten healthy subjects measured by qPCR. The cells were incubated with or without LPS for 0, 2, 4, 6, 8 and 20 hours, respectively. The IL-1 α expressions were normalized to the reference gene β_2 -microglobulin by using the Pfaffl equation. The expression was presented as the fold change compared to a non-stimulated, non-incubated (0 hours) control. The results illustrate great inter-individual variability, thus a high increase in expression is observed within 2-4 hours.

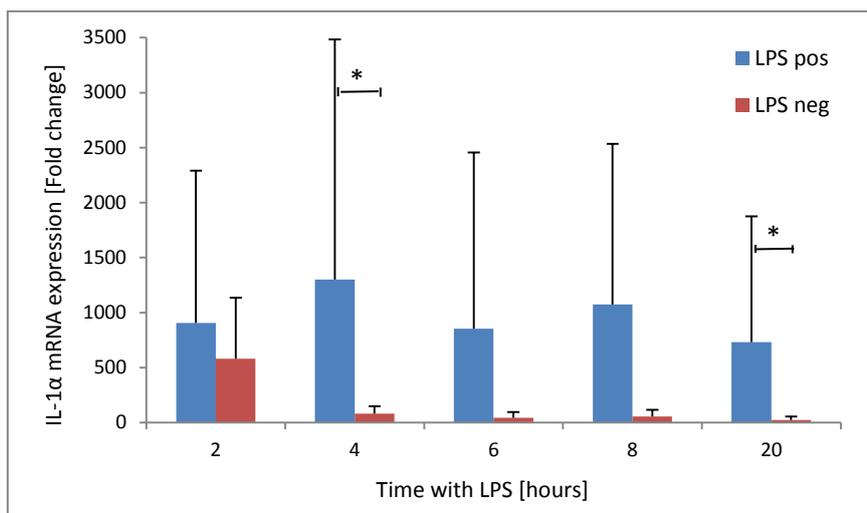


Figure 13: Mean data of the fold change in IL-1 α mRNA expression of blood macrophages from ten subjects measured by qPCR. Pfaffls equation was used to calculate the fold change in expression from a non-stimulated control (0 hours). Cells were stimulated with LPS or incubated for 0, 2, 4, 6, 8 and 20 hours, respectively. Blue columns indicate LPS-stimulated cells, while red columns indicate non-stimulated cells. Significant difference was observed at 4 and 20 h, when comparing LPS-stimulated cells with non-stimulated cells. No significant difference was observed between the times in the stimulated cells. * indicates statistical significant difference with $P < 0.05$.

4.5 Validation of FITC Conjugated Antibody for Flow Cytometry

Initially, immunofluorescence staining was conducted in order to validate the FITC conjugation of MAb 1.3.2 antibody against IL-1 α propiece. The FITC to antibody ratio was 3.2:1. Staining was performed on macrophages stimulated with LPS for 4 hours. The antibody caused a positive staining primarily of the cell nucleus in the LPS stimulated cells, while no staining was present in the non-stimulated cells as seen in figure 14A and 14B, respectively. This confirms the results from figure 6, that IL-1 α propiece is expressed in the nucleus of macrophages following LPS stimulation. Thus, the antibody was suggested to be suitable for identifying IL-1 α propiece expression by flow cytometry. Additionally, the strong staining indicated that a concentration of 2 μ L/mL of the antibody was a proper concentration for staining. Though, the staining was weaker than when two layers were used with the secondary antibody shown in figure 6A.

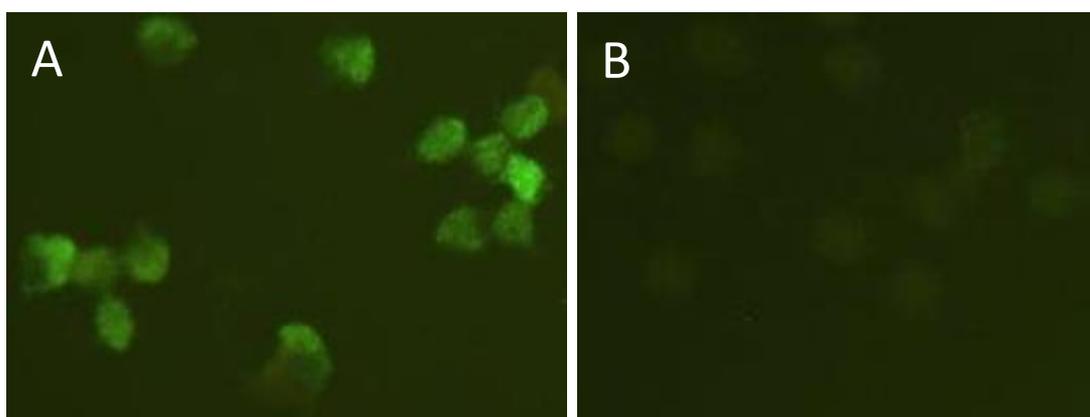


Figure 14: Immunofluorescence staining of macrophages with 2 μ L/mL FITC conjugated MAb 1.3.2 IL-1 α propiece following 4 hours of incubation with or without LPS. Green fluorescence color indicates expression of IL-1 α propiece. A: Macrophages stimulated with LPS, which demonstrates that IL-1 α propiece is present in the nucleus following stimulation. This showed that the FITC conjugated antibody was valid. B: Non-stimulated macrophages without IL-1 α propiece staining. Microscopy was performed with a Leica DM5500 fluorescence microscope with a Q imaging retiga 2000R camera and a 40xobjective.

4.6 IL-1 α Propiece Expression by Flow Cytometry

The expression of IL-1 α propiece in LPS stimulated macrophages was examined by flow cytometry. The concept of estimating IL-1 α expression by measuring the equivalent IL-1 α propiece by flow cytometry, have never been done before with the used antibody. Cells stimulated with LPS in a varying time period were stained with FITC conjugated MAb1.3.2 Propiece IL-1 α antibody in order to investigate the kinetics of IL-1 α propiece. Double staining with APC anti-human antibody against the specific surface marker CD68 was performed in order to identify macrophages. Data were generated by the Flow Cytometer and was analyzed in in software FlowJo. Initially, the cells were gated in order to isolate macrophages for further analysis. This was done by gating the cell population according to expression of CD68 and their relative cell size expressed by forward scatter height (FSC-H), see figure 15. High fluorescence intensity of APC equaled presence of CD68, thus the CD68 positive population was located at the right side of the plot, as gated for in figure 15. Subsequently, the CD68 positive cells were investigated for the expression of IL-1 α propiece, which was demonstrated by a histogram as seen in figure 16. The histogram illustrated that macrophages were divided into an IL-1a positive and an IL-1a negative population. The IL-1a positive population was located to the right since the higher fluorescence intensity of FITC represented a greater amount of IL-1 α propiece. In order to investigate the kinetics of IL-1 α production in LPS stimulated macrophages, histograms of cells stimulated in a different amount of times were fused together, as seen in figure 17. Gating of cells for these histograms was done with the attempt of getting approximately the same amount of cells at the different time points, but without affecting the IL-1a positive population. Therefore, the IL-1a negative population probably contained more cells than illustrated thought the fluorescence intensity was representative of the population. The histograms demonstrated that macrophages expressed an increasing amount of IL-1 α when stimulated with LPS for a longer period of time. This was indicated by the increasing fluorescence intensity of FITC which caused movement of the IL-1 α positive population to the right in the histogram. Furthermore, the height of the IL-1 α positive population indicated that a larger amount of macrophages expressed IL-1 α when stimulated for a longer period of time. The non-stimulated control cells stained after 0 hours, demonstrated a large number of cells but a low fluorescence intensity which indicated no presence of IL-1 α .

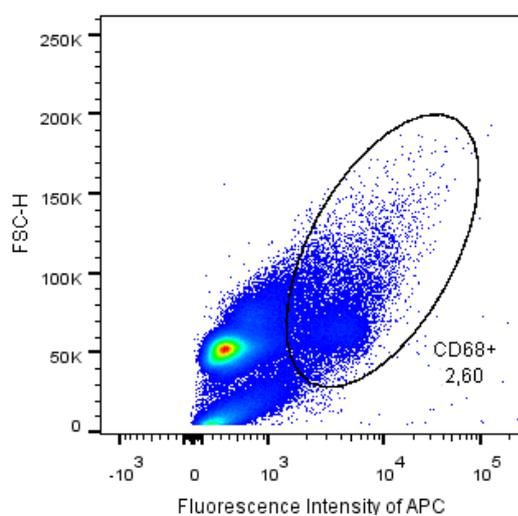


Figure 15: Pseudocolor plot with gating strategy of CD68 positive (CD68+) cells in a mononuclear cell population. The black circle indicates the isolation of a CD68+ population. The cells were extracted from donor IA36 and were stimulated with LPS for 4 hours. FACS was performed with a BD FACS Canto™. SSC-A: 346, FSC-H: 44.

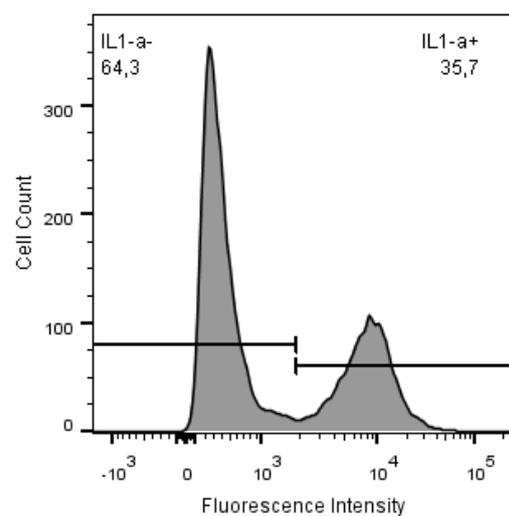
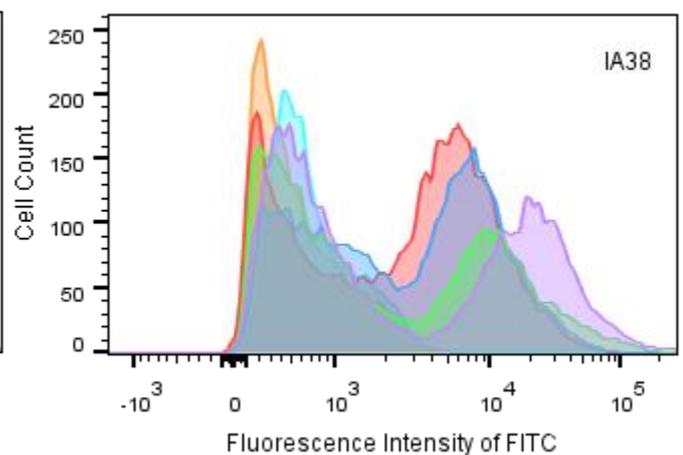
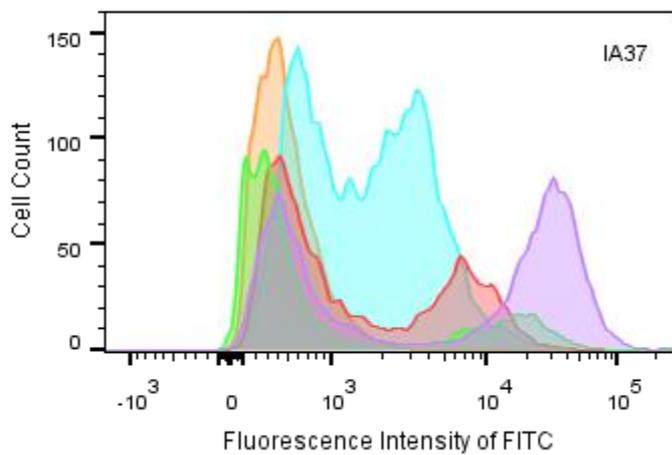
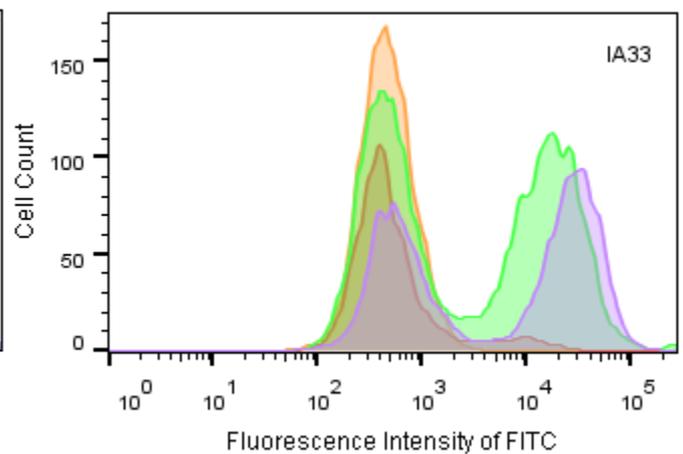
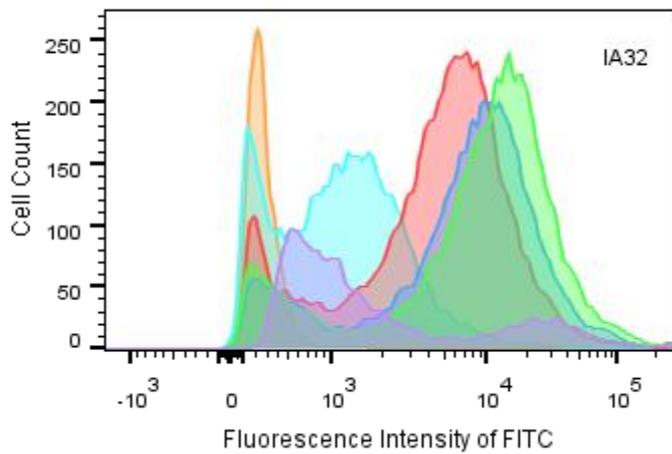
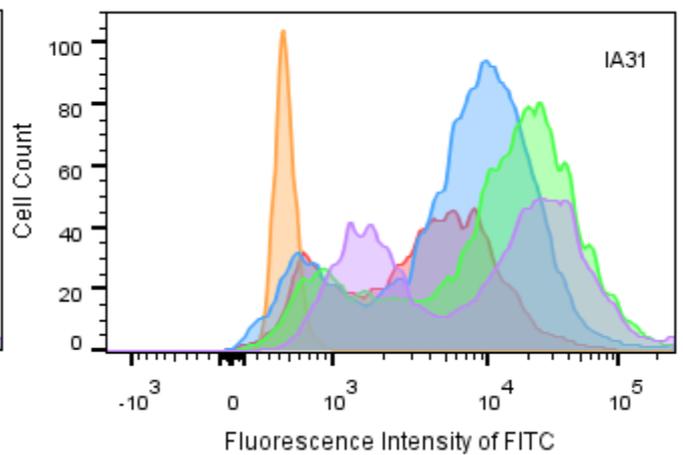
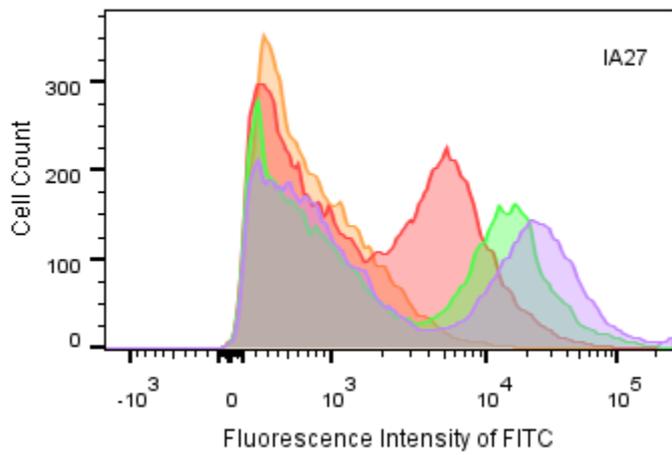
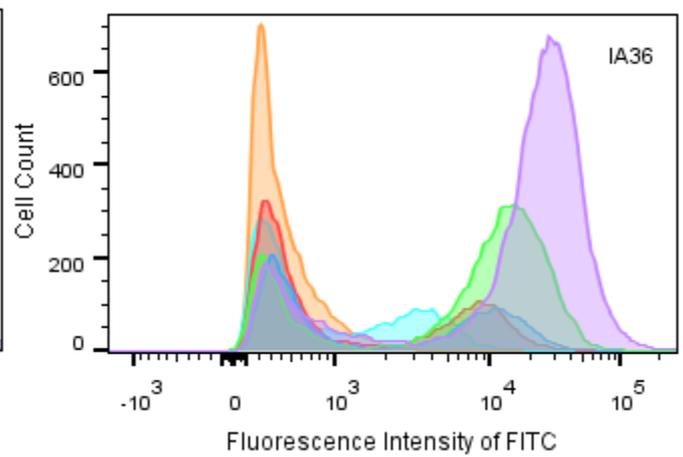
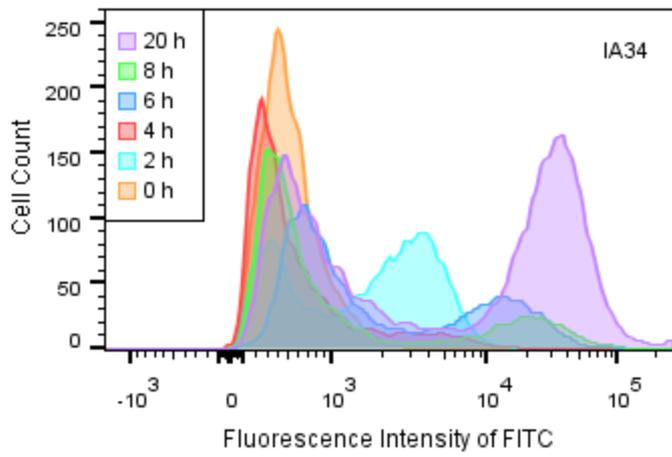


Figure 16: Histogram showing the fluorescence intensity of FITC against IL-1 α in a CD68 positive population. The black lines indicates an IL-1 α negative (IL-1a-) and an IL-1 α positive (IL-1a+) subpopulation. The cells cells in the histogram were stimulated with LPS for 4 hours. FACS was performed with a BD FACS Canto™. SSC-A: 346, FSC-H: 44.



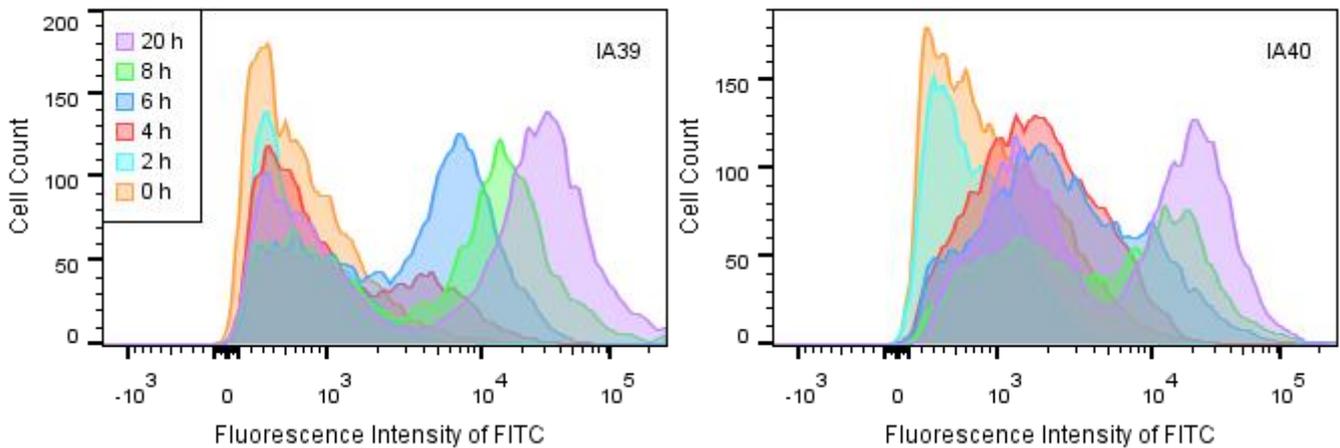


Figure 17: Histogram overlay of the IL-1 α expression in macrophages stimulated with LPS for varying times in ten healthy subjects. Orange color indicates non-stimulated cells (0 h) while the rest was stimulated with LPS for 2, 4, 6, 8 and 20 hours, indicated by light blue, red, dark blue, green and purple color, respectively. In some subjects the population at 2 or 6 h lacks due to insufficient amount of cells. The cell populations were all gated for being CD68 positive and illustrate the fluorescence intensity of FITC antibody against IL-1 α . The overlay indicates that a longer period of stimulation with LPS increases the fluorescence intensity and thus the expression of IL-1 α .

For statistical analysis of the IL-1 α response the median fluorescence intensity of the IL-1 α positive population was extracted from FlowJo. This was done by dividing the populations at the lowest point between the two peaks as seen in figure 16. In the non-stimulated cells (0 h), the median fluorescence intensity was extracted from the IL-1 α negative population since no positive population was present. The mean of the median fluorescence intensity for the ten subjects at each time point was calculated and presented in figure 18. It is clearly demonstrated that the fluorescence intensity of the cells increases with the amount of time stimulated with LPS. Statistical analysis was performed only for the medians at 0, 4, 8 and 20 hours, since data for 2 and 6 hours were missing in some individuals due to inadequate amount of cells. Statistical significance existed when comparing the non-stimulated cells (0 h, 586.7 ± 219.6) with cells stimulated for 4, 8 and 20 hours (4: 5962.5 ± 1159.8 , 8: 15110.7 ± 2108.8 , 20: 27015.8 ± 3589.1 , all $P < 0.000$). Furthermore, when comparing the stimulated cells with each other, the median fluorescence intensity was statistical different at 4 and 8 h as well as 8 and 20 h (both $P < 0.000$).

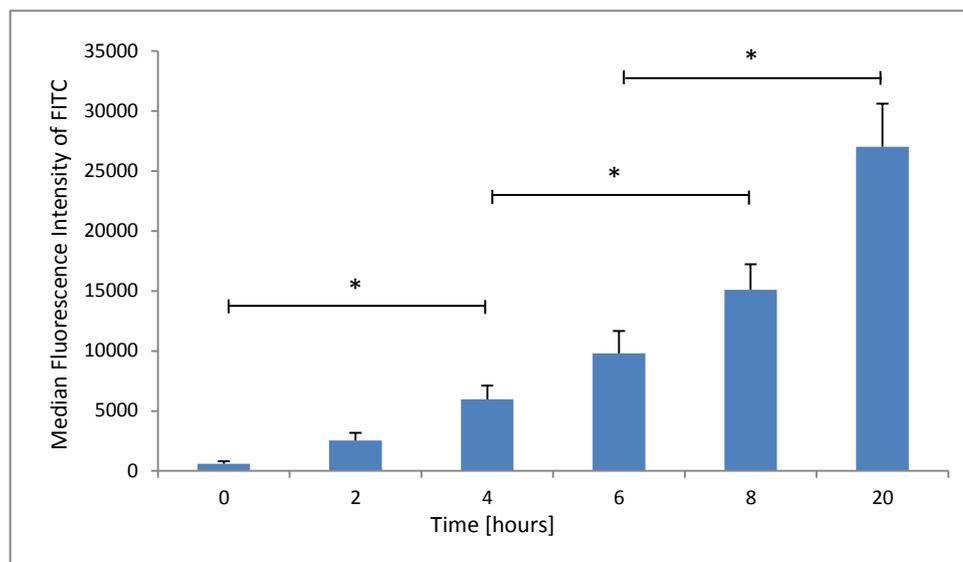


Figure 18: Mean data of the median fluorescence intensity of FITC antibody against IL-1 α propiece in blood macrophages from ten subjects. The cells were stimulated with LPS for 0, 2, 4, 6, 8 and 20 hours, respectively. It is clearly demonstrated that fluorescence intensity of FITC increases with the amount of time that cells were stimulated with LPS, indicating higher protein synthesis of IL-1 α . Significant difference was observed when comparing 0 to 4 h, 4 to 8 h, and 8 to 20 h.* indicates statistical significance with $P < 0.05$.

For each subject a negative control, incubated for 20 h, was conducted in order confirm that the LPS stimulation was responsible for the IL-1 α response. The results were visualized by a histogram as seen in figure 19, and gating strategy was similar to that in figure 17. One random histogram was presented, since all the subjects had highly similar results. The histogram demonstrated that the non-stimulated cells which were incubated for 20 h have equal fluorescence intensity of FITC as the non-stimulated cells at 0 h, while only the stimulated cells expressed an IL-1 α positive population. This was verified by statistical analysis of the median fluorescence intensity of FITC, where no significant difference was seen between the two non-stimulated cell groups (0 h: 555.0 ± 28.7 vs. 20 h: 606.8 ± 40.0 , $P=0.688$). When comparing the IL-1 α positive population in the LPS stimulated cells (38418.8 ± 3822.8) with the IL-1 α negative population in any of the two non-stimulated cell groups (0 or 20 h), a statistically significant difference was seen in the median fluorescence intensity ($P<0.000$).

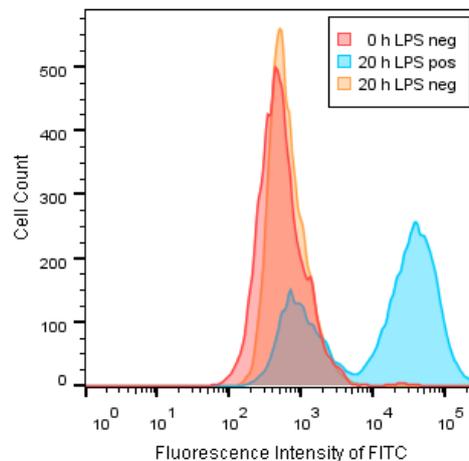


Figure 19: Histogram of the IL-1 α expression in a CD68 positive population. The blue population of cells was stimulated with LPS for 20 hours. The orange and red populations were non-stimulated cells incubated for 20 and 0 h, respectively. The LPS stimulated cells were the only group of cells which had an IL-1 α positive population.

Finally, isotype controls were conducted IL-1 α and CD68 antibodies in order to identify any unspecific binding. The isotype control used for the antibody against IL-1 α was IgG1, while the one used for CD68 antibody was IgG2b. No unspecific binding was observed since the population with the isotype control was located along with the IL-1 α and CD68 negative population, as seen in figure 20A-B.

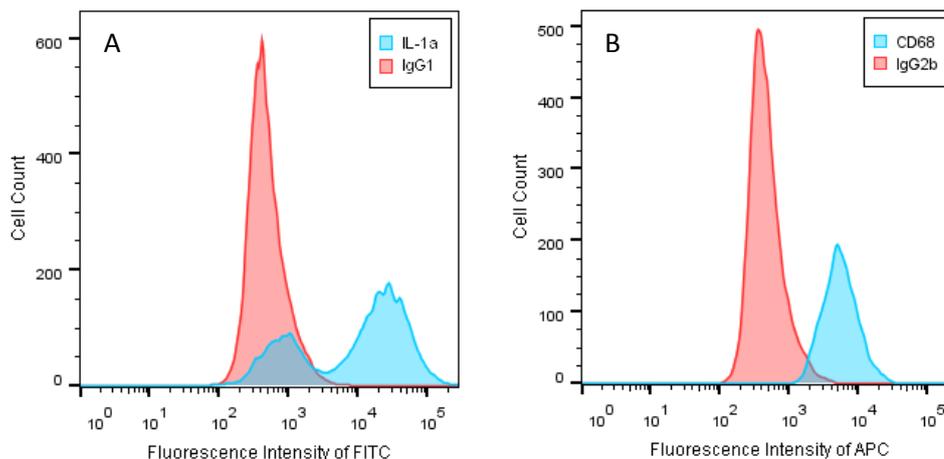


Figure 20: Histograms of isotype controls compared to staining for IL-1 α and CD68, respectively. The red population of cells shows the isotope control while the blue populations indicate expression of IL-1 α and CD68, respectively. No unspecific binding exists since the fluorescence intensity of the isotype controls is similar to the negative population of IL-1 α /CD68. A: IgG1 Isotype control and FITC conjugated MAB1.3.2 mouse-anti-human antibody against IL-1 α propiece. B: IgG2b Isotype control and APC anti-human antibody against CD68.

4.7 Comparison of IL-1 α Translation and Transcription

The kinetics of IL-1 α in response to LPS stimulation was investigated in two levels, the transcription by qPCR and the translation by flow cytometry. The combined results are illustrated in figure 21. Looking at the IL-1 α expression of the two methods together, it was revealed that the amount of mRNA increased rapidly and stayed elevated, while the protein synthesis was slowly but significantly enhanced over time.

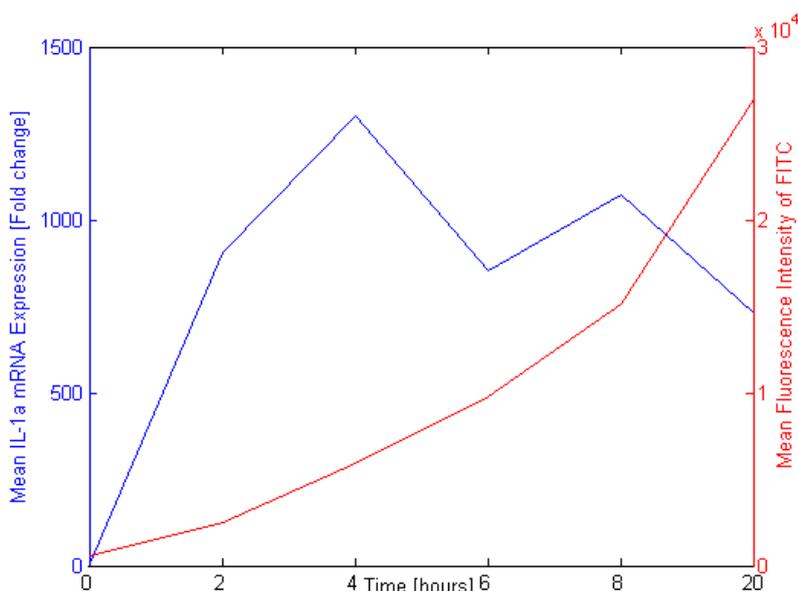


Figure 21: The association between the transcription and the translation of IL-1 α , indicated by the blue and the green line, respectively. Macrophages were stimulated with LPS for a different amount of time and investigated for IL-1 α expression. The mRNA expression increased rapidly while the protein synthesis slowly but significantly increased in response to transcription. The mRNA expression was measured by qPCR and was presented as fold change in comparison to a non-stimulated control. Protein synthesis was investigated by flow cytometry by measuring the median fluorescence intensity of FITC antibody against IL-1 α protein.

4.8 PCR Product for DNA Sequencing

Primers used for PCR were placed outside the coding DNA sequence of *IL1A*, with an expected PCR fragment length just under 1000bp. PCR was performed on cDNA from LPS-stimulated mononuclear cells with the purpose of DNA sequencing the IL-1 α gene. In the experimental setup cells were stimulated with LPS for a varying amount of time, thus the optimal condition for DNA sequencing was investigated in two random subjects. PCR was conducted on cDNA from cells stimulated with LPS for 0 (non-stimulated control), 2, 4, 8 and 20 h, respectively. The PCR product was visualized by gel electrophoresis as seen in figure 22. The gel confirms that IL-1 α was expressed in LPS-stimulated cells, while no IL-1 α product was demonstrated in the non-stimulated control cells (0 h). The gene marker (M) shows that the PCR products were migrated to a position corresponding to a length of 1000 base pairs, which corresponds to the expected size. The thickness of the bands indicates that the greatest IL-1 α expression was seen in the cells stimulated for 4 hours. Thus, in the rest of the subjects, PCR was restricted to cells stimulated with LPS for 4 h for subsequent DNA sequencing. PCR products from each individual were visualized by gel electrophoresis before DNA sequencing in order to confirm that the samples contained the PCR product of 1000 bp.

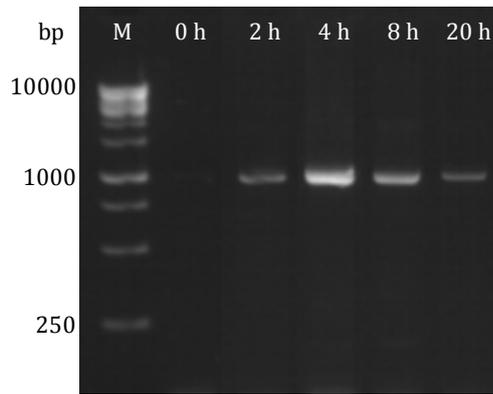


Figure 22: Gel containing PCR product of IL-1 α DNA from mononuclear which were stimulated with LPS for 0*, 2, 4, 8 and 20 hours (h), respectively. The DNA marker (M) is a 1 kb ladder, which shows that the DNAs are migrated to a position corresponding to a length of 1000 base pairs (bp). The thickness of the band indicates that the greatest amount of DNA is present after 4 h of stimulation with LPS. The image was carried out with a MiniBIS Pro. * This was a non-stimulated control.

4.9 SNP in the IL-1 α Gene

DNA sequencing was performed in order to investigate the existence of any SNPs in the IL-1 α gene of healthy individuals of Caucasian origin. Ten subjects were examined, and one similar SNP was identified in six individuals, as seen in figure 23. Figure 24 demonstrates the difference in the mRNA sequence of one subject with the SNP and one subject without. In all cases the SNP was heterozygous causing the conflict code K (G/T). A consequence of the SNP is a substitution of the amino acid number 114 from guanine (G) to thymine (T) in the IL-1 α gene. The SNP was identified using the software BLAST as having the genomic location NC_000002.12 with base pair number 112779646. The whole sequenced IL-1 α gene is available in appendix A.



Figure 23: A cut of the mRNA from IL1A of the ten subjects which was sent to GATC Biotech AG (Konstanz, Germany) for DNA sequencing. An identical SNP was demonstrated in six of the ten subjects, indicated by the conflict code k which represents a replacement in one base from G to T. The red X in the translation sequence indicates that the SNP results in a change in the amino acid adenine. Results from sequencing were displayed through CLC Genomic Workbench 6.5.1.



Figure 24: A cut of the cDNA sequence of IL-1 α from two subjects. The sequence from IA33 contains a SNP, demonstrated by the conflict code K, while the sequence from IA34 does not have a SNP. It is illustrated that the SNP is heterozygote since the sequence contains both the base G and the base T, which is indicated by the black and the red bump, respectively, at the SNP location. DNA sequencing was performed by GATC Biotech AG (Konstanz, Germany). Results from sequencing were displayed through CLC Genomic Workbench 6.5.1.

5. Discussion

The aim of this study was to investigate the IL-1 α response with the use of LPS as inducer. LPS is a pathogen widely used for activation of the innate immune system and as expected this study confirmed its ability. Immunofluorescence staining, qPCR and flow cytometry all demonstrated that LPS stimulation causes macrophages to express IL-1 α , which is discussed in the following.

5.1 Expression of mRNA

In this study, qPCR was performed to investigate if any changes in the transcription of IL-1 α occurred over time. RNA was extracted from mononuclear cells, and the quantity was measured by a Nanophotometer. The measurement with the Nanophotometer seemed rather variable and the quality of RNA was not secured since the device could not distinguish between RNA and DNA. A measurement following cDNA synthesis would have provided a more accurate knowledge about the quantity for qPCR. Though, normalization to a reference gene should account for inconsistency in the cDNA concentration. In this study B₂-M was used as a reference gene to measure the relative quantity of IL-1 α presented as the fold-change compared to a non-stimulated control at 0 h. B₂-M is a protein which exists in a relative stable concentration of most cell types and was therefore used to correct for variation in the quantity of applied cDNA. Result with a Ct value more than 30 in the B₂-M sample was excluded from the analysis since this indicated an inadequate amount of cDNA.

The results of qPCR revealed great inter-individual IL-1 α response ranging from less than a 200 fold increase to a several thousand fold enhancement in the expression. Though, in general it was indicated that transcription of IL-1 α was highly increased in LPS-stimulated macrophages within the first 2-4 hours of stimulation, and that an enhanced expression stayed throughout the 20 hours of investigation. This was verified by statistically significant higher expression at 4 and 20 hours when comparing the LPS stimulated cells with the non-stimulated control cells at the corresponding times ($P < 0.05$). No significant increase was observed in the stimulated cells at 2, 6 and 8 h, which could be due to the high inter-individual variation, the high expression in non-stimulated cells or to the fact that data was missing for some of the subject at these times and might have caused inadequate statistical power. No statistical difference was found in the expression between the LPS-stimulated cells over time, highly because of the individual variation. However, in the majority of the subjects the lowest expression was observed at 20 h indicating a regression of the response at this point. The high expression observed at 2 hours demonstrated that the IL-1 α response was initiated very shortly after LPS stimulation and more time intervals in the beginning was needed for further investigation of the onset. This was confirmed in a study by Turner et al.⁴⁷ who found an increase in IL-1 α mRNA expression within 30 min after LPS stimulation of mononuclear cells.

Contrary to expectations, the non-stimulated cells also revealed increase in transcription of IL-1 α , though the expression in general was substantial lower than in the LPS stimulates cell. Especially at 2 hours of incubation the non-stimulated cells were positive, which might indicate contamination at this time point. Initially all the non-stimulated cells showed to be contaminated, possible due to contamination of the macrophage media, and thus the samples were redone with better cautions. Though, due to time limitations it was not managed to redo the results from IA31 and IA39 which might explain the positive expression in cells from these subjects. Besides contamination, an explanation for the higher expression in the control cells might be that they reacted by being outside their familiar surroundings or that handling of the cells caused a stressful situation. The fact that the

non-stimulated cells at some times have caused a greater response than the stimulated cells, suggests that handling the cells have affected the results. This might also have affected the great differences between the subjects in the LPS-positive cells.

For the study, mononuclear cells were extracted from the donors, why it would be uncertain to state that macrophages were the only type of cells responsible for the IL-1 α expression demonstrated by qPCR. It would have been optimal to run additional samples with a macrophage specific protein to examine if macrophages were the cell type primarily responsible for the results, or if other cell types were influencing. Primers for CD68 were tested but showed inadequate efficiency and were excluded for further use in the study. Primers for IL-1 β were also tested for this study, but had likewise inadequate efficiency. The IL-1 β response would have been a good comparison to IL-1 α for evaluating the innate immune response.

5.2 IL-1 α Propiece

Flow Cytometry was used for determine the protein expression of IL-1 α following LPS stimulation. A FITC conjugated antibody against IL-1 α propiece was used as marker of IL-1 α synthesis, since the proteins are synthesized in equivalent amount from the same precursor. The advantage of staining for IL-1 α propiece instead of IL-1 α itself is that IL-1 α propiece is an intracellular protein while IL-1 α can be released from the cell. Thus, measuring IL-1 α propiece provides the most accurate result. Prior to flow cytometry the antibody against IL-1 α propiece was tested by immunofluorescence staining. The results showed clear staining of the LPS-stimulated cells while nothing was seen in the control cells, thus the antibody was valid for IL-1 α propiece.

In order to verify that the IL-1 α expression occurred in macrophages, the cells were double stained with CD68. Gaiting for CD68 positive cells were done prior to the analysis of IL-1 α expression. The amount of IL-1 α propiece was presented relatively by the fluorescence intensity of FITC. A clear pattern in the translation of IL-1 α was observed in all of the subjects. The fluorescence intensity of FITC increased radically over time with the highest intensity at 20 hours of stimulation, representing that the greatest amount of IL-1 α in each cell existed at this time. Statistical analysis was only performed on results from 0, 4, 8 and 20 h, since data was not available for all subjects at 4 and 6 h and one-way repeated measures ANOVA did not allow comparison of groups with unequal amount of subjects. The analysis revealed that significant difference occurred between 0, 4, 8 and 20 hours of stimulation ($P < 0.000$), indicating that the synthesis of IL-1 α was initiated early and kept increasing with a peak at 20 h. This increasing amount of IL-1 α propiece indicated that the protein was accumulated in the cells which were consistent with existing literature^{37,38}. Despite that a similar amount of IL-1 α was synthesized in the macrophages of all subjects an inter-individual response was observed by differences in the amount of activated cells in each subject. This confirms the findings by qPCR were a great difference in mRNA expression was observed between subjects.

A non-stimulated control was performed with cells incubation for 20 hours, to ensure that the positive results were a consequence of LPS stimulation and not due to other factors. The fluorescence intensity in the non-stimulated controls at 20 h were similar to the ones in the non-stimulated control at 0 hours ($P = 0.688$), while a distinct difference was observed when comparing the stimulated cells at 20 h with the non-stimulated cells ($P < 0.000$). This indicates that LPS was the only factor responsible for the IL-1 α synthesis. However, this was inconsistent with the result from qPCR, were a positive response

was observed in several of the non-stimulated cells. This supports a theory of uncertainty in the execution of qPCR and the coherent procedures.

Isotope controls were performed for both IL-1 α and CD68 to make sure that the high fluorescence intensity was due to presence of the proteins rather than unspecific binding of the antibodies. The fact that the fluorescence intensity of the isotope controls was similar to the protein-negative population of cells and not the positive population, confirmed that no unspecific binding existed.

4.3 The IL-1 α Response

When combining the results of qPCR and flow cytometry it was observed that mRNA expression increased rapidly while the protein synthesis was slowly enhanced over time. This corresponds to the knowledge that transcription of genes occurs prior to translation. Furthermore, the transcription remains high throughout the 20 hours of investigation, which besides LPS stimulation could be explained by increasing synthesis of IL-1 α causing autocrine and paracrine stimulation. If IL-1 α itself is partly responsible for the maintenance of self-transcription could be investigated by application of IL-1R signaling inhibitors such as IL-1Ra. The early activation and consistent stimulation of IL-1 α in macrophages indicates that IL-1 α have an essential role in the inflammatory response and might be involved in the development of diseases caused by gram-negative bacteria such as ReA.

The overall inter-individual variability which was observed in this study demonstrated that the IL-1 α response to LPS stimulation can be highly individual. However, a similarity in the response also existed and it was rather the intensity than the pattern of the response that was individual.

5.4 SNP in the IL-1 α Gene of Healthy Subjects

In this study it was investigated if any SNPs existed in the IL-1 α gene of ten healthy individuals of Caucasian origin. This was done in order to examine if SNPs in the gene affected the IL-1 α response to LPS stimulation and to compare these with other eventual SNPs in a patient population in future studies. In six of the ten subjects one identical SNP in the IL-1 α gene was identified. The SNP showed to have the genomic location NC_000002.12 base pair number 112779646. By using the search tool BLAST, it was revealed that the identified SNP was identical with the SNP known as rs17561. In this study all the SNPs were heterozygote, but a homozygote variant also exists in healthy individuals^{48,49}. This was demonstrated by the prevalence of the SNP in the control group of an AS study in a German population⁴⁸. It showed that 10.1 % of the healthy population had a homozygote SNP, 36.5 % had a heterozygote while 55.7 % did not express the SNP. These results are different than the 60 % with heterozygote SNP and 40 % without SNP that was observed in this present study. However, this variation in prevalence is possibly due to the low number of subjects in this study.

A consequence of the SNP is the replacement of amino acid number 114 in the IL-1 α gene from alanine to serine, but it is uncertain if the SNP have any biological effect. Several studies have identified the rs17561 SNP and a possible association with different types of SpA has been investigated, but with conflicting results⁵⁰⁻⁵³. As an example, a prospective meta-analysis⁵⁰, studying 2675 AS patients, revealed a strong association ($P=0.000019$) between rs17561 and AS. In contrast, other studies failed to demonstrate a significant association between this particular SNP and AS^{51,54}. A study by Kawaguchi et al.⁵⁵ investigated an eventual consequence of the rs17561 SNP in skin fibroblasts from systemic sclerosis patients. They demonstrated a higher efficiency in the cleavage of pro-IL-1 α by calpain in fibroblast from patients with a homozygote SNP compared to fibroblast from patients without a SNP.

This might result in more or faster cleavage of pro-IL-1 α following translation with the formation of IL-1 α propeptide and mature IL-1 α , causing greater autocrine and paracrine stimulation as described earlier. Thus, the study indicates that individuals with an rs17561 SNP may produce a severe inflammation than individuals without. If these findings are correct it could be expected that a greater IL-1 α response occurred in subjects with the SNP in this present study. However, it was not possible to find any immediate association between the IL-1 α response and a SNP in the gene. This could be explained by the fact that the association in the study by Kawaguchi et al.⁵⁵ was related to a homozygote SNP, while the SNP in the present study was heterozygote. Furthermore, the study was performed in fibroblasts from a Japanese population which might cause another reaction than macrophages from a Caucasian population. These inconsistencies indicate that further studies are essential for better knowledge of the consequence of rs17561.

5.5 Future study

In this study the IL-1 α response was characterized in a young healthy population. Though, the chosen times limited the ability to get a full overview of the response. In future studies it would be preferable to perform measurement of mRNA within the first two hours of stimulation to capture the initiation of the transcription. Furthermore, measurement of both mRNA and IL-1 α protein should be conducted following 20 h in order to investigate the time of regression for the translation and transcription, respectively. This study may function as a pilot study for a larger scale study of the IL-1 α response in both health and disease. The ages of this study corresponds to the young age of patients with SpA, thus the subjects of this study can be used as a part of a control group of a larger case-control study. It would also be interesting to study the eventual connection between IL-1 α response and SNPs in SpA patients. A limitation of the methods of the present study might be that measurements from qPCR and flow cytometry were both relative which might cause uncertainties when comparing with results from patients. As an example, the results from qPCR was presented as fold change from a starting value, but patients might already express IL-1 α at the beginning which will not be reflected in the fold change over time. Therefore, it would be essential to obtain an absolute quantitative measurement in order to get a full impression of the disease and its difference from healthy subjects.

6. Conclusion

In this study the IL-1 α response in human macrophages was investigated by stimulation with LPS for a varying amount of time. The mRNA expression showed great inter-individual variability, though in general a rapid increase was observed within 2-4 hours following LPS stimulation. The amount of mRNA remained highly elevated throughout the 20 h investigated compared to non-stimulated control cells. The protein synthesis of IL-1 α revealed a slow but significant enhancement over time. This corresponds to the need of an increased gene translation in order to enhance protein synthesis. DNA sequencing of the gene demonstrated a known SNP in six of the ten subjects, but no association was found between the presence of the SNP and the IL-1 α response. This study revealed that an IL-1 α response to LPS stimulation contains both individual variations and general characterizations, and it forms the basis for further study of IL-1 α in health and disease.

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