Master Project R.A. van der Leij (S4071549)

The signaling pathways of lipopolysaccharides and monophosphoryl lipid A in M1 and M2 macrophages

ABSTRACT

The main effectors in chronic liver inflammation are Kupffer cells, which exist in a pro- and anti-inflammatory form. Kupffer cells are activated by binding of substances like LPS to the toll-like receptor-4 (TLR4). LPS has been described in full detail as an inflammatory agent and can therefore be linked to the progression of inflammation in the liver. LPS gains its toxicity through the lipid A part. It was discovered that the phosphate group from LPS can be removed, leading to a detoxification of the endotoxin. The lipid A part of LPS without one of the two phosphate groups is called monophosphoryl lipid A (MPLA). Different studies suggest different signaling pathways depending on the way that MPLA is delivered to the cell and the cell type. This research aims to examine if there is a difference between the signaling pathways of LPS and MPLA in inflammatory M1 macrophages and anti-inflammatory M2 macrophages which play a key role in the inflammatory process of liver diseases. 264.7 RAW cells were polarized to either M1 or M2 and stimulated with LPS or MPLA at different concentrations to investigate the signaling pathways. The hypothesis was that LPS primarily leads to an activation of pro-inflammatory pathways and MPLA to the activation of anti-inflammatory pathways. By means of NO assays, this research has shown that MPLA leads to the production of cell damage mediator nitric oxide in both M1 and M2 macrophages, but to a lesser extent in M2 macrophages and with a lower effectivity in both M1 and M2 macrophages. To investigate the cytokine expression, qPCRs were performed which show that MPLA does not lead to an activation of anti-inflammatory pathways. LPS leads to the production of pro-inflammatory cytokines. MPLA also induces this production, but to a lesser extent than LPS. Western blots show that MPLA and LPS both lead to the activation of the pro-inflammatory NF-κB and MAPK pathways, but it's surprising that MPLA activates the pathway to the same extent or faster than LPS. To further confirm this, the anti-inflammatory pathway should be investigated. Unfortunately, this research could not make any conclusions on the anti-inflammatory AKT pathway. In conclusion, this research shows that there is a difference in the signaling pathways of LPS and MPLA. MPLA activates the pro-inflammatory pathway, but to a lesser extent than LPS. More research on the activation of the anti-inflammatory pathway is still necessary.

INTRODUCTION

The inflammatory process of chronic liver diseases

Chronic liver diseases remain a large burden on global health, leading to 2 million deaths each year worldwide and a high increase in disabilities and healthcare utilization (Moon et al., 2020). These diseases are mainly caused by alcohol consumption, obesity and hepatitis B and C infections (Pimpin et al., 2018). To this day, no clear cure for chronic liver diseases has been found, highlighting the importance of research into the mechanisms of these diseases and its progression. Chronic inflammation of the liver will eventually lead to liver cirrhosis. Kupffer cells are the resident macrophages of the liver which play an important role in this inflammatory process. They localize within the lumen of the liver sinusoidal where they make up 30% of the sinusoidal cells (Bouwens et al., 1986). When injured, they become activated and start to express cytokines and signaling molecules. Depending on which signal activates the Kupffer cell, it will differentiate to either a pro-inflammatory M1 Kupffer cell or the anti-inflammatory M2 Kupffer cell. The inflammatory process is regulated by a balance between the M1 and the M2 Kupffer cells (Wan et al., 2014). The Kupffer cells recognize the substances that they are exposed to via pattern-recognition receptors (PRRs), with the most important one being toll-like receptors (TLRs). One of the substances that is recognized, is the gut microbiota-derived bacterial product lipopolysaccharide (LPS). The role of LPS in chronic liver diseases is generally acknowledged (Soppert et al., 2023). LPS binds to the TLR-4 receptor, leading to the production of pro-, but also anti-inflammatory cytokines (Koyama & Brenner, 2017).

Next to the Kupffer cells, recruited bone marrow-derived macrophages also play an important role in the inflammatory process of the liver. Like the Kupffer cells, they can be divided into the M1 and the M2 phenotype. The M1 macrophages are induced by IFN-γ, LPS and TNF-α and lead to the expression of pro-inflammatory cytokines, whereas the M2 macrophages are induced by IL-4, IL-10 and IL-13 and initiate an anti-inflammatory/wound healing effect (Mosser & Edwards, 2008).

LPS as an agent in chronic liver diseases

LPS is found in the outer membrane of most gram-negative bacteria. It consists of three main components: the hydrophobic lipid A part, a hydrophilic inner core, comprising of a short polysaccharide chain of KDO sugars and a large repeating hydrophilic oligosaccharide side chain, referred to as the O-antigen (Bertani & Ruiz, 2018). LPS is also present in our gut microbiome. The intestinal epithelium works as a barrier to prevent the translocation of LPS into the bloodstream (Schoeler & Caesar, 2019). However, pathological conditions like bowel diseases, liver diseases or infections, will lead to additional release of LPS into the circulation because there is an increased vascular permeability caused by cytokines, prostaglandins, leukotrienes and other inflammatory mediators (Schwabe & Greten, 2020). The liver receives 75% of its blood through the portal vein, so the liver receives a lot of the pathogens and bacterial-derived factors in case of translocation (Arab et al., 2018). Once LPS is released in aquatic environments like the intestinal lumen or blood, it will form a micelle with other LPS molecules due to its hydrophobic lipids and hydrophilic sugar moieties (Park & Lee, 2013). However, mediated by cofactors like the LPS-binding protein (LBP) and cluster of differentiation 14 (CD14), single LPS molecules can be separated from their micelle form and transported to the TLR4/MD2 complex to activate a signaling cascade (Park & Lee, 2013). As previously mentioned, LPS binds to the TLR-4 receptor on

macrophages where it can lead to the production of pro-inflammatory cytokines. The process of translocation of LPS from the gut microbiome can therefore be linked to the development and the progression of inflammatory liver disease (Pradere et al., 2010).

The TLR4/LPS signaling pathway

Upon binding of LPS to TLR4, a dimerization is induced. This brings the TIR domains into close proximity of each other which leads to the formation of a platform for signaling through TIR domain-containing adaptor molecules (**figure 1**) (Li et al., 2013). TLR4 has two distinct signaling pathways: the MyD88-dependent and the MyD88-independent/TRIF-dependent pathway. It leads to the induction of pro-inflammatory cytokines through the activation of mitogen-activated protein (MAP) kinase and nuclear factor kappa-B (NF-κB) activation (Li et al., 2013). Another pathway that is activated in macrophages is that of phosphatidylinositol-3 kinase/protein kinase B (PI3K/AKT), which is triggered by receptor-interacting serine/threonine-protein kinase (RIP) and causes inhibition of the pro-inflammatory cytokines and/or an enhancement of the production of anti-inflammatory cytokines (Wallet et al., 2012).

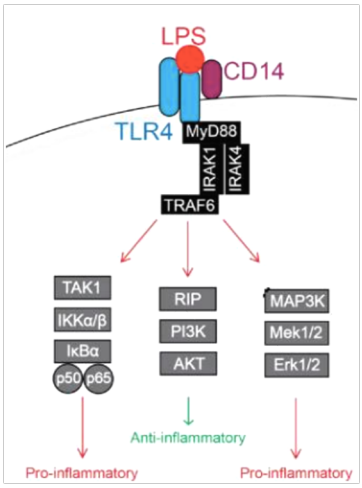

Figure 1: The TLR4-induced macrophage activation regulated by NF-kB, PI3K/AKT, and MAPK signaling pathways. *doi: 10.1189/jlb.0911447*

For the MyD88-dependent pathway (**figure 2**), MyD88 recruits IL-1 receptor-associated kinase-4 (IRAK-4). IRAK-4 is responsible for the recruitment, activation and eventually degradation of IL-1 receptor-associated kinase-1 (IRAK-1)(Lu et al., 2008). This leads to the activation of TNF receptor-associated factor 6 (TRAF-6),

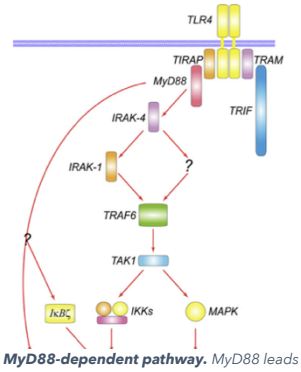

Figure 2: The MyD88-dependent pathway. *MyD88 leads to the activation of transcription factors NFκB and MAPK which leads to the production of proinflammatory cytokines. doi:10.1016/j.cyto.2008.01.006*

which in turn activates transforming growth-factor-β-activated kinase 1 (TAK1)(Lu et al., 2008). This leads to the activation of transcription factor NFκB. The activation of IκB kinase by TAK1 leads to the formation of a complex of IKKα, IKKβ and IKKγ which causes phosphorylation of IκB proteins (Lu et al., 2008). This leads to the degradation of these IκB proteins which causes the translocation of the transcription factor NFκB, causing production of pro-inflammatory cytokines (Lu et al., 2008). TRAF-6 also activates the MAP3K proteins which leads to a downstream activation of Mek1/2 and Erk1/2 (Wallet et al., 2012), also leading to the production of pro-inflammatory cytokines.
The MyD88-independent pathway (**figure 3**) makes use of TIR-domain-containing adaptor-inducing beta interferon (TRIF). TRIF activates receptor-interacting protein 1 (RIP-1), which leads to NFκB-activation (Lu et al., 2008). Next to this, TRIF also recruits TNF receptor-associated factor 3 (TRAF3) to activate interferon regulatory factor 3 (IRF3) (Lu et al., 2008). The activation of NFκB and IRF3 leads to the transcription of type I interferons which are important for anti-viral and anti-bacterial responses (Lu et al., 2008).

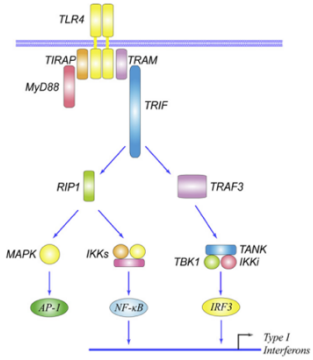

Figure 3: The MyD88-independent/TRIF-dependent pathway. *TRIF leads to the activation of transcription factors NFκB and IRF3 which leads to the production of Type I Interferons.*

Apart from the pro-inflammatory effects of TLR4 signaling, it is also suggested that there is an anti-inflammatory signaling due to a shift in de adaptor molecules (**figure 4**). PI3K has an isoform, p110δ, which is involved in the internalization of TLR4. Upon activation of TLR4, this isoform will cause a phosphorylation of phosphatidylinositol 4,5-bisphosphate (PIP_2) into (phosphatidylinositol-3,4,5-triphosphate) PIP_3 which causes dissociation and degradation of MyD88 adaptor-like protein (MAL) which is essential for the MyD88-pathway (Li et al., 2013). This leads to a shift towards the MyD88-independent/TRIF-dependent pathway, causing the production of anti-inflammatory cytokines like IL-10

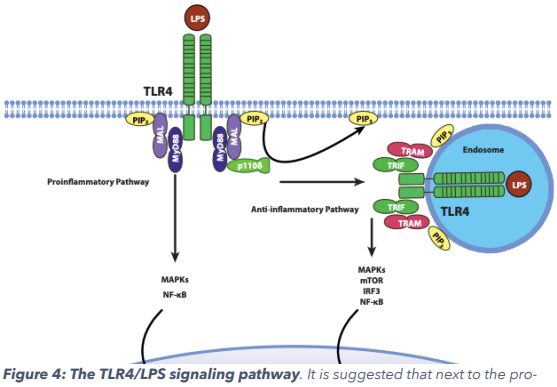

Figure 4: The TLR4/LPS signaling pathway*. It is suggested that next to the pro-inflammatory pathway, there also is an anti-inflammatory pathway induced by an internalization of TLR4 which causes a shift to the TRIF-dependent pathway. doi:10.3389/fimmu.2013.00347*

and type 1 interferons through NFκB, MAP kinases and IRF3 (Li et al., 2013).

Monophosphoryl lipid A in TLR4 signaling

The pathways that LPS activates upon binding to the TLR4 receptor have been described in full detail. LPS leads to the activation of different signaling pathways and thereby elicits its effect in macrophages, which is the production of all kinds of pro- and anti-inflammatory cytokines. However, it remains unclear when the stimulation of LPS leads to activation of pro-inflammatory or on the other hand anti-inflammatory processes. The answer to this question might lie in the structure of LPS. The structure of LPS gains its toxicity through the lipid A part (**figure 5**) (Bentala et al., 2002). Lipid A has two phosphate groups attached to diglucosamine, which is crucial for its biological activity. It was discovered that the phosphate group from LPS can be removed by adding alkaline phosphatase, leading to a detoxification of the endotoxin (Poelstra et al., 1997). The lipid A part of LPS without one of the two phosphate groups is called monophosphoryl lipid A (MPLA) (**figure 5**). Some researchers suggests that MPLA doesn't elicit the same toxic effect as lipid A does, and some even say that it has a protective effect over LPS. MPLA is currently even used in vaccines as a non-toxic adjuvant (Casella & Mitchell, 2008).

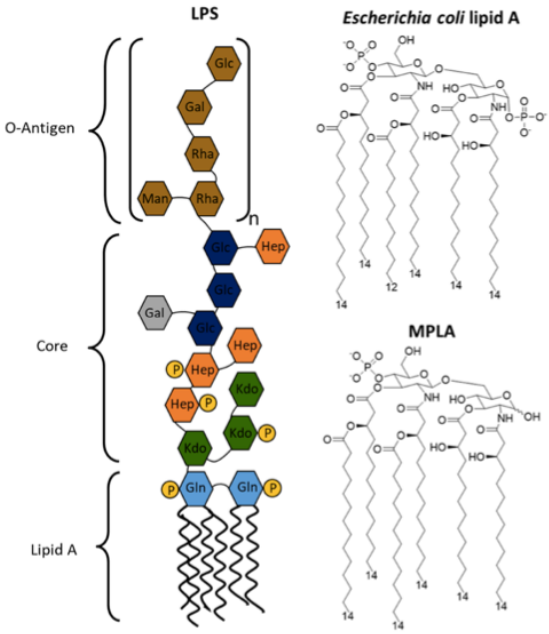

Figure 5: The structure of lipopolysaccharide (LPS), the lipid A part of LPS and monophosphoryl lipid A (MPLA). *doi: 10.1021/acsomega.3c05363*

Because of the protective and/or non-toxic effect that MPLA might elicit, it is thought that there is a bias towards the MyD88-independent/TRIF-dependent signaling cascade (Cekic et al., 2009). LPS and MPLA both lead to TRIF-dependent responses like the production of IL-10 in bone-marrow derived monocytes, but MPLA does not lead to MyD88-dependent production of IL-6 (Bohannon et al., 2013). However, there are also studies indicating that MPLA is still able to induce both the MyD88-dependent and the TRIF-dependent signaling pathway in macrophages (Owen et al., 2022). There are also studies which indicate that MPLA does not follow the CD14-mediated pathway in dedritic cells and may involve another receptor like TLR9 (Kolanowski et al., 2016; Yang et al., 2019). From these studies it can be concluded that MPLA possibly follows different pathways than LPS depending on the cell type, but a clear pathway for the effect of MPLA on TLR4 has not been found.

The aim of this research

The TLR4/LPS signaling pathway in macrophages is known, but little is known about the response that MPLA elicits when binding on the TLR4 receptor. Moreover, different studies suggest different signaling pathways depending on the way that MPLA is delivered to the cell and the cell type. Therefore, this research aims to examine the differences between the signaling pathways of LPS and MPLA in inflammatory M1 macrophages and anti-inflammatory M2 macrophages which play a key role in the inflammatory process of liver diseases.
To do this, different methods will be used to get more insight into the pathways that LPS and MPLA activate (**figure 6**). First, NO assays will be performed to examine whether MPLA leads to the same production of cell damage mediator NO as LPS in M1 and M2 polarized 264.7 RAW cells. To study the gene expression, qPCRs will be performed. It is investigated whether there are differences in gene expression of several genes encoding for pro- and anti-inflammatory cytokines upon stimulation with different concentration of LPS or MPLA in M1 or M2 polarized 264.7 RAW cells. To investigate the pathways that LPS and MPLA activate, western blots will be performed. Again, LPS and MPLA are added to 264.7 RAW cells to look at the phosphorylation of the transcription factors involved in the three pathways that are primarily involved in TLR4-induced macrophage activation: NFκB, PI3K/AKT and MAPK. From these experiments a conclusion will be made about whether there is a difference in the activation of the signaling pathways or the effects that MPLA elicit compared to LPS.

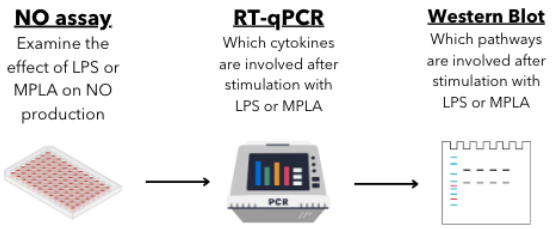

Figure 6: Overview of methods that are used in this research*. First NO assays will be performed to examine the effect of LPS and MPLA on NO production, after which qPCRs will be performed to examine which cytokines are involved and western blots to examine which pathways are involved.*

METHODS & MATERIALS

Culturing of the RAW cells

RAW 264.7 cells, a murine macrophage cell line, were cultured in Dulbecco's Modified Eagle Medium (DMEM, high glucose, GlutaMAX™ Supplement, sodium pyruvate) (catalog no. 31966-021 from Gibco™), supplemented with 10% fetal bovine serum (FBS) and 50 mg/ml gentamicin. The cell culture was detached from the bottom of the flask by scraping, after which the medium containing the cells was put in a tube. The mixture was centrifuged for 5 minutes at 300 rpm to obtain a cell pellet. The old medium was removed from the tube, after which 10 ml fresh medium was added in which the pellet was resuspended. From this mixture, a cell count was performed after which the cells were plated on 6-, 12- or 96-wells plates in different densities depending on the experiment. The cells were incubated at 37°C, with 5% CO_2. After plating for 24 hours, the medium was removed and fresh medium with polarization agents, LPS or MPLA was added depending on the experiment.

Culturing of the 3T3 cells

For the positive control of the western blot experiment, 3T3 cells were cultured. 3T3 fibroblasts were obtained from mice and cultured in Dulbecco's Modified Eagle Medium (DMEM, high glucose, GlutaMAX™ Supplement, HEPES) (catalog no. 32430-027 from Gibco™) supplemented with 10% Fetal Bovine Serum (FBS), pyruvate and antibiotics (penicillin and streptomycin). The medium was taken off the cell culture and washed twice with phosphate buffered saline (PBS). Since the cells were stuck to the bottom of the flask in which they were cultured, trypsin was added, and the flask was shaken carefully to release them. Then, culture medium was added. This mixture was put in a tube and centrifuged for 5 minutes at 300 rpm to obtain a cell pellet. The medium on top of the pellet was removed, and the pellet was resuspended in 10 ml fresh medium. From this mixture, a cell count was performed after which the cells were plated on 6-wells plates with a density of 1.10^6 cells/well in a volume of 2 ml 3T3 culture medium. The cells were incubated at 37°C, with 5% CO_2. After 24 hours, the medium was removed and fresh medium containing PDGF was added to obtain the effect necessary for the positive control.

Stimulation agents

The cells were stimulated with lipopolysaccharides from Escherichia coli O55:B5 (catalog no. L2880 from Sigma-Aldrich) or monophosphoryl lipid A from Salmonella minnesota R595 (catalog no. #401 from List Labs). LPS was directly diluted and aliquoted to 100 µg/ml stock solutions in UP water. MPLA was first diluted in 10% DMSO and then aliquoted to 100 µg/ml stock solutions in UP water. The aliquots were kept at -20°C until ready for use. About 15 minutes before use, the aliquot was put in a water bath at 37°C in the incubator to prevent the formation of micelles. The aliquots and the dilutions of the LPS or MPLA in medium were also never vortexed to prevent the formation of micelles.
For the positive control that was used for the western blot experiment, murine PDGF-BB was used (catalog no. 315-18 from PeproTech). A stock solution of 10 ug/ml in H_2O was diluted in 3T3 cell medium to obtain a concentration of 40 ng/ml.

Polarization agents

To polarize the 264.7 RAW cells towards the M1 or the M2 phenotype, polarization agents were used. Polarization towards the inflammatory M1macrophages was done with murine IFNγ (catalog no. 315-05 from PeproTech). A stock of 100 µg/ml in PBS was diluted in culture medium to obtain a concentration of 40 ng/ml. Polarization towards M2 was done with murine IL-4 (catalog no. 214-14 from PeproTech) and murine IL-13 (catalog no. 210-13 from PeproTech). A stock of 10 µg/ml IL-4 in PBS and a stock of 10 µg/ml IL-13 in PBS were diluted in culture medium to obtain a concentration of 20 ng/ml.

NO assay

The production of NO was measured in the supernatant of stimulated RAW 264.7 cells (**figure 7**). Cells were seeded in a 96-wells plate at a density of 1.10^5 cells/well in a volume of 200 µL RAW cell medium and grown overnight at 37°C, with 5% CO_2. After 24 hours, the old medium was removed. Fresh medium was added containing the M1 polarization agent IFNλ to polarize the cells towards M1. M2 polarization agents IL-4 or IL-13 were used to polarize the cells towards M2. After 1 hour, the medium was removed again and fresh medium containing varying concentrations (0 ng/ml, 0,1 ng/ml, 0,3 ng/ml, 1 ng/ml, 3 ng/ml, 10 ng/ml, 30 ng/ml, 100 ng/ml, 300 ng/ml, 400 ng/ml and 500 ng/ml) of LPS or MPLA were added to the cells in triplo and incubated at 37°C, with 5% CO_2. After another 18 hours of incubation, 100 µL of the supernatants was plated onto a new 96-wells plate. To the supernatant, 100 µL Griess reagent which was freshly made by adding equal amounts of Griess reagent A (2 g sulphanilamide + 5 mL phosphoric acid in a total volume of 100 ml Milli-Q water) and Griess reagent B (200 mg N-naphthyl ethylene diamine in 100 ml Milli-Q water) was added. The absorbance was measured at 550 nm in a BioTek Synergy H1 microplate reader. NaNO2 diluted in culture medium was used to create a standard curve. After measuring, the results were processed by means of Excel by interpolating the absorbance values of the samples into the standard curve to get the concentration of NO in µM. The experiment was repeated three times, after which the results were grouped together by means of Excel. See **appendix 1** for the protocol that was used.

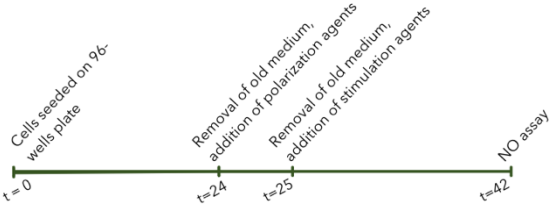

Figure 7: Experimental design for the NO assay experiments. *Cells were seeded on a 96-wells plate at a density of 1.10^5 cells/well and after 24 hours fresh medium with the polarization agents was added. At t=25 the medium was removed again and fresh medium containing the stimulation agents was added. At t=42 the NO assays were performed with the medium of the cell culture.*

Real-time quantitative PCR

By means of real-time quantitative PCR (qPCR), it was measured whether the expression of genes encoding for anti- and pro-inflammatory cytokines would be different between LPS and MPLA stimulated RAW cells (**Figure 8**). Cells were seeded in 12-wells plates at a density of 4.10^4 cells/well in a volume of 1 ml RAW cell medium and grown overnight at 37°C, with 5% CO_2. After 24 hours, the old medium was removed. Fresh medium was added containing the M1 polarization agent IFNλ to polarize the cells towards M1, or the M2 polarization agents IL-4 and IL-13 to polarize the cells towards M2. After 1 hour, the medium was removed again and fresh medium containing 0, 3 or 300 ng/ml of LPS or MPLA was added to the cells in duplo and incubated at 37°C, with 5% CO_2. These concentrations were chosen based on the results of the NO assay.
After 18 hours of incubation with LPS or MPLA, the medium was removed, and the cells were washed with PBS. To examine whether MPLA and LPS elicit the same effect on the cells as in the NO assay, NO assays were performed with the removed medium before any further experiments were performed. After washing with PBS 200 µL of pre-chilled homogenization buffer containing 1-thioglycerol (20 µL/ml) from the Maxwell® 16 LEV simplyRNA Tissue Kit was added to the wells. The plates were then stored at -80°C until further use or the samples were immediately homogenized with a pipet. After homogenization, the samples were stored in RNase free 1.5 ml tubes on ice. RNA isolation was performed using the using the Maxwell® 16 LEV simplyRNA Tissue Kit (catalog no. AS1280 from Promega). The protocol is described in detail in **appendix 2**. After the RNA isolation, the elution tubes were stored at -80°C until further use or the tubes were put on ice and used for the next step, which was the cDNA conversion. The elution tubes were centrifuged briefly before further use.

Table 1: primer sequences of the primers that were used for RT-qPCR

Gene	Forward primer	Reverse primer
β-actin	5'-ATCGTGCGTGACATCAAAGA-3'	3'-ATGCCACAGGATTCCATACC-5'
IL-10	5'-ATAACTGCACCCACTTCCCAGTC-3'	3'-CCCAAGTAACCCTTAAAGTCCTGC-5'
IL-10R-α	5'-CCAAACCAGTCTGAGAGCACCT-3'	3'-CAGGACAATGCCTGAGCCTTTC-5'
TNF-α	5'-CATCTTCTCAAAATTCGAGTGACAA-3'	3'-GAGTAGACAAGGTACAACCC-5'
TGF-β	5'-AGGGCTACCATGCCAACTTC-3'	3'-GTTGGACAACTGCTCCACCT-5'
IL-6	5'-TGATGCTGGTGACAACCACGGC-3'	3'-TAAGCCTCCGACTTGTGAAGTGGTA-5'
IL-1β	5'-GCCAAGACAGGTCGCTCAGGG-3'	3'-CCCCCACACGTTGACAGCTAGG-5'

Before the cDNA conversion, the RNA yield was determined by measuring the amount of RNA in ng/µL. Measurement was done on a Take3 micro-volume plate on the BioTek Synergy H1 microplate reader. This concentration was then used to dilute the RNA to a concentration of 250 ng/µL RNA in 5 µL RNase free water. An RT-mix containing RT buffer (catalog no. M531A from Promega), dNTP mix (catalog no. U151A from Promega), reverse transcriptase (catalog no. M170A from Promega), RNasin (catalog no. N251B from Promega), random hexamers (catalog no. C118A from Promega) and RNase-free water was prepared according to **appendix 3** and added to each sample. Extra samples were made for the standard curve. The cDNA conversion was done with the T100 Thermal Cycler from Bio-Rad, see **appendix 3** for the setting of the machine. See **appendix 4** for a detailed protocol of the preparation of the standard curve, the preparation of the cDNA dilution, primermix, mastermix and the qPCR reaction and plate lay-out.
After the cDNA conversion was performed, the tubes were briefly centrifuged to collect the condensed water from the lids and then stored at -20°C until further use. Before use, the sample cDNA was diluted by adding 90 µL of RNase-free water to each sample. The standard curve was prepared as described in **appendix 4**. For each gene of interest, a primermix was prepared containing the forward and reverse primers in RNase-free water. The primers used are presented in **table 1.** A mastermix was prepared containing the primermix, RNase-free water and a Sybr Green Mix (catalog no. A600A from Promega). Then, the qPCR reaction was prepared by adding 2 µL of the standard curve and samples in triplo onto a 384-wells plate. A positive control (sample with a known Ct-value) and a negative control (RNase-free water) were also pipetted onto the plate. Then 8 µL of the mastermix was added to each well. A seal was put on the plate and the plate was briefly centrifuged to make sure all the samples and mastermix were at the bottom of the plate. The real-time qPCR was performed using the Biorad CFX 384 machine, of which the settings can be found in **appendix 4**.
The expression of the housekeeping gene, β-actin, was first measured before the genes of interest were measured. The obtained quantities for the genes of interest were normalized for the quantity of housekeeping gene β-actin of the same sample. This was only done if the β-actin values were around an equal value for each sample. The amplification curve and standard curve were obtained from the machine's software, QuantStudio real-time PCR software v1.3, and the Ct-values from the samples were interpolated into the standard curve to get the quantity of the genes of interest. The qPCR of the gene of interest was considered successful if the standard curve showed an efficiency between 90 and 110% and no more than one of the three triplo's had to be omitted. The results were processed and grouped by means of Excel once an n=3 was obtained for each gene of interest. Statistical analysis of the results was performed with a t-test (Excel). $\rho < 0.05$ was considered as the minimum level of significance.

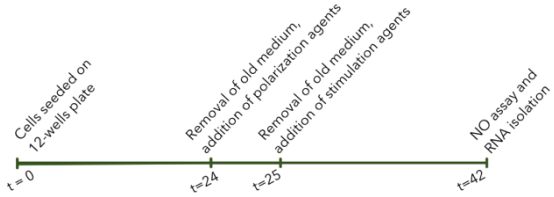

***Figure 8: Experimental design of the qPCR experiments**. Cells were seeded on a 12-wells plate at a density of 4.10⁴ cells/well and after 24 hours fresh medium with the polarization agents were added. At t=25 the medium was removed again and fresh medium containing the stimulation agents was added. At t=42 NO assays were performed, and RNA isolation took place.*

Western Blot

By means of western blots, the differences in pathway activation upon stimulation with LPS or MPLA were investigated (**figure 9**). The following pathways were investigated: the AKT-pathway, the NFκB-pathway and the MAPK-pathway. For each pathway, a different experimental set-up was used. Cells were seeded in 6-wells plates at a density of 1.10^5 cells/well in a volume of 2 ml RAW cell medium and grown overnight at 37°C, with 5% CO_2. After 24 hours, the old medium was removed.
For the NFκB-pathway and the MAPK-pathway, cells were stimulated for either 10 or 60 minutes with either LPS or MPLA. As a positive control for the MAPK-pathway, 3T3 cells were used to which PDGF was added as was described earlier. PDGF is a known activator of the MAPK-pathway in fibroblasts (Moens et al., 2013). As a positive control for the NFκB-pathway, a sample from an earlier experiment which showed a positive result was used.
For the AKT-pathway, cells were stimulated with 3 or 300 ng/ml MPLA for either 1, 2, 5, 10 or 60 minutes. As a positive control, again 3T3 cells were used to which PDGF was added as was described earlier.
See **appendix 5** for the protocol of the protein isolation with RIPA-TBS. The stimulation of the cells was stopped by removing the medium, washing twice with PBS and immediately adding RIPA-TBS to the cells. The cells were then stored at -80°C until further use or the protein isolation was performed while keeping the plates on ice.
See **appendix 6** for the western blot protocol where the electrophoresis, blotting, antibody incubation and quantification is described in detail. After the protein isolation, a gel was made, and the samples were loaded onto the gel to perform the electrophoresis. After electrophoresis, the blot was made. After blotting, the membranes were incubated with the antibodies of interest. The following primary antibodies were used: rabbit pAKT (1:1000, catalog no. 9275S from Cell Signaling Technology), rabbit pNFκB (1:1000, catalog no. 3033S from Cell Signaling Technology), rabbit p-p44/42 (1:1000, catalog no. 9101S from Cell Signaling Technology) and rabbit GAPDH (1:10.000, catalog no. 5174S from Cell Signaling Technology). As a secondary antibody, goat anti-rabbit horseradish peroxidase (1:1000, catalog no. P0448 from DakoCytomation) was used. Measurements and imaging were done with the G:BOX and GeneSnap from Syngene.

***Figure 9: Experimental design of western blot experiments**. Cells were seeded on a 6-wells plate at a density of 1.10^5 cells/well and after 24 hours LPS or MPLA was added at different timepoints. At t=25 the protein isolation was performed.*

RESULTS

The effect of LPS and MPLA on the nitric oxide (NO) production in M1 or M2 polarized macrophages

For the raw data of this experiment, see the shared drive from the NDT department > Rick van der Leij > 23-RL-10 > 23-RL-10.xls

The effect of increasing concentrations of LPS and MPLA on the nitric oxide (NO) production in M1 or M2 polarized RAW264.7 cells was investigated first.

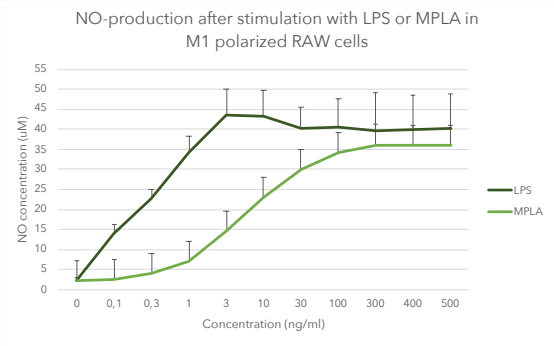

***Figure 10: The nitric oxide production after stimulation with LPS or MPLA in M1 polarized RAW cells.** A similar maximum for both LPS and MPLA is observed, but the maximum is reached at a higher concentration for MPLA which indicates a lower effectivity.*

For the NO production in M1 polarized macrophages (**figure 10**), it is observed that a maximum of 45 µM NO is reached at roughly 3 ng/ml after stimulation with LPS. MPLA also reaches this maximum, but at a higher concentration (roughly 300 ng/ml). There is a shift to the right for MPLA, which indicates a lower effectivity. From these results it can be concluded that MPLA does elicit an effect in M1 macrophages, but to a lesser extent and with lower NO production than LPS.

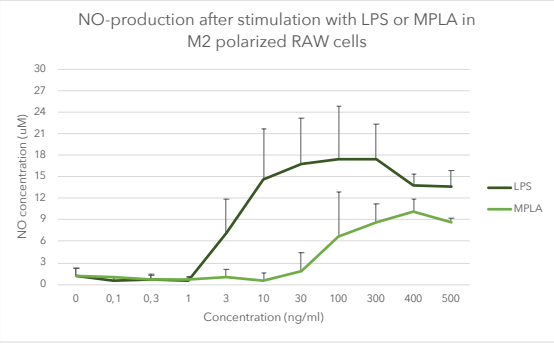

***Figure 11: The nitric oxide production after stimulation with LPS or MPLA in M2 polarized RAW cells.** There is an overall lower NO production, with a higher maximum for LPS than for MPLA. MPLA shows a shift to the right.*

For the NO production in M2 polarized macrophages (**figure 11**), it is observed that there is an overall lower production of nitric oxide compared to the M1 polarized macrophages. It is also observed that the maximum NO production after stimulation with LPS is reached at a higher concentration, roughly around 300 ng/ml. The maximum NO production after stimulation with MPLA is lower than that of the LPS stimulation, but there still is a shift to the right. This observation was also made in the M1 macrophages. From these results the conclusion can be made that there is an overall lower production of NO in M2 macrophages and that MPLA also has a lower effectivity to eliciting an effect in M2 macrophages as was also observed in the M1 macrophages.
To see which cytokines and pathways are involved in this process, qPCRs and western blots were performed next.

The effect of LPS and MPLA on the expression of pro- and anti-inflammatory cytokines in M1 or M2 polarized macrophages

For the raw data of this experiment, see the shared drive from the NDT department > Rick van der Leij > 23-RL-09 > 23-RL-09 combined data.xls

To look more closely into if there is a difference between the pathways that LPS and MPLA activate, a real-time qPCR was performed. The gene expression of several pro- and anti-inflammatory cytokines in M1 or M2 polarized macrophages after stimulation with LPS and MPLA was investigated.
See **appendix 7** for examples of the standard curves and amplification plots that were obtained for each gene or the raw data file on Drive for all the standard curves and amplification plots.
There is a significant increase in expression of IL-10 in M1 macrophages at 300 ng/ml for LPS compared to MPLA, while in M2 macrophages there is a higher expression of IL-10 after stimulation with 300 ng/ml MPLA (**figure 12A, 12B**). In M2 polarized macrophages, stimulation with LPS or MPLA leads to a decrease in IL-10 expression compared to the control. Especially with 300 ng/ml LPS and MPLA. For IL-10R-α, a significant increase in expression is observed in M1 macrophages (**figure 12C**). LPS leads to a higher expression than MPLA at both 3 and 300 ng/ml. There is a significant decrease in IL-10R-α at 3 ng/ml MPLA compared to 3 ng/ml LPS in M2 macrophages (**figure 12D**). The overall expression of IL-10R-α doesn't increase as much compared to the control. From the results of IL-10 and its receptor, it is interesting to note that IL-10 is decreased in M2 macrophages upon stimulation with LPS while the receptor expression is increased. For the M1 receptor this is not the case. There is an increased expression of IL-10 and its receptor upon stimulation with LPS.
For the other anti-inflammatory cytokine, TGF-β, it appears that LPS lowers the expression while MPLA has the same expression as the control (**figure 12E**). At 3 ng/ml LPS there is a significant decrease in expression of TGF-β compared to 3 ng/ml MPLA. There is no significant change in the TGF-β expression upon stimulation with LPS or MPLA in M2 macrophages (**figure 12F**).
For the anti-inflammatory cytokines, the overall conclusion can be made that LPS leads to their induction, mostly in the inflammatory macrophages. MPLA does not lead to a significant increase in anti-inflammatory cytokines in either inflammatory or anti-inflammatory macrophages. The next step is to see if these results are

reflected in the results of the anti-inflammatory pathways of the western blot experiment.
There is a significant increase in pro-inflammatory TNF-α expression in M1 macrophages upon stimulation with 300 ng/ml LPS compared to MPLA (**figure 12G**). For M2 macrophages, there is a significant increase in TNF-α expression upon stimulation with LPS, whereas MPLA does not lead to an increase in the expression (**figure 12H**).
For IL-6, the expression appears to follow the same pattern in M1 and M2 macrophages, but the expression is a tenfold lower in M2 macrophages (**figure 12I, 12J**). In both M1 and M2 macrophages, LPS leads to a high expression of IL-6 at higher concentration. MPLA also leads to an increase in the expression of IL-6 in M1 and M2, but not to the same extent as LPS.
For IL-1β, the results show a similar expression pattern to TNF-α and IL-6 (**figure 12K, 12L**). There is a significant increase in IL-1β expression in M1 and M2 macrophages at 300 ng/ml LPS. MPLA leads to an increase in the expression of IL-1β in both M1 and M2 macrophages, but again not to the same extent as LPS.
Overall, M1 has a higher production of pro-inflammatory cytokines TNF-α, IL-6 and IL-1β in response to LPS compared to a lower production in M2 macrophages. MPLA shows a much lower expression of these cytokines compared to LPS, but there is an increase compared to the control situation. Therefore, we could conclude that MPLA activates the pro-inflammatory pathway, but to a lesser extent than LPS. Since the cytokine production is only an effect of the pathway activation, it is necessary to investigate the pathways themselves. For this, the western blot experiments were performed.

The effect of LPS and MPLA on TLR4-induced macrophage activation

For the raw data of this experiment, see the shared drive from the NDT department > Rick van der Leij > 24-RL-02 for the NFκB-pathway; 24-RL-05 for the AKT-pathway; 24-RL-06 for the MAPK-pathway.

To investigate the differences in pathways that are involved in TLR4-induced macrophage activation upon stimulation with LPS or MPLA, western blots were performed. For the NFκB-pathway and the MAPK-pathway, cells were stimulated with either 3 or 300 ng/ml LPS or MPLA for either 10 or 60 minutes (**figure 13**).

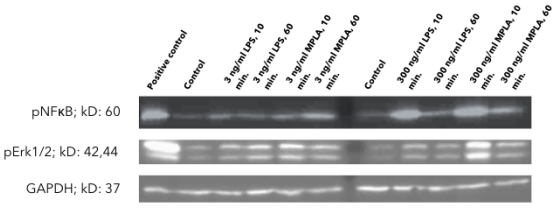

***Figure 13: Western blot results for NFκB- and the MAPK-pathway**. RAW cells were stimulated with either 3 or 300 ng/ml LPS or MPLA for either 10 or 60 minutes.*

At 3 ng/ml, the highest activation of NFκB can be observed for MPLA after 60 minutes. LPS also leads to an activation of NFκB, but this appears to happen at 10 minutes and decreases after 60 minutes. At higher concentration, 300 ng/ml, both LPS and MPLA lead to an activation of NFκB after 10 minutes. There is a decrease in activation after 60 minutes for both LPS and MPLA. The intensity of the band appears to be higher for LPS than for MPLA. This confirms the previous conclusion from both the NO assay as the RT-qPCR that MPLA does activate the inflammatory pathway, but to a lesser extent and with lower effectivity.
Surprisingly, the MAPK-pathway appears to be activated more and faster by MPLA than by LPS. At lower concentration, MPLA leads to a faster activation than LPS after 10 minutes of stimulation. For the higher concentration, the highest activation can be observed for MPLA after 10 minutes as well. From this it can be concluded that MPLA activates the anti-inflammatory pathway to the same extent or even faster than LPS.
For the AKT-pathway, the results were inconclusive: both in the control situation as in the samples which were stimulated with LPS or MPLA, no bands were observed. Therefore, no clear conclusion can be made about the activation of the anti-inflammatory AKT pathway. See the Drive for the results of this experiment.

DISCUSSION

The effect of LPS and MPLA on the nitric oxide (NO) production in M1 or M2 polarized macrophages

The effect of LPS and MPLA on the nitric oxide production in M1 or M2 polarized macrophages was investigated by means of NO assays. The presence of nitric oxide was measured since this is a mediator in the process of cell damage. Unregulated NO production can cause cell death through oxidative stress, DNA damage, a disruption of the energy metabolism and so on (Murphy, 1999). This was done to investigate the claims from literature that MPLA has a protective or less damaging effect than LPS and to investigate if there is a difference in NO production between inflammatory M1 and anti-inflammatory M2 macrophages.
The overall NO production is higher in M1 than in M2 macrophages. This is as expected, since M1 is an inflammatory macrophage and would therefore show more of this mediator, while M2 is an anti-inflammatory macrophage and would therefore protect against damage and show less NO (Wan et al., 2014). Previous research also shows that when M1 or M2 macrophages are stimulated, there is a higher expression of inducible nitric oxide synthase (iNOS) in M1 macrophages than in M2 macrophages (Cui et al., 2022).
For MPLA, there is a shift to the right in both M1 and M2 macrophages. This indicates a lower effectivity of the macrophages to induce the production of NO upon stimulation with MPLA than for LPS. Moreover, for the M2 macrophages, it can be observed that MPLA doesn't reach the same maximum as LPS. This indicates that MPLA does activate the production of NO, but to a lesser extent in M2 than in M1.
Although there is a difference between the M1 and the M2 macrophages, it must be considered that the medium with the polarization agents was removed, and that new medium without the polarization agents was added after 1 hour of stimulation with the polarization agents. M1/M2 polarization is a dynamic process, and the phenotype of polarized macrophages can be reversed (Wang et al., 2014). Particularly LPS stimulation of TLR4 drives macrophages to the M1 phenotype (Wang et al., 2014). Because the M2 polarization agents were removed, and LPS was added to the cells without new polarization agents, the phenotype of the M2 macrophages might have (partly) switched back towards the M1 phenotype.

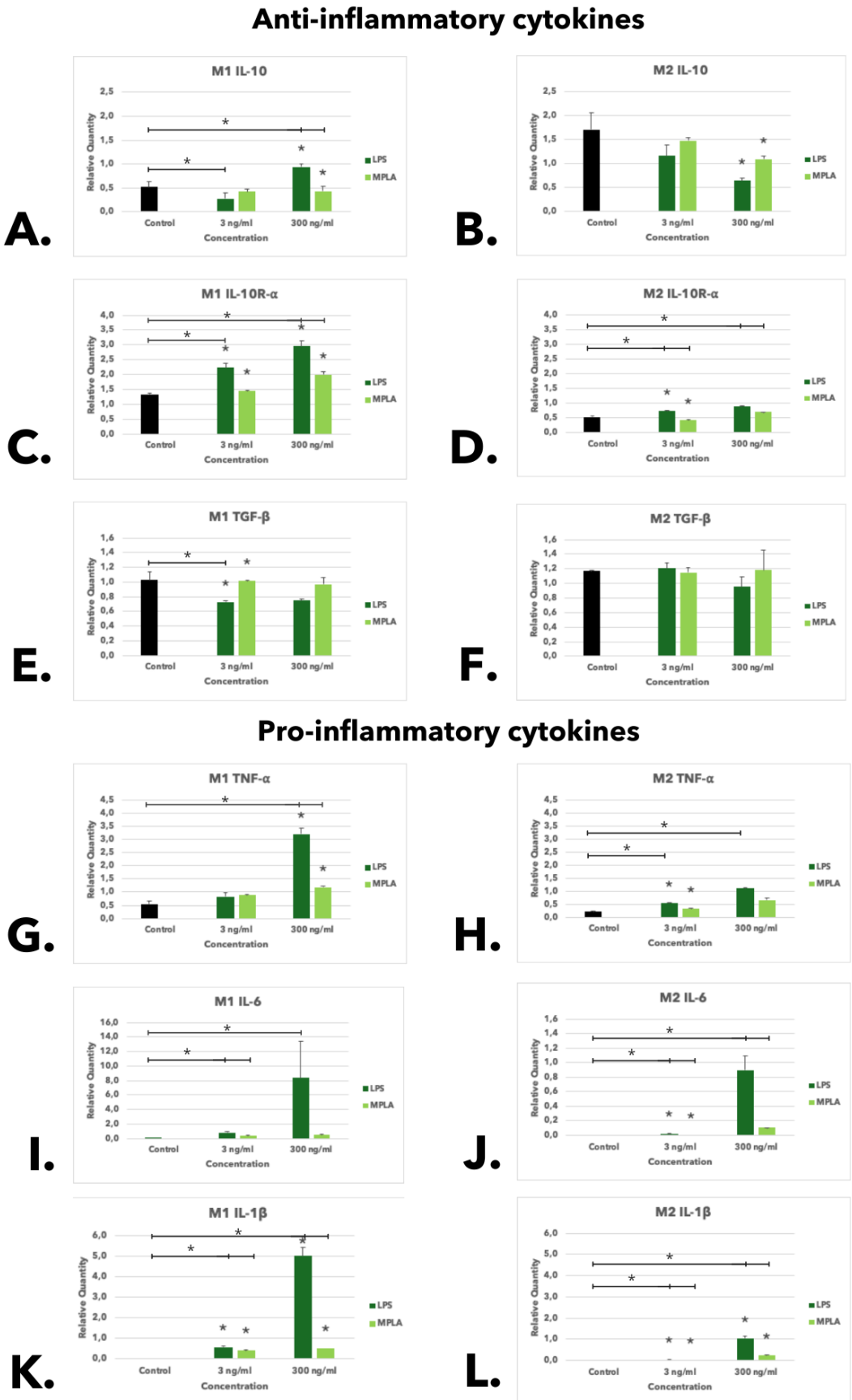

Figure 12: Gene expression of several anti- and pro-inflammatory cytokines after stimulation with LPS and MPLA in M1 or M2 polarized macrophages. *(**A, B**) The effects of LPS or MPLA on the expression of IL-10 in M1 and M2 polarized macrophages, respectively. (**C, D**) The effects of LPS or MPLA on the expression of IL-10R-α in M1 and M2 polarized macrophages, respectively. (**E, F**) The effects of LPS or MPLA on the expression of TGF-β in M1 and M2 polarized macrophages, respectively. (**G, H**) The effects of LPS or MPLA on the expression of TNF-α in M1 and M2 polarized macrophages, respectively. (**I, J**) The effects of LPS or MPLA on the expression of IL-6 in M1 and M2 polarized macrophages, respectively. (**K,L**) The effects of LPS or MPLA on the expression of IL-1β in M1 and M2 polarized macrophages, respectively. (* = $p < 0.05$)*

The overall conclusion for the NO experiment is that MPLA leads to a lower NO production in M2 macrophages, and that MPLA has a lower effectivity to induce NO production in both M1 and M2 macrophages as is confirmed by the shift to the right in both graphs. An explanation for this could be that MPLA has a weaker binding to the TLR4-MD2 receptor complex than LPS because it is different from the lipid A structure in LPS (Saha et al., 2022). It could however also be that the removal of polarization agents played a role in the effect. This needs to be investigated further in future research, where the polarization agents remain present in the medium upon stimulation with LPS or MPLA. It is also advised to perform MTT-assays in future research, since this assay could really show the effect of MPLA or LPS on cell damage (Kumar et al., 2018).
The NO-assays showed that there is indeed a difference between M1 and M2 macrophages when it comes to the production of NO by LPS or MPLA, indicating that the pathways might be activated differently. To investigate where this difference lies, real-time quantitative PCR was performed to look at cytokine production and western blotting was done to look at the pathway activation.

The effect of LPS and MPLA on the expression of pro- and anti-inflammatory cytokines in M1 or M2 polarized macrophages

There appears to be a switch between M1 and M2 macrophages in the expression of anti-inflammatory IL-10 after stimulation with LPS and MPLA. In the inflammatory M1 macrophages, LPS leads to a higher expression of IL-10 compared to MPLA. While in the anti-inflammatory M2 macrophages, MPLA leads to the higher expression of IL-10. From this, it could be concluded that LPS stimulates an anti-inflammatory process in M1 macrophages and that MPLA does not enhance this activation in the M1 macrophages. In the M2 macrophages there appears to be a decrease in the expression of IL-10 expression upon addition of LPS compared to MPLA. Reason for this might be that MPLA is less toxic and therefore produces less IL-10, activating different pathways. The reason that LPS doesn't elicit the same effect as in M1, could be because LPS is not challenged in the M2 macrophages with the result that LPS does not elicit the same effect.
The expression of IL-10R-α is different than the expression of IL-10 itself. More receptor expression does not necessarily mean more expression of the cytokine. It can be observed that LPS leads to more expression of IL-10R-α in the inflammatory M1 macrophages than MPLA. Little to no change in expression is observed in the M2 macrophages between LPS and MPLA. From this the conclusion can again be made that LPS plays a role in activating the anti-inflammatory pathway in M1 macrophages, whereas this effect is smaller in the M2 macrophages. MPLA does not elicit the same effect as LPS in both M1 or M2, indicating that MPLA might indeed follow a different pathway.
Another anti-inflammatory cytokine that was investigated, was TGF-β. For the M2 macrophages, the conclusion can be made that there is no activation of the anti-inflammatory pathway upon stimulation with either LPS or MPLA. For the M1 macrophages, only LPS leads to an increase in the expression of TGF-β. This again shows the activation of the anti-inflammatory pathway by LPS in M1 macrophages and further confirms the assumption that MPLA does not activate the anti-inflammatory pathway.
It is known that LPS leads to the expression of anti-inflammatory cytokines by activation of the TLR4-receptor (Lu et al., 2008). From the differences between LPS and MPLA, it can be concluded that MPLA does not necessarily lead to a higher expression of anti-inflammatory cytokines. MPLA might activate the anti-inflammatory pathways in a different way or to a different extent. The western blot experiments are done to further confirm this.
It is expected that LPS leads to an overall higher expression of pro-inflammatory cytokines since LPS is a promoter of inflammation (Paik et al., 2003). This is the case when looking at the results.
In both the M1 and M2 macrophages, the expression of TNF-α is more increased upon stimulation with LPS, compared to MPLA. The expression of IL-1β and IL-6 follow the same pattern as the TNF-α expression. There is a significant increase in expression with LPS compared to MPLA, while MPLA still elicits some production. In the M2 macrophages, the overall cytokine production is lower. From the results, the assumption could be made that MPLA seems to activate the same pro-inflammatory pathway as LPS, but to a lesser extent. This is also in line with the results of the NO assay, where it was observed that MPLA has a lower effectivity to inducing the production of nitric oxide. An explanation for this could again be that MPLA has a weaker binding to the TLR4-MD2 receptor complex than LPS (Saha et al., 2022). The overall lower production of the pro-inflammatory cytokines in M2 macrophages can be explained by the anti-inflammatory nature of the M2 macrophages, which causes them to have a protective effect against the inflammatory cytokine (Yunna et al., 2020).
It should again be noted that the polarization only took place for one hour, after which the polarization agents were removed. This might have caused the M2 macrophages to switch to M1, leading to different results.
From the qPCR results, the overall assumption can be made that MPLA does not lead to an activation of the anti-inflammatory pathway but might lead to an activation of pro-inflammatory pathways. However, the activation is to a lesser extent than that of LPS. To further confirm these assumptions, western blots were performed.

The effect of LPS and MPLA on TLR4-induced macrophage activation

To investigate which pathways are activated upon binding of LPS or MPLA and if there is a difference in the activation, western blots were performed. To see which pathways are activated, the phosphorylated forms of transcription factors were investigated. In response to stimuli from the environment like LPS or MPLA, the signal transduction pathways target the transcription factors leading to their phosphorylation by protein kinases which leads to their activation and subsequent transcription of pro- and anti-inflammatory cytokines which were investigated in the qPCR experiment (Whitmarsh & Davis, 2000).
The pro-inflammatory pathways that were investigated were that of NFκB and MAPK, for which the

phosphorylated form of NFκB or Erk1/2 (p44/42) was investigated for each pathway respectively. For the NFκB pathway, it is observed that both LPS and MPLA lead to its activation. From these results, the conclusion could be made that both LPS and MPLA activate the NFκB pathway, but at different rates. LPS appears to lead to a strong and fast activation. This is also confirmed in other research, where LPS indeed induces a fast activation of the NFκB pathway in macrophages (Bagaev et al., 2019). MPLA on the other hand has a slower activation at lower concentration but does lead to a fast activation at higher concentration. This confirms the conclusion that was made for the pro-inflammatory cytokines of the qPCR experiment that MPLA does activate the pro-inflammatory pathway but not as strong as LPS.
MPLA shows a faster activation of the MAPK pathway than LPS, which is remarkable. MPLA appears to lead to a fast activation at high concentration, while LPS does not activate the pathway to the same extent. This is remarkable, as it interferes with the previous conclusion that MPLA does lead to an activation but to a lesser extent. MPLA activates the pathways to the same extent (NFκB) or even faster (MAPK) than LPS. However, it should also be noted that both the MAPK and the NFκB pathway are also involved in the anti-inflammatory pathway (Chi et al., 2006; Lawrence, 2009). To confirm whether the anti-inflammatory pathway is activated or not, the AKT pathway was investigated. However, the experiment to show the differences in activation of the AKT pathway failed, as the antibody that was used did not lead to reproducible results (see raw data on Drive). Therefore, no clear conclusion about the activation of the anti-inflammatory pathway can be made. This is something that has to be investigated in future research. It should also be noted that the western blot experiments were performed in native RAW cells, and not in M1 and M2 polarized RAW cells. This might have influenced the way in which the activation of the macrophages took place. For future research it might be interesting to investigate the activation in M1 and M2 polarized macrophages.

CONCLUSION

The aim of this research was to examine the differences between the signaling pathways of LPS and MPLA in inflammatory M1 macrophages and anti-inflammatory M2 macrophages. The hypothesis was that MPLA would lead to an activation of the anti-inflammatory pathway, whereas LPS would lead to an activation of the pro-inflammatory pathway. The production of cell damage mediator NO was measured upon stimulation with LPS or MPLA in M1 and M2 polarized macrophages. There was an overall lower production in M2 macrophages and MPLA has a lower effectivity to elicit NO production in both M1 and M2 macrophages. To investigate which cytokines play a role in this and whether the difference was caused by a blockage of the inflammatory pathway or a stimulation of the anti-inflammatory pathway, RT-qPCRs were performed. It was concluded that MPLA does not lead to an increase in anti-inflammatory cytokines but does lead to an increase in pro-inflammatory cytokines. However, the increase in pro-inflammatory cytokines was less for MPLA when compared to LPS. Together with the results from the NO assay, the assumption was made that MPLA activates the same pro-inflammatory pathways as LPS, but to a lesser extent. To further investigate this, western blots were performed to investigate the pathway activation. It was concluded that MPLA activates the NFκB and the MAPK pathway faster than or to the same extent as LPS.

A few things must be considered in this research. First, the cells were only polarized for one hour after which the polarization agents were removed and fresh medium with only LPS or MPLA was added. This might have caused the M2 macrophages to polarize back towards their native or the M1 phenotype. Also, for the western blot experiments no polarization was performed. This might be interesting to look at for future research, as this might influence the pathway activation. Also, no conclusions could be made on the activation of the anti-inflammatory pathway as the results for this experiment were inconclusive.
In conclusion, LPS is very potent as a pro-inflammatory molecule. The removal of one phosphate group leads to a strong decrease in the effects. MPLA has little effect but does lead to an activation of the signaling cascade. MPLA activates the NFκB and the MAPK pathway faster than or to the same extent as LPS. Both pathways are involved in both the anti-inflammatory pathway as well as the pro-inflammatory pathway. The hypothesis was made that MPLA leads to an activation of the anti-inflammatory cascade, but this research was not able to prove this. For the future, more research on the anti-inflammatory pathways is necessary to investigate this.

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Appendix 1: Protocol NO assay

Materials:

- 100 mM NaNO2 stock solution
- 96 well plate
- 1,5 ml tubes for the standard curve
- Medium of the cells
- Griess solutions:
  - Griess A and Griess B

Calibration curve of Sodium Nitrite ($NaNO_2$):

1. Prepare stock-solution: 100 mM $NaNO_2$-solution in MQ (0.69 g/100 ml) *(Stock-solution stored in vials at -20°C)*
2. Dilute stock-solution 100x in culture medium (= 1 mM solution). Pipet 10 ul 100 mM NaNO2 in 1 ml medium ➔1 mM NaNO2
3. Make the standard curve:

[NaNO2] (uM)	**V NaNO2**	**V medium**
100	100 ul 1 mM	900 ul
50	500 ul 100 uM	500 ul
25	500 ul 50 uM	500 ul
12.5	500 ul 25 uM	500 ul
6.3	500 ul 12,5 uM	500 ul
3.1	500 ul 6.3 uM	500 ul
1.6	500 ul 3,1 uM	500 ul
0.8	500 ul 1,6 uM	500 ul
0	-	500 ul

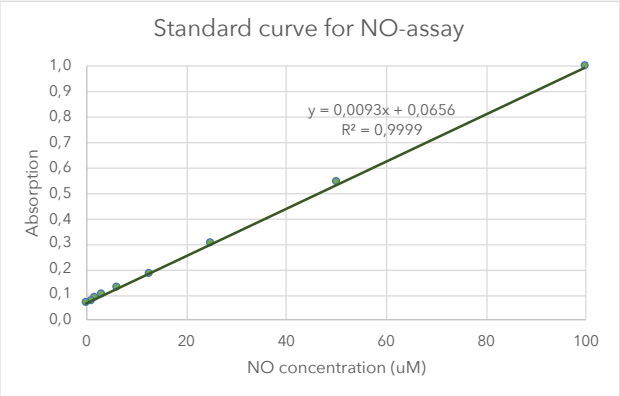
The reaction:

1. Pipet 100 ul of the standard curve samples in triplo in a 96 well plate
2. Pipet 100 ul of your experimental samples in empty wells
3. Make fresh Griess reagent by mixing equal volume of Griess A and Griess B
4. Pipet 100 ul of this fresh prepared Griess to all the standards and samples
5. Remove the bubbles out of the wells (they disturb the readout)
6. Measure the plate at 550 nM

Griess reagens:
Griess A: 2gr Sulfanilamide en 5 ml fosforzuur in total volume of 100ml MQ
Griess B: 200mg N-(1-Naphthyl)ethylenediamine dihydrochloride in 100ml MQ
1:1 mengen vlak voor gebruik

$NaNo_2$ stock
0.69 g $NaNo_2$/100 mL MiliQ water
$NaNo_2$ = #1772 in weighing room

Standard curve



The standard curve that was used were all similar to the standard curve pictured over here. The standard curve should have a maximum absorption of 1,0 at 100 µM and a linear increase in the absorption with increasing concentration.

Appendix 2: Protocol for RNA isolation using the Maxwell® LEV simplyRNA Cells Kit

Prepare before starting:
HB solution: Add 20 ul 1-Thioglycerol per 1 ml of Homogenization Solution.

Harvest the samples:
Wash the cells twice with PBS and discard the PBS
Add 200 ul pre-chilled HB to each well, homogenize them with the pipet
Place the samples in RNase free 1.5 ml tubes on ice.

Prepare Maxwell for isolation:
Place the cartridge (RNA LEV Simple) in the black holder
Strip off the covers
Place plungers in position 8
Add 5 ul DNase (stored at -20) to position 4 (yellow solution), and the solution will turn green
Place 0.5 ml tubes (from the kit!) in the FRONT row (firmly press tubes)
Add 50 ul RNase free water in the 0.5 ml tubes *(Check if there are NO air on the bottom of the tubes)*
.

Lyse the samples:
Add per sample 200 ul lysis buffer and vortex immediately for 15 seconds.
Pipet the sample straight in its position in the RNA cartridge.

Start Isolation:
Turn on the Maxwell → click RUN →
Choose program 1 → RNA → Simply RNA
Choose Run (green button) → open the door → place the cartridge in position

Appendix 3: RNA conversion to cDNA

Precaution:
tubes, tips en water must be RNase free.
You yourself are the source of Rnase

RT mix:

RT buffer	2.0 ul	
dNTP(=A,G,C,T)mix (10 mM)	0.1 ul	
Rnasin	0.25 ul	(=10 units)
Rev Transcriptase	0.5 ul	(=100 units)
Random Hexamers	0.5 ul	(=0.5 ug)
RNA	0.5 ug	(preferably in 5 ul)
H_20	1.65 ul	(to get total vol. of 10 ul)
	-------- +	
Total volume	10 ul	

NB: *Add (6) extra samples for the standard curve !!*

Converting RNA tot cDNA:
10 min 20 °C
30 min 42 °C
10 min 20 °C
5 min 99 °C
5 min 20 °C

Place the tubes in the machine
Start the file *MLVCDNA*

After the reaction is completed:
Spin the tubes (condensed water from the lids)
Store the samples at -20.

Appendix 5: qPCR protocol for creating the standard curve; preparing the cDNA; preparing the primermix; plate design; preparing the Taq MasterMix; preparing the qPCR reaction; qPCR protocol

Creating Standard Curve			
STD (rel)	**V (ul)**	**H2O (ul)**	
STD4	60 ul of pooled cD	90	
STD2	60 ul of STD4	60	
STD1	60 ul of STD2	60	
STD0.5	60 ul of STD1	60	
STD0.25	60 ul of STD0.5	60	
Prepare the cDNA			
Dilute the cDNA samples after the conversion 10 times			
Add 90 ul Rnase free H2O to the cDNA samples			
Prepare 10 uM Primermx F+R			
20 ul of 50 uM primer For			
20 ul of 50 uM primer Rev			
60 ul H2O			

Prepare the qPCR reaction		
1. Pipet 2 ul of the standard in duplo in the 384 wells plate		
2. Pipet 2 ul of the diluted samples in duplo in the 384 wells plate		
3. Add a PC and NC to the plate		
4. Add 8 ul of the Taq Mastermix to all the wells		
5. Place a seal on the plate and tight it well		
6. Go to the PCR machine and start PCR		

PCR protocol			
Stage 1	10 min	95	
Stage 2	15 sec	95	
	30 sec	60	
	40 cycli		
Stage 3	15 sec	95	
	1 min	60	
	Gradient van 0.05/sec naar 95		

Plate-design for the 384-wells plate for the qPCR reaction

Plate design	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	STD4	STD4	STD2	STD2	STD1	STD1	STD0.5	STD0.5	STD0.25	STD0.25	NC	PC												
B	STD4		STD2		STD1		STD0.5		STD0.25															
C	S1	S1	S2	S2	S3	S3	S4	S4	S5	S5	S6	S6	S7	S7	S8	S8	S9	S9	S10	S10	S11	S11	S12	S12
D	S1		S2		S3		S4		S5		S6		S7		S8		S9		S10		S11		S12	
E	S13	S13	S14	S14	S15	S15	S16	S16	S17	S17	S18	S18	S19	S19	S20	S20	S21	S21	S22	S22	S23	S23	S24	S24
F	S13		S14		S15		S16		S17		S18		S19		S20		S21		S22		S23		S24	
G	S25	S25	S26	S26	S27	S27	S28	S28	S29	S29	S30	S30												
H	S25		S26		S27		S28		S29		S30													
I	STD4	STD4	STD2	STD2	STD1	STD1	STD0.5	STD0.5	STD0.25	STD0.25	NC	PC												
J	STD4		STD2		STD1		STD0.5		STD0.25															
K	S1	S1	S2	S2	S3	S3	S4	S4	S5	S5	S6	S6	S7	S7	S8	S8	S9	S9	S10	S10	S11	S11	S12	S12
L	S1		S2		S3		S4		S5		S6		S7		S8		S9		S10		S11		S12	
M	S13	S13	S14	S14	S15	S15	S16	S16	S17	S17	S18	S18	S19	S19	S20	S20	S21	S21	S22	S22	S23	S23	S24	S24
N	S13		S14		S15		S16		S17		S18		S19		S20		S21		S22		S23		S24	
O	S25	S25	S26	S26	S27	S27	S28	S28	S29	S29	S30	S30												
P	S25		S26		S27		S28		S29		S30													

Appendix 5: Protocol for protein isolation with RIPA-TBS
PROTEIN ISOLATION WITH RIPA-TBS

Keep your protein samples on ice!!!

RIPA-TBS

Conc:	**50 ml:**
50 mM Tris-Cl pH=8.0	2.5 ml 1M Tris.CL pH=8.0
150 mM NaCl	2.5 ml 3M NaCl
1% Igepal Ca 630	500 ul Igepal ca-630
0.5% Sodium Deoxycholate	250 mg Sodium Deoxycholate
1.0% SDS	2.50 ml 20% SDS
	Fill up to 50 ml with H_2O

Just before use add:

20 ul protease inhibitor cocktail per ml *-20 draw WB*
+ 10 µl Phospho stop per ml *-20 draw WB*
+ 10 µl NaF (1M) per ml *-20 draw WB*

NB: without protease inhibitor cocktail, the RIPA can be stored for long time at 4 degree.

!!!Keep your protein on ice !!!

Method:
Cell culture:

1. Suck off medium
2. Wash the cells 1-2 times with PBS
3. Add 125 ul RIPA on celculture in 6 well (10 cm2)
4. Scrape all the cell lyses from the bottom, collect it in 1,5 ml tube
5. Shear DNA with a insuline needle (5* forced trough needle)
6. Add 40 ul loading buffer for WB
7. boil the sample for 5 minutes
8. Bring 15 ul on PAA gel
9. The rest of the sample can be stored at -20.
10. After thawing reboil sample for 1 minute before WB.

Appendix 6: Protocol for electrophoresis, blotting, antibody incubation and quantification of Western Blot

Electrophoresis.

Create a gel:

Pour a PAA gel with a acrylamide concentration that fits your need to perfectly separate proteins with different sizes.

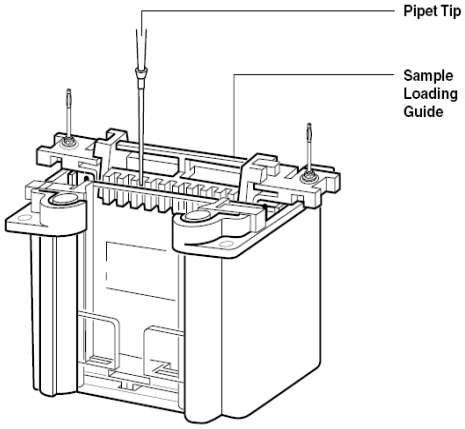
1. Start with creating a glasplate construction in which your gel can be made.
2. Prepare a separation gel according to the table
   NB: ***Poly Acryl Amide is highly toxic! Don't mesh around with it!!***
   Straight after adding TMED and APS to the solution, mix it and pour the solution between the glass plate construction (until 2 cm beneath the top of the smallest glass plate.
3. Add about 1 ml 2-butanol on top of the separation gel to create a airtight and straight topline of your gel.
4. Let the solution polymerise for about 30 minutes
5. When the gel is solid, take of the butanol and wash off the butanol with H2O. Take care that you take off the water.
6. Prepare a stack gel solution according to the table.
   Also here: as soon as you added TMED and APS, pour the stack on top of the solid separation gel until the top of the small glass plate
7. Take a comb with the correct dept (1 of 1.5 mm) and place it in the stack.
8. Let the solution polymerize for about 30 min.

Prepare gel system:

1. Choose the precise protein gel needed for your job
2. Create electrophorese system:
3. Fill the inner chamber with ~125ml electrophoresis buffer until the level reaches halfway between the tops of the taller and shorter glass plates of the gel cassettes.
4. Add ~200ml of electrophoresis buffer to the outer chamber
5. Rinse the slots with buffer.

Sample loading.

1. Create samples: take 25 ug protein and add appropriate amount of loading buffer, heat the samples for 5 minutes at 95 degree in a dry bath
2. Cool sample to RT (no ice), short spin the sample.
3. Load the samples into the wells.



4. Run the system: 100 V, 1-3 hours, depending on the size of the protein.

Gel removal.

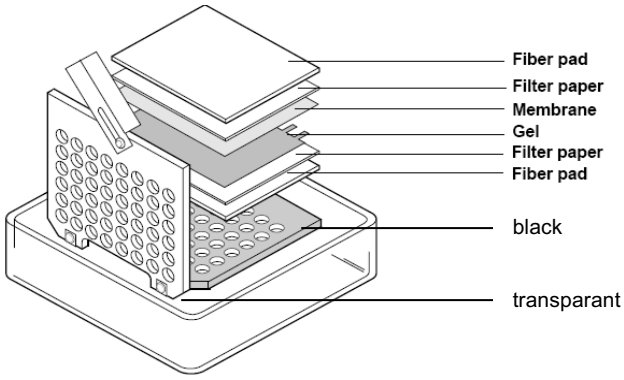
5. After electrophoresis is complete, turn off the power supply and disconnect the electrical leads.

6. Remove the tank lid and carefully lift out the inner chamber assembly.
7. Remove the gels from the gel cassette by gently separating the two plates of the gel cassette. The green, wedgeshaped, plastic Gel Releaser may be used to help pry the glass plates apart.

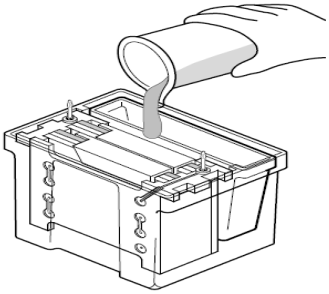
Blotting.

8. Cut the membrane and the filter paper to the same size as the gel. Always wear gloves when handling membranes to prevent contamination. Soak the membrane, filterpaper and fiber pads in blot buffer.
9. Prepare the gel sandwich
    a. Place the cassette, with the black side down, on a clean surface.
    b. Place one pre-wetted fiber pad on the gray side of the cassette.
    c. Place a sheet of filter paper on the fiber pad.
    d. Place the gel on the filterpaper.*
    e. Place the pre-wetted membrane on the gel.*
    f. Complete the sandwich by placing a piece of paper on th e membrane, and add the last fiber pad.

    * Remove any airbubble (important for good results)



10. Close the cassette firmly with the white latch. Place the cassette in module. (black facing black and transparant facing Red)
11. Add the frozen Bio-Ice cooling unit. Place in tank and completely fill the tank with buffer.



12. Put the tank in box with ice.
13. Run for 1 hours at 300 mA (0.3A).
14. You can check if blotting is completed by looking if the marker is completely transferred from gel to the membrane.
15. Check with Ponceau if the proteins are on the membrane.
16. Mark your membrane with a pencil and cut off any excess membrane

Incubation with antibodies.

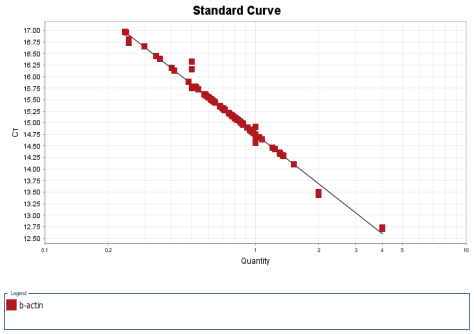
17. Block the membrane for 20 minutes with the blocking buffer (5 g skimmed milk/ 100 ml TBS + Tween 20)
18. Dilute the primary antibody in 5 ml blocking buffer (per 2 membranes)
19. Incubate primary antibody via shaking (3 hours in room temperature or overnight in the cold room)
20. Wash the membrane three times with TBS + Tween 20 (5 minutes each time)
21. Dilute the secondary antibody in 5 ml blocking buffer (per 2 membranes)
22. Incubate the secondary antibody also by shaking (1 hour at room temperature or overnight in cold room)
23. Wash the membrane three times with TBS + Tween 20 (5 minutes each time)
24. Wash the membrane 2 times with TBS.

Quantification.

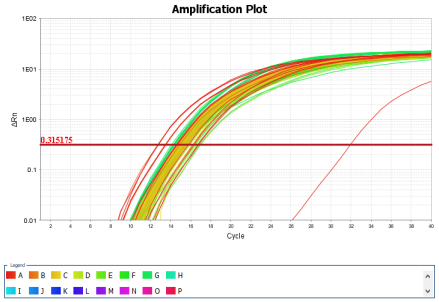
25. Mix supersignal 1 and supersignal 2 (ratio 1:1). Pour the SS mixture over the whole membrane (plastic slide cleaned with 70% EtOH)
26. Put the membrane (on a plastic slide) on the drawer into the machine.
27. Select the timepoints at which you want the machine to make a picture and run the program with 'No light'
28. Make a white light picture
29. Merge the two images
30. Save picture as….
31. Quantificate the results in GeneTools.

Appendix 7: Standard curve and amplification plot for each gene of interest of the qPCR experiment

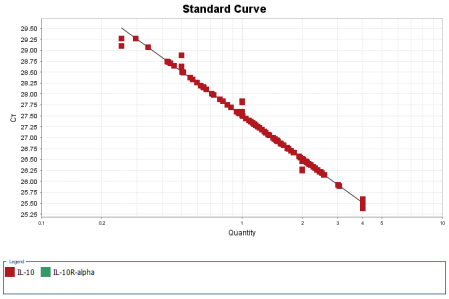
Standard curve and amplification plot of β-actin



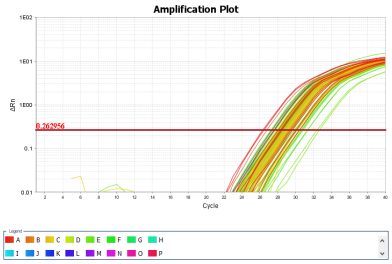
Eff: 90%



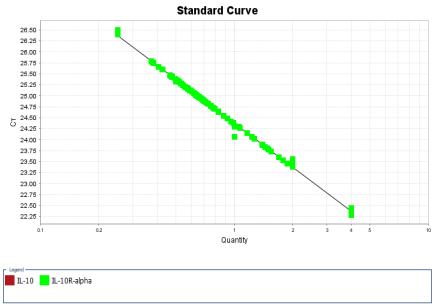
Standard curve and amplification plot of IL-10



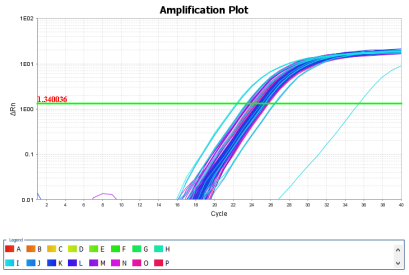
Eff: 109%



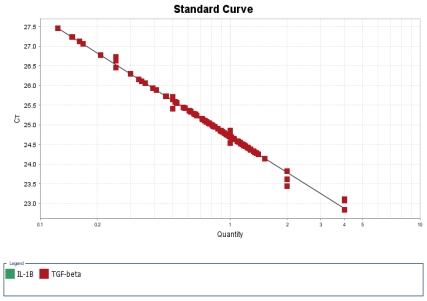
Standard curve and amplification plot of IL-10R-α



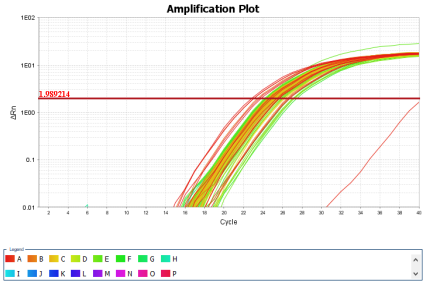
Eff: 99%



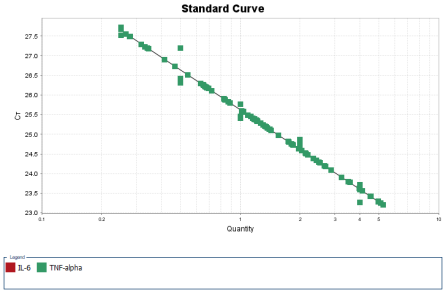
Standard curve and amplification plot of TGF-β



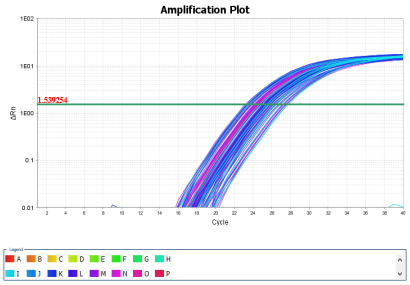
Eff: 107%



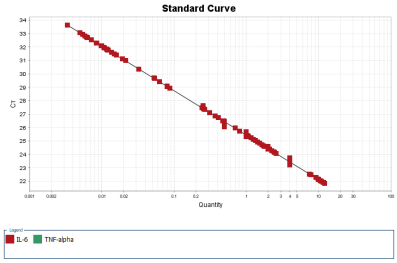
Standard curve and amplification plot of TNF-α



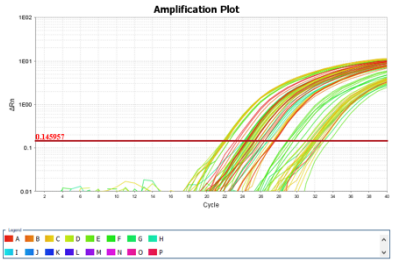
Eff: 98%



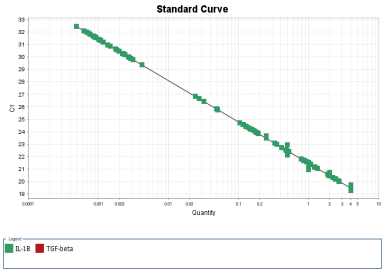
Standard curve and amplification plot of IL-6



Eff: 100%



Standard curve and amplification plot of IL-1β



Eff: 100%