Inflammatory effect of IL-10 and IL-10 peptide derivatives P1, P2 and P3 on liver fibrosis

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Abstract

In the search for novel therapies in the treatment of liver fibrosis, interleukin-10 (IL-10) has gained particular attention due to its anti-inflammatory properties. However, when liver fibrosis is virus-induced anti-inflammatory compounds cannot be used as they suppress the immune system. The treatment of virus-induced liver fibrosis therefore requires an anti-fibrotic compound with no anti-inflammatory properties. Smaller, specific IL-10 peptide derivatives could offer tremendous potential. This study analyzed the anti-inflammatory effect of IL-10 and used this to test the effect of IL-10 peptide derivatives P1, P2 and P3 on RAW 264.7 cells. Cytokine effect was determined by the production of nitric oxide (NO Assay) and relative TNF-α gene expression (qPCR). Cells were pre-stimulated with LPS and a combination of LPS and IFN-γ, pre-stimulated with cytokine (IL-10, P1, P2 or P3) or co-stimulated with both LPS and cytokine. IL-10 showed to have minimal to no anti-inflammatory effect on the production of NO. Only pre-stimulation with IL-10 and co-stimulation with IL-10 and LPS at t = 22 hr after cytokine stimulation showed a reduction in NO production. P1, P2 and P3 had no effect on the production of NO and thereby do not affect inflammation through this mechanism. However, IL-10 did significantly reduce the relative expression of pro-inflammatory cytokine TNF-α in RAW 264.7 cells when pre-stimulated with IL-10. In contrast, P1, P2, and P3 increased relative TNF-α expression suggesting pro-inflammatory properties. In summary, IL-10 exhibits anti-inflammatory properties primarily through the inhibition of pro-inflammatory cytokine gene activation while P1, P2 and P3 show no anti-inflammatory properties.

Introduction

Liver fibrosis

Cirrhosis, an advanced stage of liver fibrosis, is a global cause of morbidity and mortality. In 2019, it accounted for 2.4% of global deaths [1]. More generally, liver fibrosis is marked by the thickening or scarring of tissue and is often the endpoint of chronic diseases [2]. Patients can survive and develop the condition over years reaching the severe stage of cirrhosis only after 15-20 years. It is often at this stage that patients develop symptoms including nausea and unexplained weight loss [3].

Liver fibrosis is the result of repeated injury to the liver. It can be categorized based on the type and site of chronic injury: hepatotoxic or cholestatic injury. Hepatotoxic injury describes cellular injuries caused by external factors such as viral hepatitis B and C, alcoholic liver disease (ADL) and non-alcoholic fatty liver disease (NAFLD) while cholestatic injury occurs when the transport of bile to the duodenum is obstructed. This can be the result of diseases such as primary sclerosing cholangitis (PSC) and biliary atresia [4]. These injuries, caused by both genetic and environmental factors, lead to the prolonged accumulation of extracellular matrix proteins (ECM) and ultimately the formation of permanent scar tissue.

Mechanism of Liver Fibrosis: Fibrotic and Inflammatory response

Although the focus of this study is liver fibrosis, the fibrotic response is similar in many organs. It involves a fibrotic and inflammatory component, both connected and crucial in understanding the development of disease.

Upon primary tissue damage, epithelial and/or endothelial cells release inflammatory mediators which stimulate the coagulation pathway. This leads to the formation of a fibrin clot and functions to reduce initial blood loss. The damaged tissue stimulates the release of cytokines and growth factors which initiate the acute inflammation phase. Innate immune cells such as neutrophils and macrophages are recruited to the site of injury. These cells activate the adaptive immune system through antigen presentation. The end of the inflammatory phase is marked by pathogen clearance and signals cells to enter the proliferation phase [2].

In the proliferation phase, ECM components are synthesized to recover the wound. In the liver, cytokines and growth factors stimulate hepatic stellate cells (HSCs) to differentiate into myofibroblasts. In a normal liver, these cells are situated in the perisinusoidal space (the space of Disse) and function primarily as a storage site for vitamin A. This is why in a normal liver they are referred to as "dormant". Upon chronic liver injury, they differentiate into myofibroblasts – the main cells responsible for ECM production. Besides the production of ECM, myofibroblasts have proinflammatory and contractile properties [5,6].

The last stage of fibrogenesis is known as the remodeling phase – a prolonged phase by which the tissue is restored through scar tissue formation and maturation. At this stage, inflammatory and fibroblast cells are no longer recruited. In liver fibrosis however, repeated injury leads to presence of activated HSCs which continuously produce ECM. The complex interplay between fibrotic HSCs and immune cells suggests inflammation plays a very important role in the development of liver fibrosis. Looking for novel therapies thereby requires a thorough understanding of the function of cytokines and immune cells in fibrogenesis. This thesis will therefore focus primarily on the inflammatory response.

Cytokines: IL-10, P1, P2 and P3

The connection between inflammatory responses and fibrosis has placed particular attention on interleukin-10 (IL-10) in the context of liver fibrosis. It's function as an anti-inflammatory cytokine classifies it as a potential anti-fibrotic therapy.

IL-10, initially thought to be produced only by T2 helper cells (Th2), is produced by various immune cells including B cells, granulocytes, and macrophages [2]. It binds to a tetrameric receptor complex with two copies of IL-10 Receptor-1 (also known as IL-10 Rα) and two copies of IL-10 Receptor-2 (also known as IL-10 Rβ). Studies show IL-10 binds with high affinity to IL-10 R1 [7]. IL-10 R2 is then recruited with minimal contribution to ligand binding. However, the binding to IL-10 R2 is crucial in signal transduction. While IL-10 R1 is expressed in low levels only by hematopoietic cells, IL-10 R2 is expressed by most cells. IL-10 R1 expression can be upregulated in various cells, including non-hematopoietic cells, in response to stimuli.

Upon binding of IL-10 to the receptor complex, the Janus tyrosine kinases JAK1 and Tyk2 are activated. JAK1 is associated with IL-10 R1 and Tyk2 with IL-10 R2. Activation of JAK1 and Tyk2 causes phosphorylation of tyrosine residues in IL-10 R1 and recruitment of STAT3. STAT3 is also phosphorylated and homodimerizes to activate gene expression in the nucleus [7].

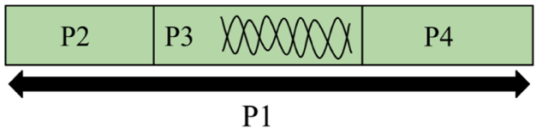

Fig. 1. Structure of IL-10 peptide derivates

For some cases of liver fibrosis, the anti-inflammatory and anti-fibrotic properties of IL-10 are ideal. However, in virus-induced liver fibrosis, IL-10 cannot be used as its anti-inflammatory properties suppress the immune system causing great harm. P1, P2 and P3 were synthesized in the hopes of finding an anti-fibrotic compound that does not reduce inflammation. P1, P2 and P3 are IL-10 R1 binding peptides mimicking the IL-10 structure. As shown in fig. 1 above, P1 is structurally identical to IL-10 and P2, P3 and P4 are different segments of P1. P4 was not analyzed in this study. P3 is known to contain the IL-10 helix. Due to patent reasons, the sequence of P1, P2 and P3 cannot be disclosed.

Inflammatory response: M1 and M2

Two important mediators in inflammatory response include the M1 and M2 macrophage, also known as classically activated and alternatively activated macrophages respectively. These macrophages, known as Kupfer cells in the context of liver fibrosis, have shown to be primary targets for IL-10's anti-inflammatory effect [2]. M1 macrophages are involved in pro-inflammatory responses and M2 macrophages in anti-inflammatory responses.

Polarization into M1/M2 phenotype is achieved through stimulation by different cytokines and other stimulants. Prior to polarization, the macrophage is termed M0. As shown in fig. 2, M1 is activated by bacterial lipopolysaccharide (LPS) and/or interferon-gamma (IFN-γ) as well as tumor-necrosis factor alpha (TNF-α). When activated, M1 macrophages rely on glycolytic metabolism. They express nitric oxide synthase (iNOS) which produces nitric oxide (NO) and citrulline from arginine [8].

M2 macrophages are activated by cytokines IL-4/IL-13 and IL-10. They rely on oxidative phosphorylation for metabolism [2]. This switch in metabolism observed in M1/M2 phenotype switch is known as metabolic reprogramming [8,9]. M2 macrophages express the arginase enzyme which converts arginine to urea and ornithine [10].

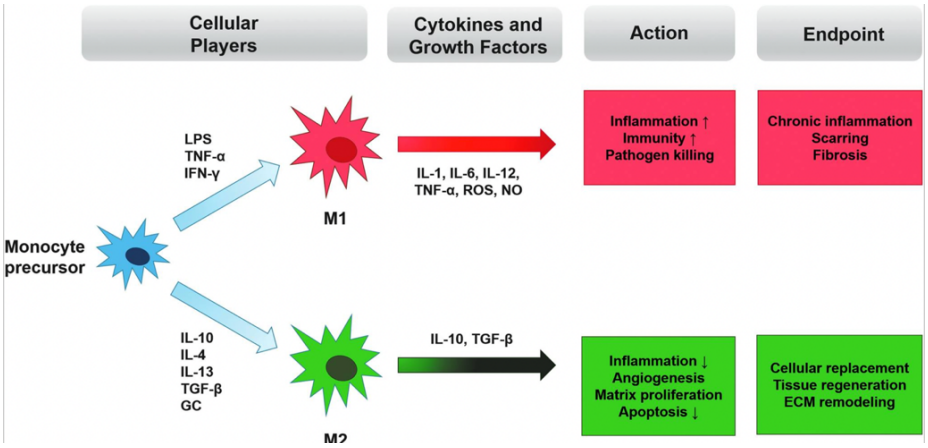

Fig. 2. *M1/M2 polarization. M1 is polarized by bacterial LPS and/or by cytokines TNF-α and IFN-γ. When polarized, M1 is involved in pro-inflammatory response. M2 is polarized when exposed to cytokines IL-10, IL-4, IL-13 and TGF-β. M2 is involved in the anti-inflammatory response [2].*

Cytokines, Inflammation and Gene Expression

In the polarization of M1 macrophage, LPS binds to co-receptor CD-14 which is either in a soluble form or linked to the cell surface by a glycosylphosphatidylinositol anchor. CD-14 aids in the binding of LPS to toll-like receptor 4 (TLR4) – MD-2 complex. LPS is then transferred to MD-2 protein and leading to the dimerization with TLR4. This activates two important signalling pathways: MyD88-dependent and MyD88-independent pathway (TRIF-dependent pathway). The MyD88-dependent pathway is responsible for early activation of nuclear factor-*k*B (NF-*k*B) and mitogen-activated protein kinases (MAPK). The MyD88-independent pathway results in the late activation of NF-*k*B [11]. These two downstream pathways induce the polarization to M1 macrophage and the expression of pro-inflammatory cytokine genes [12]. They also trigger the transcription of the iNOS gene leading to the production of NO.

The anti-inflammatory effects of IL-10 have been studied extensively. However, the molecular mechanism remains widely debated. Despite the contradiction, IL-10 is known to inhibit the production of pro-inflammatory cytokines such as interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α). These two pro-inflammatory cytokines are central in the immune response [13]. IL-10 inhibits the production of these cytokines through inhibition of multiple signalling pathways including NF-*k*B and p38 MAPK [14]. As previously mentioned, theses pathways are activated by LPS in the pro-inflammatory response.

Therefore, this study aims to determine the optimal method of measuring the anti-inflammatory effect of IL-10 and use this to analyze the effect of IL-10 peptide derivaties P1, P2 and P3 on LPS-stimulated macrophages. Quantities analyzed include the production of NO with NO assay as well as pro-inflammatory cytokine gene expression with quantitative PCR.

Materials and Methods

Reagents

Cytokines (obtained from PeproTech™) and stimulants: Recombinant murine IFN-γ (CAT: 315-05), recombinant murine IL-10 (CAT: 210-10), Lipopolysaccharides from Escherichia coli (Merck, CAT: L4391), IL-10R1-binding peptide named P1 and three parts of IL-10R1-binding peptide named P2, P3 and P4 (created by prof. doc. K. Poelstra, synthesized by Nunzianna Doti, Institute of Biostructures and Bioimaging, Napels, Italy).

Cell culture

The cell line used for all experiments were RAW 264.7 murine macrophages. They were cultured in DMEM (DMEM, CAT: 32430-027) + 10% FBS at 37 °C and 5% CO_2.

NO Assay

In all experiments RAW 264.7 cells were cultured for 24 hr in a 96 well plate (10^5 cells/well) to adhere to the bottom. In experiment 1.1, they were then stimulated with 100 ng/mL LPS, 20 ng/mL IFN-γ or a combination of both for 24 hr. In experiment 1.2, RAW 264.7 cells were pre-stimulated for 24 hr with a combination of 100 ng/mL LPS and 20 ng/mL IFN-γ followed by a 2 hr stimulation of 30 ng/mL IL-10, P1, P2 or P3. NO concentration was measured at t = 0, 2 and 24 hr relative to cytokine stimulation. This was achieved by freezing samples in - 20 °C. For experiment 1.3, a combination of previous stimulations were chosen including pre-stimulation with 100 ng/mL LPS for 24 hr followed by stimulation with 30 ng/mL IL-10 for 2 hr, pre-stimulation with 30 ng/mL IL-10 for 2 hr followed by stimulation with 100 ng/mL LPS for 24 hr and co-stimulation with both 30 ng/mL IL-10 and 100 ng/mL LPS. NO Assay was done at t = 24 and t = 26 hr after pre-stimulation (with either LPS or cytokine). Lastly, in experiment 3.1, RAW 264.7 cells were pre-stimulated with 30 ng/mL IL-10, P1, P2 or P3 for 2 hrs followed by stimulation with 100 ng/mL LPS for 24 hrs. NO concentration was determined at t = 26 hr. All NO Assays were conducted according to the NO Assay Protocol (Appendix H). Raw NO concentrations were standardized to percentages relative to the positive control (either LPS or a combination of LPS and IFN-γ). This allowed averaging of different samples and presentation in a single figure.

MTT Assay

MTT Assay was conducted on the same RAW 264.7 cells from experiment 1.1 and 1.2 at t = 24 hr to determine % cell viability. MTT assay quantifies cell viability by the ability of cellular enzymes to convert the tetrazolium dye (MTT) to purple formazan. Absorbance was recorded at 550 nm according to the MTT Assay Protocol (Appendix I). From absorbance measurements, cell viability was calculated according to the following formula:

$$\%\ cell\ viability = \frac{abs_{sample} - abs_{negative\ control}}{abs_{positive\ control}} * 100$$

Whereby the negative control is the MTT control (background noise) containing dead cells and the positive control contains untreated cells.

Quantitative Polymerase Chain Reaction (qPCR)

RAW 264.7 cells ($4x10^5$ cells/well) were cultured for 24 hr in a 12 well plate. They were pre-stimulated with 30 ng/mL IL-10, P1, P2 or P3 for 1 hr followed by LPS stimulation at different concentrations for 2 hr. LPS concentrations used include 10 ng/mL, 30 ng/mL and 100 ng/mL. Co-stimulation of 30 ng/mL IL-10 and 100 ng/mL LPS for 3 hr was also analyzed. RNA was extracted from the cells using the Maxwell® 16 LEV simplyRNA Cells Kit. RNA

concentration was determined using the nanodrop. cDNA was generated using cDNA reverse transcriptase (RT) kit from Promega. qPCR was proceeded using the Promega SYBR® Green Mix. The conditions for qPCR were set to 5 min at 95 °C followed by 15 sec at 95 °C and 30 sec at 60 °C for 40 cycles and 15 sec at 95 °C, 1 min at 60°C with a gradient of 0.05 °C /sec to 95 °C. TNF-α gene expression was quantified relative to beta-actin housekeeping gene. The primer sequences were beta-actin forward 5'- ATCGTGCGTGACATCAAAGA-3' and reverse 5'-ATGCCACAGGATTCCATACC-3'; TNF-α forward 5'-CATCTTCTCAAAATTCGAGTGACAA-3' and reverse 5'-GAGTAGACAAGGTACAACCC-3'. In all qPCR experiments, raw data was presented by equating the positive control (LPS) to 100% and expressing TNF-α as a percentage relative to LPS.

Results

Since M1 macrophages can be stimulated by both LPS or LPS in combination with IFN-γ it was important to determine the optimal response. A study published in *Inflammation* suggested the best results concerning stimulation of RAW 264.7 cells were observed with LPS in combination with IFN-γ [15]. The concentrations used in the study were 100 ng/mL LPS and 20 ng/mL IFN-γ. These were chosen as a reference for the conducted experiments.

1.1: NO and MTT Assay stimulation with IFN-γ, LPS or a combination of both

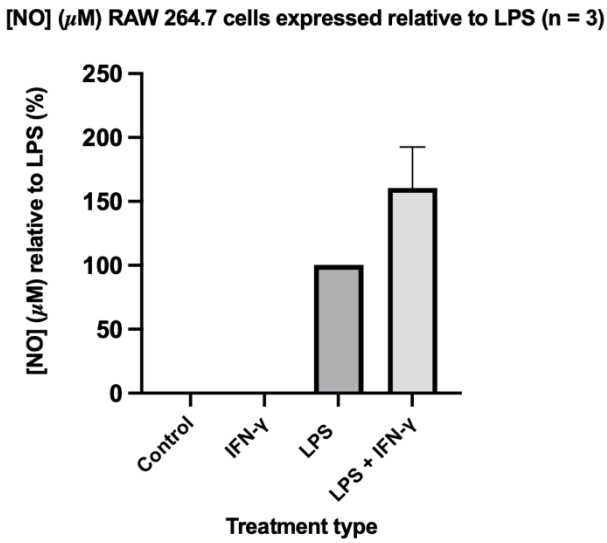

Fig. 3. *[NO] (μM) expressed as a percentage relative to LPS control (n = 3). RAW 264.7 cells were left unstimulated or stimulated with 20 ng/mL IFN-γ, 100 ng/mL LPS, or a combination of 20 ng/mL IFN-γ and 100 ng/mL LPS for 24 hr. NO concentration was measured at t = 24 hr.*

In experiment 1.1, the effect of co-stimulation with IFN-γ and LPS was analyzed to determine the optimal response for the following experiments. As shown above, there is a significant increase in NO produced by RAW 264.7 cells when they are stimulated with both LPS and IFN-γ when compared to only stimulating with LPS. Cells left unstimulated or stimulated only with IFN-γ showed no production of NO relative to the LPS control.

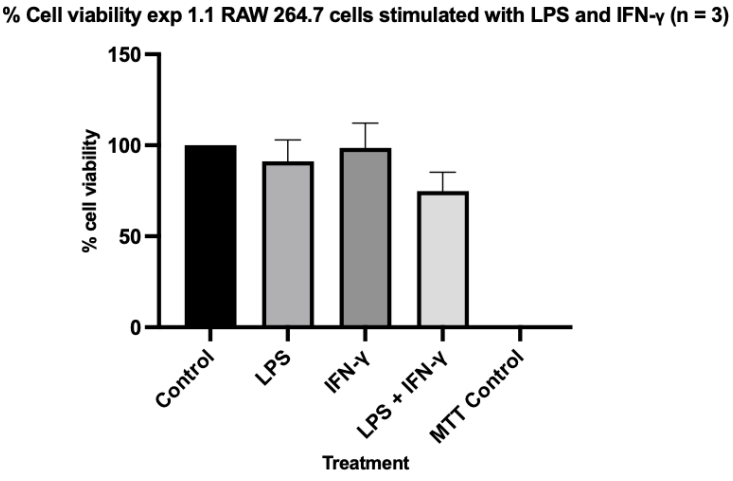

Fig. 4. *% Cell viability at t = 24 hr after stimulation with LPS, IFN-γ or a combination of both (n = 3). The MTT Assay was done on the cells corresponding to experiment 1.1.*

When looking at the production of NO it is important to also analyze cell viability through an MTT assay. NO response can be largely influenced by the number of proliferating cells. Fig. 4 shows the MTT assay done on RAW 264.7 cells stimulated with LPS, IFN-γ or a combination of both. When compared to the control (100% cell viability), stimulation with LPS or IFN-γ for 24 hr does not influence the number of living cells. A reduction of % viability is observed after co-stimulation with LPS and IFN-γ. However, considering the increase in NO production seen in fig. 3, the decrease in viability had no negative influence on the NO response.

The combination of LPS and IFN-γ was therefore used to analyse the effects of cytokines IL-10, P1, P2 and P3 on NO response and thereby inflammation in the following experiments.

1.2: NO and MTT Assay stimulation IL-10, P1, P2, P3 at t = 0 hr, t = 2 hr and t = 24 hr after cytokine stimulation

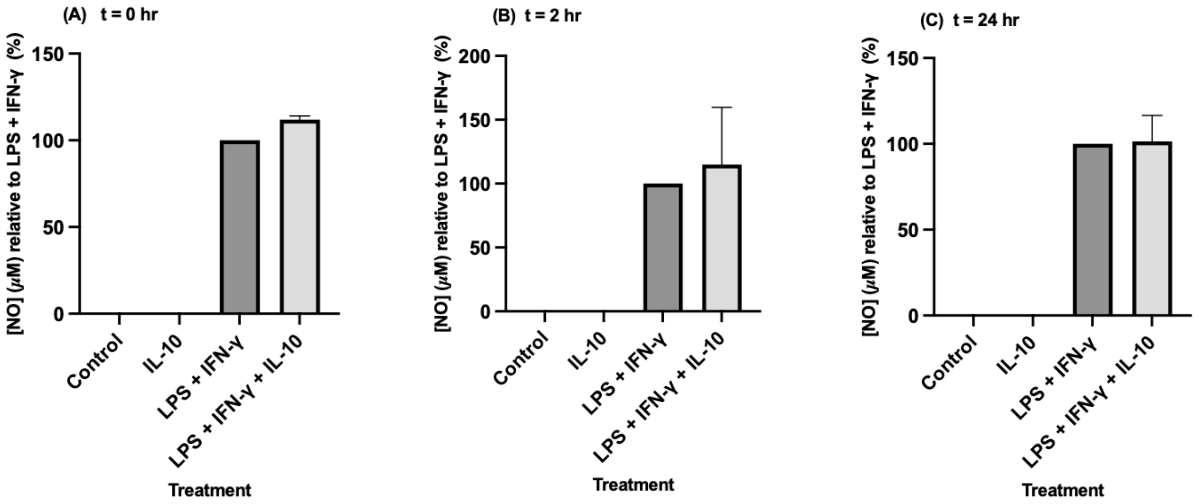

Fig. 5*. [NO] (μM) expressed as a percentage relative to LPS + IFN-γ control (n = 2) after IL-10 stimulation. RAW 264.7 cells were left unstimulated or stimulated with 20 ng/mL IFN-γ and 100 ng/mL LPS for 24 hr. After pre-stimulation, 30 ng/mL IL-10 was added. (A) Relative [NO] at t = 0 hr in the stimulation with IL-10. (B) Relative [NO] at t = 2 hr after stimulation with IL-10. (C) Relative [NO] (uM) at t = 24 hr after stimulation with IL-10.*

As shown in Fig.5 the effect of IL-10 on NO production was measured after LPS + IFN-γ pre-stimulation since this showed the greatest NO production in fig.3. At all three time points, unstimulated cells (control) and cells stimulated with only IL-10 showed no response. Treatment with IL-10 after pre-stimulation with LPS and IFN-γ did not significantly increase or decrease NO concentration at t = 24 hr. An increase is observed at t = 2 hr but this could not be stated with certainty due to the large error bar.

For all cytokines tested in experiment 1.2, time point 0 hr should show similar response between LPS + IFN-γ and LPS + IFN-γ + cytokine. This is because at t = 0 hr no cytokine is added. Slight deviations are discussed in the discussion section and could be explained by slight variation in cell count between samples.

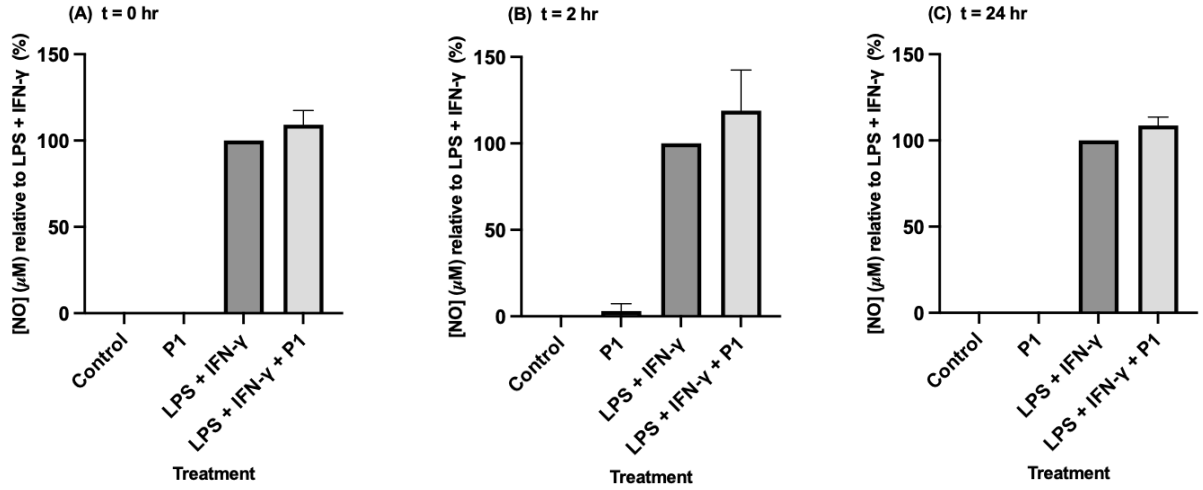

Fig. 6*. [NO] (μM) expressed as a percentage relative to LPS + IFN-γ control (n = 2) after P1 stimulation. RAW 264.7 cells were left unstimulated or stimulated with 20 ng/mL IFN-γ and 100 ng/mL LPS for 24 hr. After pre-stimulation, 30 ng/mL P1 was added. (A) Relative [NO] at t = 0 in the stimulation with P1. (B) Relative [NO] at t = 2 hr after stimulation with P1. (C) Relative [NO] at t = 24 hr after stimulation with P1.*

Similar to Fig.5, the effect of P1 on pre-stimulated cells was measured at different time points. As shown in Fig.6, at all three time points unstimulated cells showed no NO response and cells stimulated only with P1 showed no response at t = 0 and t = 24 hr. Treatment with P1 after pre-stimulation had no significant effect on NO production at t = 24 hr. At t = 2 hr there is a slight increase in NO production; this is also observed in the P1 control at t = 2 hr. These two data points also have large error bars due to large deviation between the two samples measured.

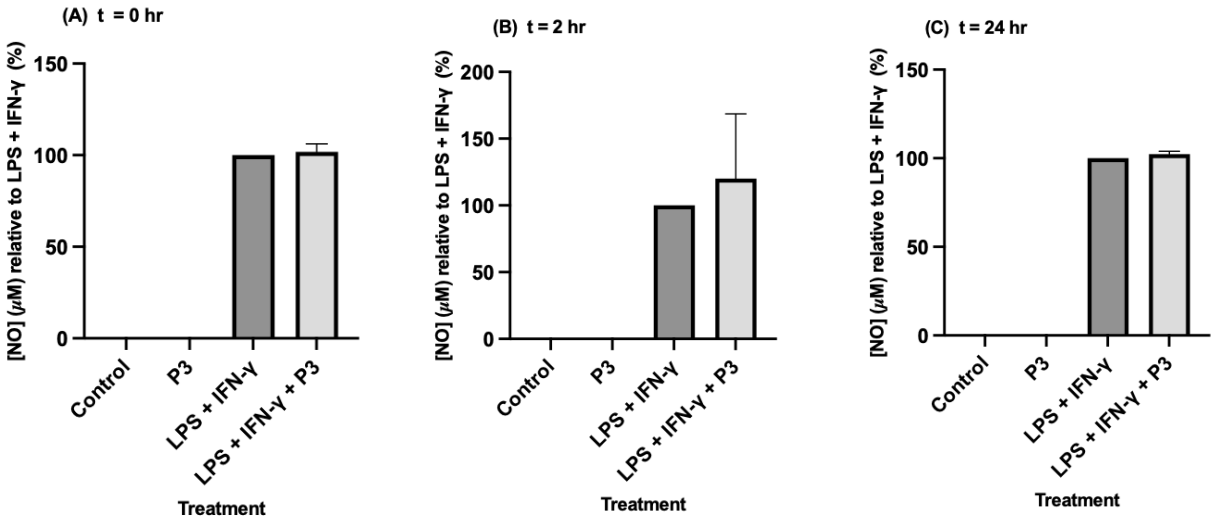

Fig. 7. *[NO] (μM) expressed as a percentage relative to LPS + IFN-γ control (n = 2) after P3 stimulation. RAW 264.7 cells were left unstimulated or stimulated with 20 ng/mL IFN-γ and 100 ng/mL LPS for 24 hr. After pre-stimulation, 30 ng/mL P3 was added. (A) Relative [NO] at t = 0 in the stimulation with P3. (B) Relative [NO] at t = 2 hr after stimulation with P3. (C) [NO] at t = 24 hr after stimulation with P3.*

Fig. 7 shows the effect of P3 on NO production after LPS + IFN-γ pre-stimulation. Unstimulated cells or cells stimulated only with P3 showed no response at t = 0, t = 2 hr, and t = 24 hr. Stimulation with P3 had no effect on NO concentration at t = 24 hr compared to LPS + IFN-γ induced response. At t = 2 hr an increase in response is seen with P3 relative to LPS + IFN-γ but this increase is accompanied by a large standard deviation – the scope of the error bar could mean both an increase and decrease in response.

The different cytokines were assigned to different students. P1 and P3 were analysed in duplo (n = 2) but P2 was analysed by one student only. The results were included in this thesis to provide a more thorough overview of the three cytokines.

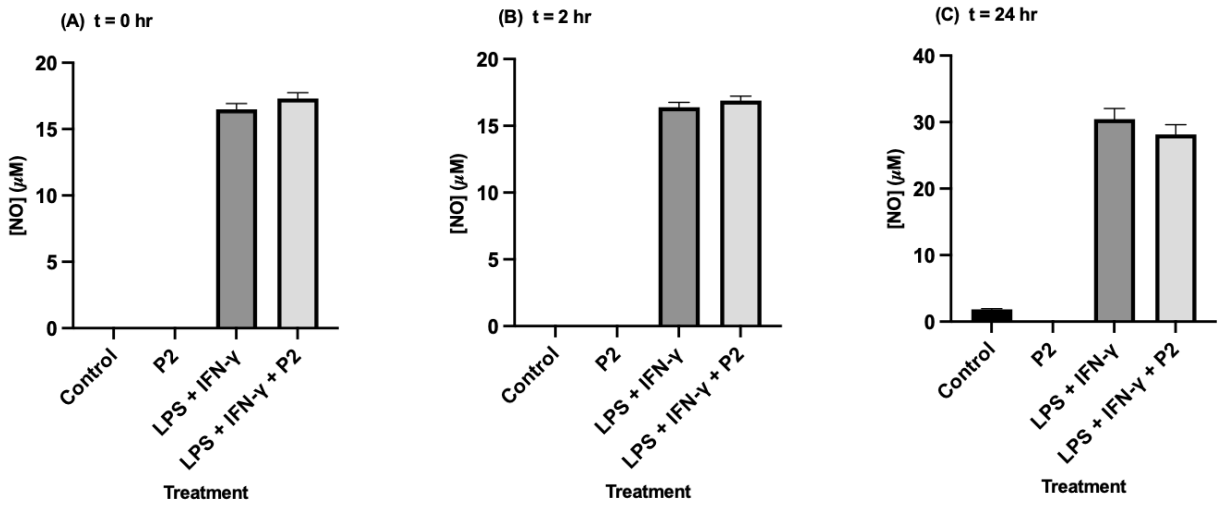

Fig. 8. *[NO] (μM) (n = 1) after P2 stimulation. RAW 264.7 cells were left unstimulated or stimulated with 20 ng/mL IFN-γ and 100 ng/mL LPS for 24 hr. After pre-stimulation, 30 ng/mL P2 was added. (A) [NO] (μM) at t = 0 in the stimulation with P2. (B) [NO] (μM) at t = 2 hr after stimulation with P2. (C) [NO] (μM) at t = 24 hr after stimulation with P2.*

As mentioned, fig. 8 represents the NO concentration measured in one experimental trial. Stimulation with P2 had no effect on NO concentration at t = 2 hr. At t = 0 and t = 2 hr unstimulated cells or cells stimulated only with P2 showed no response. At t = 24 hr there is a slight decrease in NO production with P2 stimulation compared to only LPS + IFN-γ. This is a decrease of only 2,3 μM. At t = 24 hr unstimulated cells also show a minimal NO response. NO production increases over time for both the LPS + IFN-γ control and LPS + IFN-γ + P2. This is shown by the two-fold increase in NO concentration at t = 24 hr compared to t = 2 hr.

Overall, no significant increase or reduction in NO response was seen with either cytokine or time point. The slight deviations are inconsistent and accompanied by large error bars. An MTT assay was done after 24 hr stimulation with cytokine to analyse the influence of cell viability on the NO response.

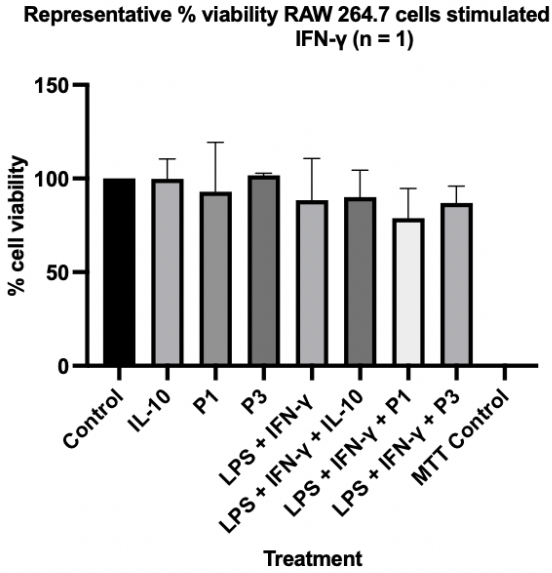

Fig. 9. *Representative figure % cell viability at t = 24 hr after pre-stimulation with LPS and IFN-γ and cytokine stimulation (n = 1). The MTT Assay was done on the cells corresponding to experiment 1.2.*

Fig. 9 shows the MTT assay done on a single sample set of experiment 1.2. This assay is therefore representative of the repeated experiments. It is clear in fig. 9 that no stimulation led to significant reduction in cell viability. Pre-stimulation with LPS + IFN-γ followed by stimulation with P1 showed the most reduction in cell viability after 24 hr. As shown in fig. 6., this did not influence the production of NO as the concentration of NO is higher in cells treated with P1 than cells only treated with LPS and IFN-γ. Overall, decrease in cell viability did not have a significant influence on NO production and the treatment types were not toxic to cells.

1.3: NO assay pre-stimulation LPS, pre-stimulation IL-10, and co-stimulation

The experiments above all involved pre-stimulation with LPS followed by stimulation with the cytokine. Studies in the past have also looked at pre-stimulation with IL-10 as well as co-stimulation. A study published in *Innate Immunity* showed significant inhibition of pro-inflammatory cytokine IL-6 with co-stimulation of RAW 264.7 macrophages [14]. Although no NO assay was done in the study it suggested co-stimulation had anti-inflammatory effects. For the following experiments, RAW 264.7 cells were either pre-stimulated with LPS, pre-stimulated with IL-10 or co-stimulated with both LPS and IL-10 to see if the form of stimulation influences response.

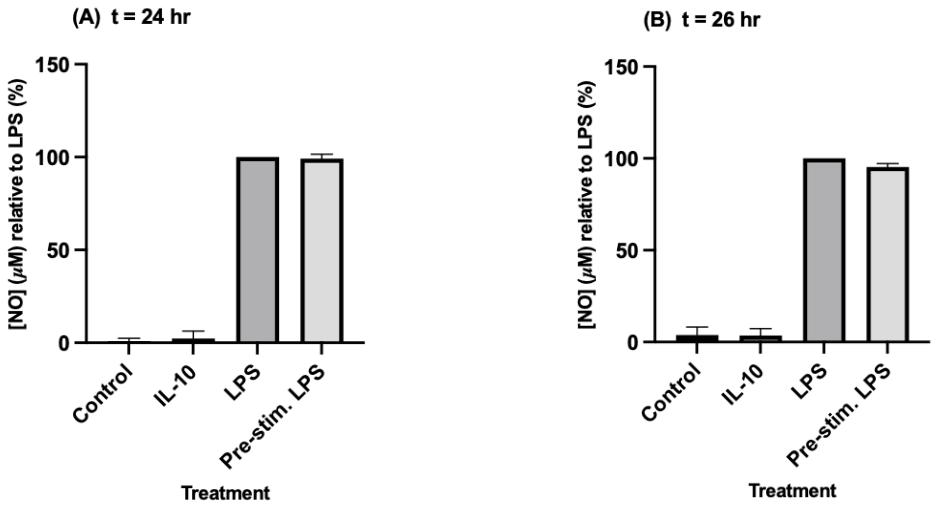

Fig. 10. *[NO] (μM) expressed as a percentage relative to LPS (n = 3) with pre-stimulation LPS. RAW 264.7 cells were left unstimulated or pre-stimulated with 100 ng/mL LPS for 24 hr. They were then stimulated with 30 ng/mL IL-10 for 2 hr. (A) Relative [NO] at t = 24 hr after stimulation with LPS. (B) Relative [NO] at t = 26 hr after stimulation with LPS.*

Fig 10. shows the effect of pre-stimulation with LPS followed by stimulation with IL-10 on NO production. At t = 24 hr no difference is seen between the LPS control response and the pre-stimulation response as cells have only been stimulated with LPS. At t = 26 hr, a slight reduction of 5% is seen in NO production relative to the LPS control.

Since the three stimulation conditions were analysed under the same experiment unstimulated cells and cells stimulated only with IL-10 show a consistent response. At t = 24 hr unstimulated cells show no response and cells stimulated only with IL-10 show minimal response. At t = 26 hr both unstimulated cells and cells stimulated with IL-10 only show minimal NO response.

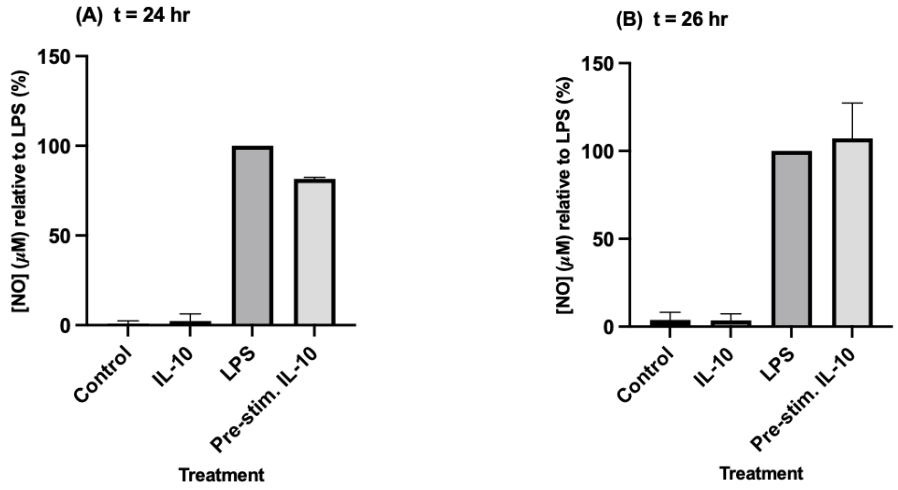

Fig. 11. *[NO] (μM) expressed as a percentage relative to LPS (n = 3) with pre-stimulation IL-10. RAW 264.7 cells were left unstimulated or pre-stimulated with 30 ng/mL IL-10 for 2 hr. They were then stimulated with 100 ng/mL LPS for 24 hr. (A) Relative [NO] at t = 24 hr after stimulation with IL-10. (B) Relative [NO] at t = 26 hr after stimulation with IL-10.*

Fig. 11 shows the effect of pre-stimulating cells with IL-10 followed by stimulation with LPS on the production of NO. As shown in fig. 11 (A) pre-stimulation with IL-10 for two hours leads to a reduction of 18.4% in relative NO concentration at t = 24 hr. Two hours later, at t = 26 hr, pre-stimulation with IL-10 shows a slight increase (7%) in NO production compared to only LPS. As shown in fig. 11 (B) this increase has high error bars.

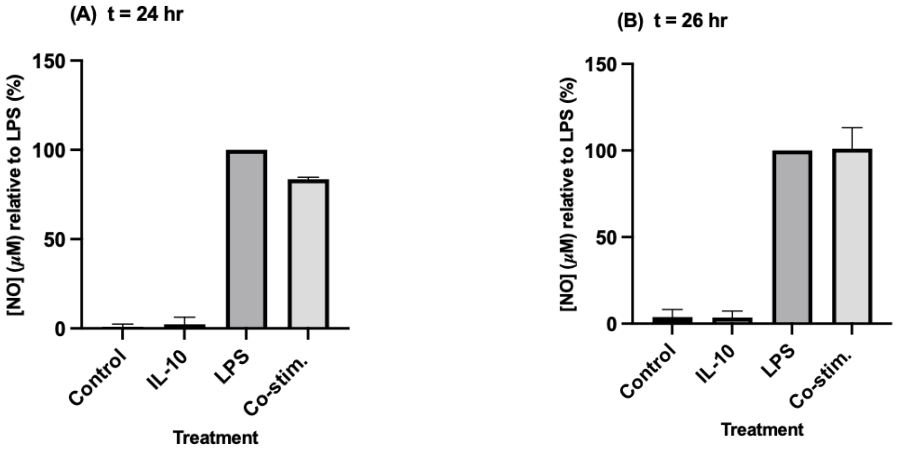

Fig. 12. *[NO] (μM) expressed as a percentage relative to LPS (n = 3) with co-stimulation of LPS and IL-10. RAW 264.7 cells were left unstimulated or stimulated with 30 ng/mL IL-10 and 100 ng/mL LPS for 24 hrs. (A) Relative [NO] at t = 24 hr after co-stimulation. (B) Relative [NO] at t = 26 hr after co-stimulation.*

Fig. 12 shows the effect of co-stimulation with IL-10 and LPS on NO concentration. At t = 24 hr after co-stimulation (fig. 12 A), there is a 16% decrease in NO production. At t = 26 hr, no effect of co-stimulation is observed on NO production when compared to the LPS control. At this time point (t = 26 hr), the response observed by co-stimulation has a relatively high error bar compared to the error bar of t = 24 hr.

Experiment 1.3 shows that different stimulation conditions have varying effect on NO production. Although the reduction is small, pre-stimulation with IL-10 and co-stimulation with IL-10 and LPS show the most reduction in NO production at t = 24 hr. These stimulation conditions were therefore chosen for qPCR in the analysis of mRNA expression of pro-inflammatory cytokines.

2.1: qPCR TNF-α mRNA expression pre-stimulation IL-10 and co-stimulation

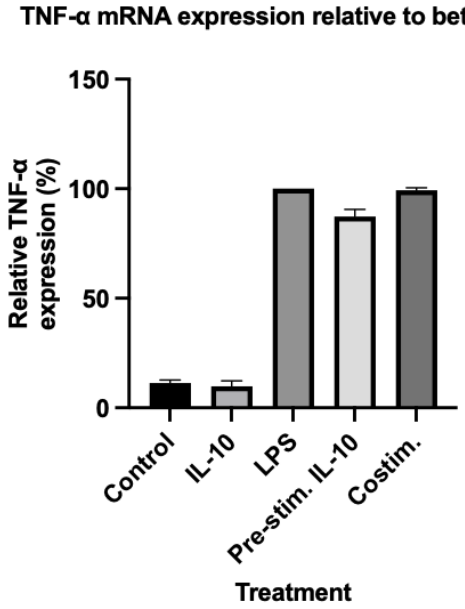

Fig. 13. *TNF-α mRNA expression relative to housekeeping gene beta-actin (n = 2) with IL-10 pre-stimulation and co-stimulation. Relative expression was quantified by equating LPS control = 100%. RAW 264.7 cells were left unstimulated, pre-stimulated with 30 ng/mL IL-10 for 1 hr followed by 100 ng/mL LPS stimulation for 2 hr or co-stimulated with both 30 ng/mL IL-10 and 100 ng/mL LPS for 3 hrs.*

Experiment 2.1 shows the effect of pre-stimulating cells with IL-10 and co-stimulating with IL-10 and LPS on pro-inflammatory cytokine, TNF-α, gene expression. As shown in fig. 13, unstimulated cells and cells stimulated only with IL-10 show low levels of TNF-α expression relative to beta-actin. TNF-α expression is upregulated when stimulated with LPS. Pre-stimulation with IL-10 followed by LPS stimulation shows a slight downregulation of the TNF-α gene when compared to LPS stimulation. This reduction in expression is around 14,6%. Co-stimulation did not show significant change in TNF-α expression when compared to LPS.

Based on literature findings regarding the anti-inflammatory properties of IL-10, a significant reduction in TNF-α expression should be observed. Since the same concentration of 100 ng/mL LPS and 30 ng/mL IL-10 had been used throughout experiments 1.2 – 1.3 with no response observed, a dose response curve was attempted to investigate whether the lack of response was related to the concentrations chosen for experiments 1.2, 1.3 as well as 2.1. New IL-10 stock was ordered and used for the following experiments.

2.2: qPCR TNF-α mRNA expression pre-stimulation IL-10 and P1 at different LPS concentrations

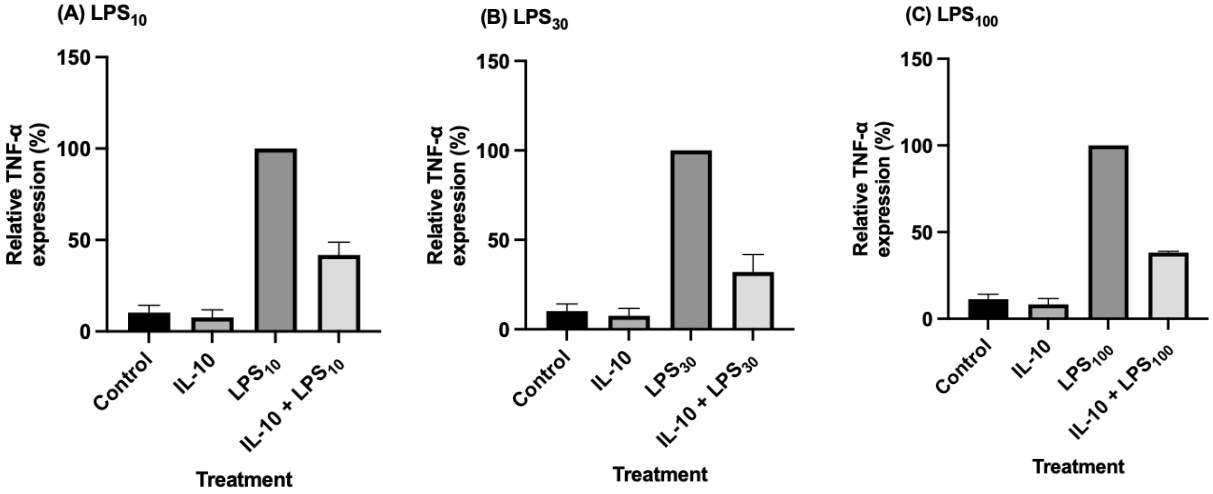

Fig. 14. *TNF-α mRNA expression relative to housekeeping gene beta-actin (n = 2) with IL-10 pre-stimulation. Relative expression was quantified by equating each LPS control = 100%. RAW 264.7 cells were left unstimulated or pre-stimulated with 30 ng/mL IL-10 for 1 hr followed by 10, 30 and 100 ng/mL LPS stimulation for 2 hr. (A) Relative TNF-α mRNA expression 10 ng/mL LPS stimulation. (B) Relative TNF-α mRNA expression 30 ng/mL LPS stimulation. (C) Relative TNF-α mRNA expression 100 ng/mL LPS stimulation.*

As shown in fig. 14, three different LPS concentrations were chosen for the analysis of the effect of LPS concentration on the reduction of relative TNF-α gene expression. Unstimulated cells and cells stimulated with only with IL-10 show low levels of TNF-α expression relative to beta-actin. This is seen for the controls at all LPS concentrations. For all three LPS concentrations a significant reduction in relative TNF-α expression is observed. This reduction, around 60%, is much higher than that observed in the previous experiment 2.1. The highest reduction was achieved with 30 ng/mL LPS as shown in fig 14 (B) by the reduction of 68%. This concentration was therefore chosen for the following experiments.

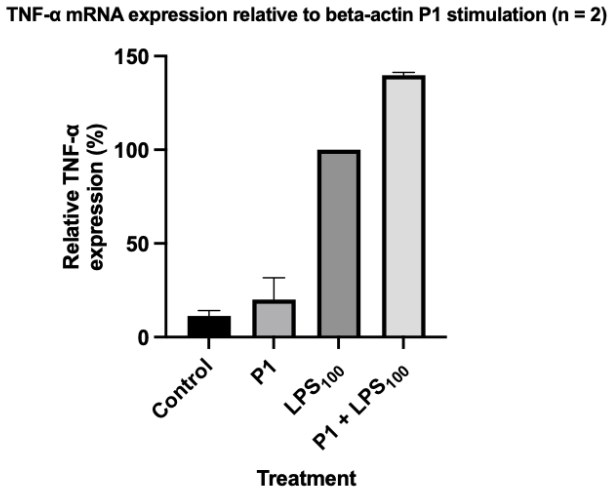

Fig. 15. *TNF-α mRNA expression relative to housekeeping gene beta-actin (n = 2) with P1 pre-stimulation. Relative expression was quantified by equating LPS_{100} control = 100%. RAW 264.7 cells were left unstimulated or pre-stimulated with 30 ng/mL P1 for 1 hr followed 100 ng/mL LPS stimulation for 2 hr.*

Besides IL-10, the effect of P1 was also analysed in this experiment. Due to lack of P1 stock solution at the time only one LPS concentration was used, 100 ng/mL. As shown in fig. 15, there is an increase of 40% in relative TNF-α expression when pre-stimulating cells with P1 compared to only with LPS. Unstimulated cells and cells pre-stimulated with only P1 showed low TNF-α mRNA expression as seen by the control and P1 bars on fig. 13.

2.3: qPCR TNF-α mRNA expression pre-stimulation IL-10, P1, P2 and P3

After observing an increase in TNF-α expression with P1 it was important to also look at the effect of P2 and P3 as these are shorter fragments of P1. The pro-inflammatory response observed in fig. 15 could potentially be achieved by the shorter fragments, providing an indication as to which structural component of P1 could explain these properties. Analysis of P2 and P3 was done following the same methods of pre-stimulation with cytokine and stimulation with 30 ng/mL LPS.

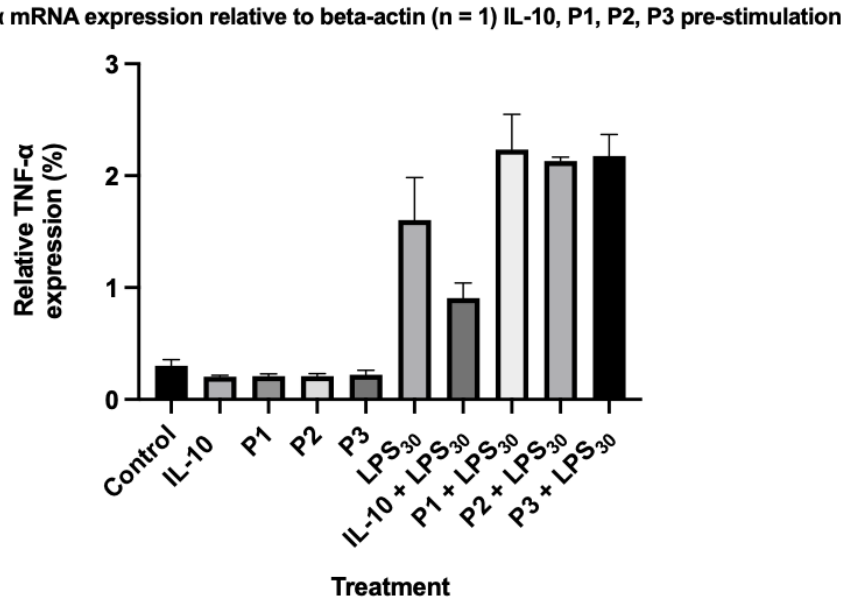

Fig. 16. *TNF-α mRNA expression relative to housekeeping gene beta-actin (n = 1) with IL-10, P1, P2 and P3 pre-stimulation. Relative expression was quantified by equating LPS_{30} control = 100%. RAW 264.7 cells were left unstimulated or pre-stimulated with 30 ng/mL IL-10, P1, P2 or P3 for 1 hr followed by 30 ng/mL LPS stimulation for 2 hr.*

As shown in fig. 16 pre-stimulation with all three cytokines P1, P2 and P3 shows an increase in relative TNF-α expression compared to LPS. P1 shows the highest increase in mRNA expression but also the highest standard deviation. Although this experiment was only done once due to time constraints (n = 1), the effect of P1 pre-stimulation on TNF-α expression lines up with the results obtained in the previous experiment (fig.15) where a concentration of 100 ng/mL LPS also led to an increase in TNF-α expression. As observed in previous experiments, pre-stimulation with IL-10 showed a reduction in relative TNF-α expression. There is a 43% downregulation with IL-10, slightly less than that observed in the experiment 2.2 with LPS_{30}.

For both NO assays 1.2 and 1.3 stock IL-10 was used that had already been prepared for a previous Master Project. As a significant reduction in TNF-α expression was observed in qPCR with the new stock IL-10 (experiment 2.2 and 2.3), the last experiment was designed to make sure the

quality of IL-10 did not influence the NO results. The new IL-10 was therefore used for a new NO Assay based on pre-stimulation with IL-10, P1, P2 and P3 instead of with LPS +/- IFN-γ.

3.1: NO Assay pre-stimulation IL-10, P1, P2 and P3

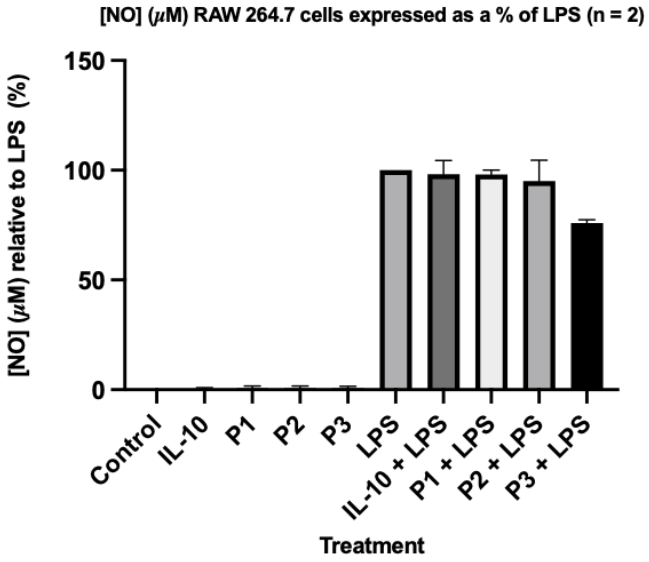

***Fig. 17**. [NO] (μM) expressed as a percentage relative to LPS control (n = 2). RAW 264.7 cells were left unstimulated or pre-stimulated with 30 ng/mL IL-10, P1, P2 or P3 for 2 hr. They were then stimulated for 24 hr with 100 ng/mL LPS. NO concentration was measured at t = 26 hr.*

Fig. 17 shows the effect of pre-stimulation with cytokines on NO concentration. Unstimulated cells or cells stimulated only with cytokines show no response. Pre-stimulation with IL-10, P1 and P2 had no effect on NO concentration as the same response is observed as when stimulating only LPS. Pre-stimulation with P3 however, does show significant reduction of 24% in NO production relative to the LPS control.

Besides the reduction observed with P3, the overall lack of reduction observed with IL-10 pre-stimulation is in line with the previously obtained results. This means it cannot be explained by the use of previously prepared IL-10.

Discussion

Effect of co-stimulation with LPS and IFN-γ on NO production

As shown in the results of experiment 1.1, stimulation of RAW 264.7 cells with LPS alone induced the production of NO. Cells stimulated only with IFN-γ showed no response and stimulation with a combination of LPS and IFN-γ led to a significantly higher NO production. This can be explained by the current paradigm of LPS/ IFN-γ mechanisms.

As mentioned previously in the introduction, LPS activates the MyD88-dependent and MyD88-indepdent pathway thorugh a combination of co-receptor CD-14, TLR-4 and MD-2 protein. This activates NF-*k*B and MAPK signaling pathways triggering the expression of iNOS and production of NO as well as pro-inflammatory cytokines. The increase in NO production after stimulation with LPS clearly illustrates the effect of LPS on iNOS gene expression.

The higher increase observed with the combination with IFN-γ suggests IFN-γ indirectly upregulates iNOS gene expression and thereby increases NO production. IFN-γ binds to the IFN-γ receptor (IFNGR) activating the JAK/STAT signalling pathway. Although this is a different receptor than LPS, a study using THP-1 macrophages showed IFN-γ upregulates CD14, TLR4, MD-2 and MyD88 expression [16,17]. This could explain the synergic effect of using a combination of both.

Experiment 1.1 therefore shows stimulation with LPS and IFN-γ leads to an increased NO production relative to LPS only induced response. In contrast to LPS, IFN-γ alone produces no NO and cannot induce M1 polarization. This can be explained by the previously mentioned indirect effect of IFN-γ on NO. The repetition (n = 3) as well as minimal error bars confirm these results. As shown in fig. 4 reduction in cell viability after stimulation also had no effect on NO production and stimulation conditions were not toxic to cells.

Effect of cytokine (P1, P2, P3 and IL-10) stimulation on NO production

Using the results from experiment 1.1, the effect of cytokines P1, P2, P3 and IL-10 could be tested in experiment 1.2. It is important to note that the time points are relative to the addition of cytokine. Therefore, at t = 0 the response of LPS + IFN-γ should be the same as LPS + IFN-γ + cytokine as only pre-stimulation with LPS was done at this point. The slight deviations at t = 0 between these two groups could be due to deviation in cell count and cell proliferation. For all four cytokines an increase in NO response is observed 2 hours after stimulation. This would suggest a pro-inflammatory effect. However, large deviation is seen between the two samples for P1, P3 and IL-10 as shown by the large error bars at t = 2 hr.

To check whether cytokines needed time to have their effect, an NO assay was also done at t = 24 hr. For the three cytokines P1, P3 and IL-10 no significant increase or reduction was observed at t = 24 hr. This was seen in both samples as suggested by the small error bars. Although P2 shows a slight reduction in NO production, this is only of 7 % and with an n = 1 not enough to conclude P2 inhibits NO production.

Therefore, experiment 1.2 shows IL-10, P1, P2 and P3 have no significant effect on NO production when RAW 264.7 cells are pre-stimulated with LPS and IFN-γ. These results are in line with the

representative MTT assay shown in fig. 9 as no stimulation condition led to cytotoxicity and significant loss of cells.

Effect of pre-stimulation LPS, pre-stimulation IL-10 and co-stimulation on NO production

Based on the previous results and literature data, it was important to rule out the possibility that the method of stimulation had an influence on NO production. To facilitate experimental procedure, cells were only stimulated with LPS as opposed to LPS and IFN-γ. Experiment 1.3 therefore investigated the three possibilities: pre-stimulation with IL-10, LPS or co-stimulation with both. In line with the results obtained in the previous experiment, pre-stimulation with LPS had no effect on NO production at any time point.

Co-stimulation and pre-stimulation with IL-10 did however both show reduction of 16% and 18% respectively after 22 hr stimulation. This reduction is seen in all three samples (n = 3). This effect is not seen two hours later as NO production is remained the same compared to the LPS control. This could mean co-stimulation and pre-stimulation with IL-10 have a short-term effect on NO production and time points before 24 hr should've been tested. Longer stimulation times could mean LPS overrules the anti-inflammatory effect of IL-10.

Relative TNF-α mRNA expression pre-stimulation IL-10 and co-stimulation IL-10 and LPS

Although a slight reduction in NO concentration was seen with co-stimulation and pre-stimulation with IL-10, a much larger reduction should've been seen to conclude IL-10 predominantly acts on the production of NO. Instead of using NO production as an indication of anti-inflammation, gene expression was used – in particular gene expression of pro-inflammatory gene TNF-α. The stimulation conditions that showed reduction in the previous NO assay were chosen as well as shorter stimulation times. Experiment 2.1 shows pre-stimulating cells with IL-10 leads to a reduction in relative TNF-α.

Although the reduction is small, pre-stimulation with IL-10 inhibits TNF-α mRNA expression while co-stimulation shows no effect. This is confirmed by the replicability of the data. The study by *Innate Immunity* previously mentioned showed co-stimulation did not downregulate MyD88 expression [14]. This could be because LPS upregulates MyD88 before IL-10 and thereby prevents downregulation by IL-10. A previous study by the same group [18] showed IL-10 could only inhibit MyD88 expression when pre-stimulating with IL-10. Since MyD88 is directly involved in pro-inflammatory TNF-α production this could explain the difference between pre-stimulation and co-stimulation.

Relative TNF-α mRNA expression pre-stimulation IL-10, P1, P2 and P3

At first, it was postulated that the limited reduction in expression occurred because of limited binding of IL-10 to receptors IL-10R1 and IL-10R2. However, analysis of the effect of varying LPS concentrations revealed much more reduction in TNF-α mRNA expression than shown in the previous experiment 2.1. This could be explained by the use of new stock IL-10. As mentioned, experiments 1.2, 1.3 and 2.1 utilized IL-10 that had been made by a previous Master student. This could've influenced the cytokine's stability as the TNF-α reduction went from 14

to 60%. For all three LPS concentrations significant reduction in TNF-α was seen with slightly more reduction with 30 ng/mL LPS. IL-10 therefore significantly inhibits the production of pro-inflammatory cytokine TNF-α.

IL-10 synthetic peptides P1, P2, and P3 all showed an increase in relative TNF-α expression as shown in fig. 15 and 16. This result is replicated for P1, as it was tested with both 30 ng/mL LPS and 100 ng/mL LPS but not for P2 and P3 limiting the credibility of the results. Therefore, peptide derivative P1 does not inhibit, and rather stimulates the production of pro-inflammatory cytokine TNF-α. The conclusion regarding P2 and P3 is limited by the lack of replicability.

Effect of new stock IL-10 on NO response

As previously mentioned, to exclude the possibility that the stability of IL-10 influenced the NO assays a final NO assay was done with P1, P2 and P3 as well. The results showed on fig. 17 confirmed that IL-10 has indeed minimal to no effect on the production of NO. The results are in line with previous conclusions that pre-stimulation with IL-10, P1 and P2 do not influence the production of NO after 26 hrs. Pre-stimulation with P3 did however, show a reduction in NO production. Since the previous experiment was replicated with n = 2 further investigation would need to be done at different points to draw any conclusions regarding inhibition of NO production by P3.

Conclusion

In conclusion, IL-10 does not have an inhibitory effect on the production of NO. Reduction in NO production was observed only with IL-10 pre-stimulation and IL-10 and LPS co-stimulation at t = 22 hrs specifically. Further investigation is needed to analyze the time-dependency of IL-10 activity and draw conclusions regarding the way IL-10 exhibits its anti-inflammatory effects through inhibition of NO production. P1, P2 and P3 being peptide derivatives of IL-10 also showed no effects on the production of NO and thereby do not affect inflammation through this mechanism. IL-10 does, however, significantly downregulate the expression of pro-inflammatory cytokine TNF-α. It therefore acts as an anti-inflammatory cytokine primarily by inhibition of pathways that promote expression of pro-inflammatory cytokines. On the other hand, P1, P2 and P3 promote the mRNA expression of pro-inflammatory cytokines and thereby do not show any anti-inflammatory properties in the given study. This suggests P1, P2 and P3 have pro-inflammatory properties.

Further Studies

The study clearly shows the anti-inflammatory properties of IL-10 and that the optimal method of quantifying this is through qPCR. Following studies should focus on investigating the effect of IL-10 on other pro-inflammatory cytokine gene expression such as IL-1β or IL-6. Since only P1 was tested with repetition, further studies on the effect of peptide derivatives P2 and P3 on pro-inflammatory cytokine expression should be done. It is important to check whether the pro-inflammatory behavior observed by P1 is due to its structure and not the form of synthesis. Peptides can be cultured in *Escherichia coli* meaning they already contain LPS, a major component of the outer membrane. The upregulation of pro-inflammatory cytokine TNF-α observed with P1 could therefore be due to the LPS already present. To determine whether this is the case, peptides P1, P2 and P3 can be scrambled and tested with qPCR. If the response is the same as observed in the non-scrambled peptides, the pro-inflammatory properties are attributed to the peptide itself as opposed to the LPS present.

Once further studies on the peptide derivatives are completed and pro-inflammatory properties are confirmed, studies can be done to investigate the anti-fibrotic properties of both IL-10 and IL-10 peptide derivatives. This can be done by qPCR on TGF-β1 gene expression. TGF- β1 is known as a pro-fibrotic growth factor. When injected subcutaneously, it stimulated the production of collagen and thereby promoted tissue fibrosis [19]. In lung fibrosis, IL-10 showed anti-fibrotic properties through inhibition of TGF- β1 production [2]. This would need to be confirmed by qPCR and used to test whether the IL-10 peptide derivatives show the same anti-fibrotic properties. This would increase the potential of IL-10 derivatives for the treatment of liver fibrosis.

Further studies could also focus on the specific binding of IL-10 to IL-10 R1 and R2 as well as P1, P2 and P3 binding. This is important in this study as an assumption is made regarding the binding affinity of IL-10 derivatives to IL-10 R1. It could be that the pro-inflammatory properties observed by the peptides in this study can be explained by their affinity to receptor-1 specifically. Fluorescence activated cell sorting (FACS) can be used to analyse this.

The findings in the current study therefore serve as a promising starting point for further studies and hopefully the discovery of an anti-fibrotic peptide derivative that does not suppress the immune system.

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Appendix

Appendix A: Raw Data NO Assay and MTT Assay Experiment 1.1

*Table 1. Absorbance and Concentration NO Assay sample 1 experiment 1.1 (**fig. 3**)*

Absorbance	Treatment			
	Control	LPS	IFN-γ	LPS + IFN-γ
1	0,060	0,174	0,053	0,219
2	0,052	0,229	0,053	0,232
3	0,058	0,187	0,060	0,227
Average	0,057	0,197	0,055	0,266
St Dev	0,004	0,029	0,004	0,007

	Treatment			
	Control	LPS	IFN-γ	LPS + IFN-γ
[NO] (uM)	0	7,76	0	9,57
St Dev	0	1,13	0	0,28
[NO] (uM) ± Sd	0 ± 0	7,76 ± 1,13	0 ± 0	9,57 ± 0,28

*Fig. 18. Calibration curve NO Assay sample 1 experiment 1.1 (**fig. 3**)*

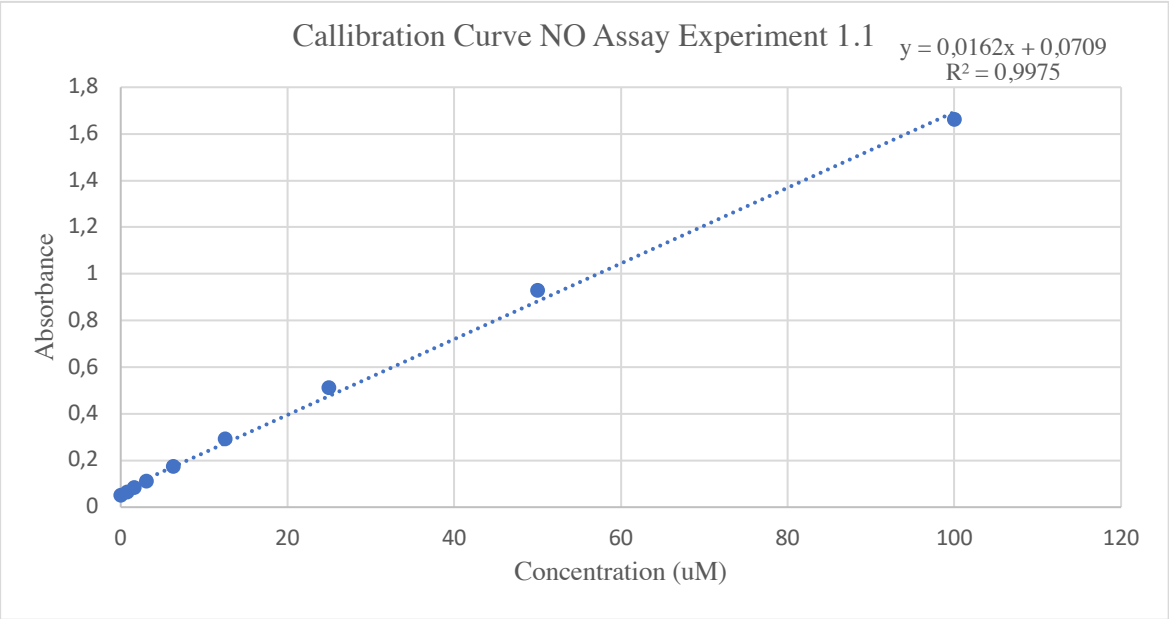


*Table 2. Absorbance and Concentration NO Assay sample 2 experiment 1.1 (**fig. 3**)*

Absorbance	Treatment			
	Control	LPS	IFN-γ	LPS + IFN-γ
1	0,053	0,199	0,051	0,306
2	0,053	0,052	0,054	0,295
3	0,051	0,204	0,051	0,3
Average	0,0523	0,2015	0,052	0,300

St Dev	0,00115	0,0864	0,00173	0,00551

	Treatment			
	Control	LPS	IFN-γ	LPS + IFN-γ
[NO] (uM)	0	10,93	0	19,38
St Dev	0	4,68	0	0,355
[NO] (uM) ± Sd	0 ± 0	10,93 ± 4,68	0 ± 0	19,38 ± 0,355

*Fig. 19. Calibration curve NO Assay sample 2 experiment 1.1 (**fig. 3**)*

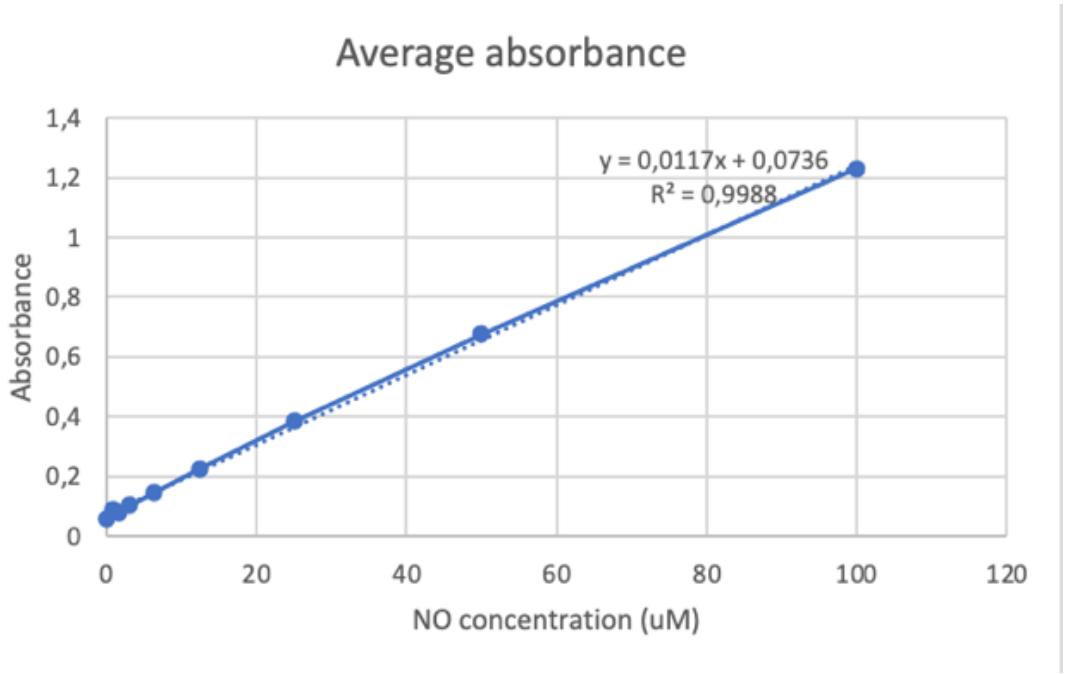


*Table 3. Absorbance and Concentration NO Assay sample 3 experiment 1.1 (**fig. 3**)*

Absorbance	Treatment			
	Control	LPS	IFN-γ	LPS + IFN-γ
1	0,056	0,185	0,059	0,279
2	0,063	0,253	0,059	0,283
3	0,054	0,174	0,058	0,296
Average	0,055	0,180	0,059	0,281
St Dev	0,001414	0,00778	0,000577	0,00283

	Treatment			
	Control	LPS	IFN-γ	LPS + IFN-γ
[NO] (uM)	0	13,2	0	23,8
St Dev	0	0,57	0	0,239
[NO] (uM) ± Sd	0 ± 0	13,2 ± 0,57	0 ± 0	23,8 ± 0,239

*Fig. 20. Calibration curve NO Assay sample 3 experiment 1.1 (**fig. 3**)*

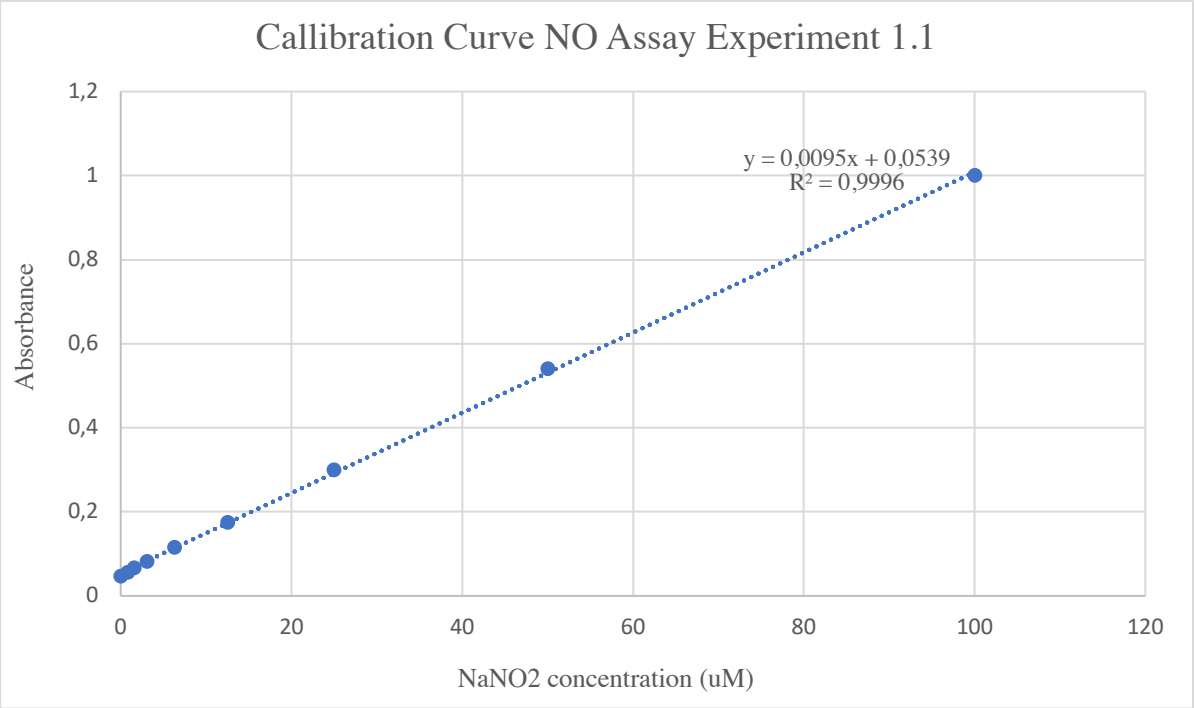


*Table 4. Absorbance and Cell Viability MTT Assay sample 1 experiment 1.1 (**fig. 4**)*

Absorbance	Treatment				
	Control	LPS	IFN-γ	LPS + IFN-γ	MTT control
1	0,605	0,271	0,378	0,479	0,086
2	0,590	0,603	0,516	0,384	0,072
3	0,414	0,429	0,528	0,564	0,067
Average	0,536	0,434	0,474	0,476	0,075
St Dev	0,106	0,166	0,083	0,090	0,010

	Treatment				
	Control	LPS	IFN-γ	LPS + IFN-γ	MTT Control
% Cell viability	100	77,89	86,49	86,85	0
St Dev	0	29,78	15,21	16,44	0
% Viability± St Dev	100 ± 0	77,89 ± 29,78	86,49 ± 15,21	86,85 ± 16,44	0

*Table 5. Absorbance and Cell Viability MTT Assay sample 2 experiment 1.1 (**fig. 4**)*

Absorbance	Treatment				
	Control	LPS	IFN-γ	LPS + IFN-γ	MTT control
1	0,677	0,684	0,992	0,688	0,099
2	0,525	0,825	0,595	0,452	0,1
3	0,493	0,596	0,809	0,504	0,103
Average	0,565	0,702	0,799	0,548	0,101
St Dev	0,174	0,204	0,352	0,219	0,002

	Treatment				
	Control	LPS	IFN-γ	LPS + IFN-γ	MTT Control
% Cell viability	100	96,31	113,47	69,11	0
St Dev	0	28,06	49,97	27,68	0
% Viability± St Dev	100 ± 0	96,31 ± 28,06	113,47 ± 49,97	69,11 ± 27,68	0 ± 0

*Table 6. Absorbance and Cell Viability MTT Assay sample 3 experiment 1.1 (**fig. 4**)*

Absorbance	Treatment				
	Control	LPS	IFN-γ	LPS + IFN-γ	MTT control
1	0,677	0,684	0,992	0,688	0,099
2	0,525	0,825	0,595	0,452	0,1
3	0,493	0,596	0,809	0,504	0,103
Average	0,565	0,702	0,799	0,548	0,101
St Dev	0,174	0,204	0,352	0,219	0,002

	Treatment				
	Control	LPS	IFN-γ	LPS + IFN-γ	MTT Control
% Cell viability	100	96,31	113,47	69,11	0
St Dev	0	28,06	49,97	27,68	0
% Viability± St Dev	100 ± 0	96,31 ± 28,06	113,47 ± 49,97	69,11 ± 27,68	0 ± 0

Appendix B: Raw Data NO Assay and MTT Assay Experiment 1.2

*Table 7. Absorbance and Concentration NO Assay sample 1 experiment 1.2 (**fig. 5,6 and 7**) t = 0, 2 and 24 hr respectively*

Absorbance t = 0 hr	Treatment							
	Control	P1	P3	IL-10	IFN-γ + LPS	IFN-γ + LPS + IL-10	IFN-γ + LPS + P1	IFN-γ + LPS + P3
1	0,046	0,043	0,045	0,042	0,133	0,135	0,141	0,129
2	0,045	0,043	0,044	0,042	0,155	0,171	0,144	0,152
3	0,049	0,044	0,046	0,042	0,15	0,172	0,163	0,153
Average	0,0467	0,0433	0,0450	0,0420	0,1460	0,1593	0,1493	0,1447
St Dev	0,00208	0,00058	0,0010	0,00	0,01153	0,02108	0,01193	0,01358

Concentration t = 0 hr	Treatment							
	Control	P1	P3	IL-10	IFN-γ + LPS	IFN-γ + LPS + IL-10	IFN-γ + LPS + P1	IFN-γ + LPS + P3
[NO] (uM)	0	0	0	0	20,64	23,42	21,34	20,37
St Dev	0	0	0	0	1,63	3,10	1,70	1,91
[NO] (uM) ± Sd	0 ± 0	0 ± 0	0 ± 0	0 ± 0	20,64 ± 1,63	23,42 ± 3,10	21,34 ± 1,70	20,37 ± 1,91

Absorbance t = 2 hr	Treatment							
	Control	P1	P3	IL-10	IFN-γ + LPS	IFN-γ + LPS + IL-10	IFN-γ + LPS + P1	IFN-γ + LPS + P3
1	0,044	0,04	0,045	0,042	0,141	0,131	0,141	0,138
2	0,041	0,044	0,047	0,043	0,192	0,156	0,157	0,155
3	0,047	0,078	0,046	0,046	0,162	0,149	0,205	0,152
Average	0,044	0,054	0,046	0,044	0,165	0,145	0,168	0,148
St Dev	0,003	0,02088	0,0010	0,00208	0,02563	0,01290	0,03331	0,00907

Concentration t = 2 hr	Treatment							
	Control	P1	P3	IL-10	IFN-γ + LPS	IFN-γ + LPS + IL-10	IFN-γ + LPS + P1	IFN-γ + LPS + P3
[NO] (uM)	0	1,48	0	0	24,60	20,51	25,16	21,13
St Dev	0	0,57	0	0	3,82	1,82	4,99	1,29
[NO] (uM) ± Sd	0 ± 0	1,48 ± 0,57	0 ± 0	0 ± 0	24,60 ± 3,82	20,51 ± 1,82	25,16 ± 4,99	21,13 ± 1,29

Absorbance t = 24 hr	Treatment							
	Control	P1	P3	IL-10	IFN-γ + LPS	IFN-γ + LPS + IL-10	IFN-γ + LPS + P1	IFN-γ + LPS + P3
1	0,056	0,043	0,046	0,042	0,174	0,15	0,172	0,174
2	0,042	0,041	0,042	0,043	0,193	0,183	0,207	0,196
3	0,056	0,043	0,043	0,044	0,203	0,197	0,214	0,205
Average	0,051	0,042	0,044	0,043	0,190	0,177	0,198	0,192
St Dev	0,00808	0,00115	0,00208	0,0010	0,01473	0,02413	0,02250	0,01594

Concentration t = 24 hr	Treatment							
	Control	P1	P3	IL-10	IFN-γ + LPS	IFN-γ + LPS + IL-10	IFN-γ + LPS + P1	IFN-γ + LPS + P3
[NO] (uM)	0	0	0	0	29,81	27,03	31,41	30,16
St Dev	0	0	0	0	2,31	3,69	3,57	0,70
[NO] (uM) ± Sd	0 ± 0	0 ± 0	0 ± 0	0 ± 0	29,81± 2,31	27,03 ± 3,69	31,41 ± 3,57	30,16 ± 0,70

*Fig. 21. Calibration curve NO Assay sample 1 experiment 1.2 (**fig. 5,6 and 7**)*

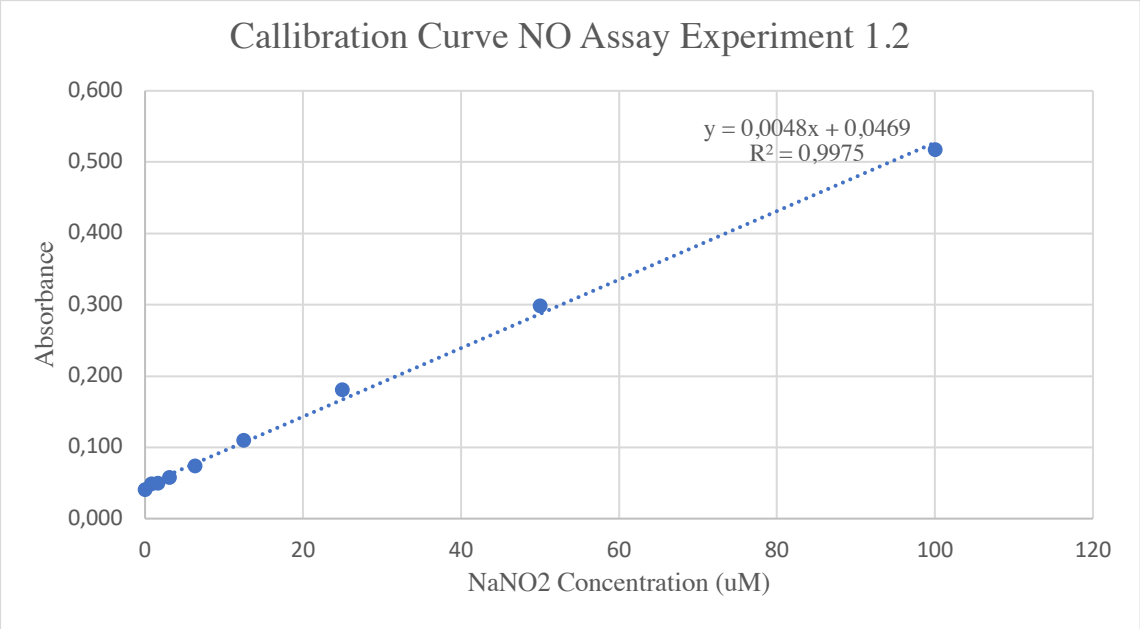


*Table 8. Absorbance and Concentration NO Assay sample 2 experiment 1.2 (**fig. 5,6 and 7**) t = 0, 2 and 24 hr respectively*

Absorbance t = 0 hr	Treatment							
	Control	P1	P3	IL-10	IFN-γ + LPS	IFN-γ + LPS + IL-10	IFN-γ + LPS + P1	IFN-γ + LPS + P3
1	0	0	0	0	0,106	0,127	0,138	0,124
2	0	0	0	0	0,15	0,148	0,154	0,135
3	0	0	0	0	0,141	0,15	0,145	0,151
Average	0	0	0	0	0,132	0,142	0,146	0,137
St Dev	0	0	0	0	0,0232	0,0127	0,00802	0,0136

Concentration t = 0 hr	Treatment							
	Control	P1	P3	IL-10	IFN-γ + LPS	IFN-γ + LPS + IL-10	IFN-γ + LPS + P1	IFN-γ + LPS + P3
[NO] (uM)	0	0	0	0	18,49	20,43	21,26	19,39
St Dev	0	0	0	0	3,25	1,84	1,17	1,93
[NO] (uM) ± Sd	0 ± 0	0 ± 0	0 ± 0	0 ± 0	18,49± 3,25	20,43 ± 1,84	21,26± 1,17	19,39 ± 1,93

Absorbance t = 2 hr	Treatment							
	Control	P1	P3	IL-10	IFN-γ + LPS	IFN-γ + LPS + IL-10	IFN-γ + LPS + P1	IFN-γ + LPS + P3
1	0	0	0	0	0,092	0,139	0,097	0,13
2	0	0	0	0	0,112	0,124	0,149	0,136
3	0	0	0	0	0,107	0,132	0,129	0,143
Average	0	0	0	0	0,104	0,132	0,125	0,136
St Dev	0	0	0	0	0,01041	0,00751	0,02623	0,00651

Concentration t = 2 hr	Treatment							
	Control	P1	P3	IL-10	IFN-γ + LPS	IFN-γ + LPS + IL-10	IFN-γ + LPS + P1	IFN-γ + LPS + P3
[NO] (uM)	0	0	0	0	12,51	18,35	16,96	19,32
St Dev	0	0	0	0	1,26	1,05	3,56	0,92
[NO] (uM) ± Sd	0 ± 0	0 ± 0	0 ± 0	0 ± 0	12,51 ± 1,26	18,35 ± 1,05	16,96 ± 3,56	19,32 ± 0,92

Absorbance t = 24 hr	Treatment							
	Control	P1	P3	IL-10	IFN-γ + LPS	IFN-γ + LPS + IL-10	IFN-γ + LPS + P1	IFN-γ + LPS + P3
1	0	0	0	0	0,165	0,196	0,188	0,181
2	0	0	0	0	0,186	0,191	0,195	0,179
3	0	0	0	0	0,184	0,197	0,201	0,189
Average	0	0	0	0	0,178	0,195	0,195	0,183
St Dev	0	0	0	0	0,01159	0,00321	0,00651	0,00529

Concentration t = 24 hr	Treatment							
	Control	P1	P3	IL-10	IFN-γ + LPS	IFN-γ + LPS + IL-10	IFN-γ + LPS + P1	IFN-γ + LPS + P3
[NO] (uM)	0	0	0	0	28,07	31,47	31,47	29,04
St Dev	0	0	0	0	1,82	0,52	1,05	0,84
[NO] (uM) ± Sd	0 ± 0	0 ± 0	0 ± 0	0 ± 0	28,07 ± 1,82	31,47 ± 0,52	31,47 ± 1,05	29,04 ± 0,84

*Fig. 22. Calibration curve NO Assay sample 2 experiment 1.2 (**fig. 5,6 and 7**)*

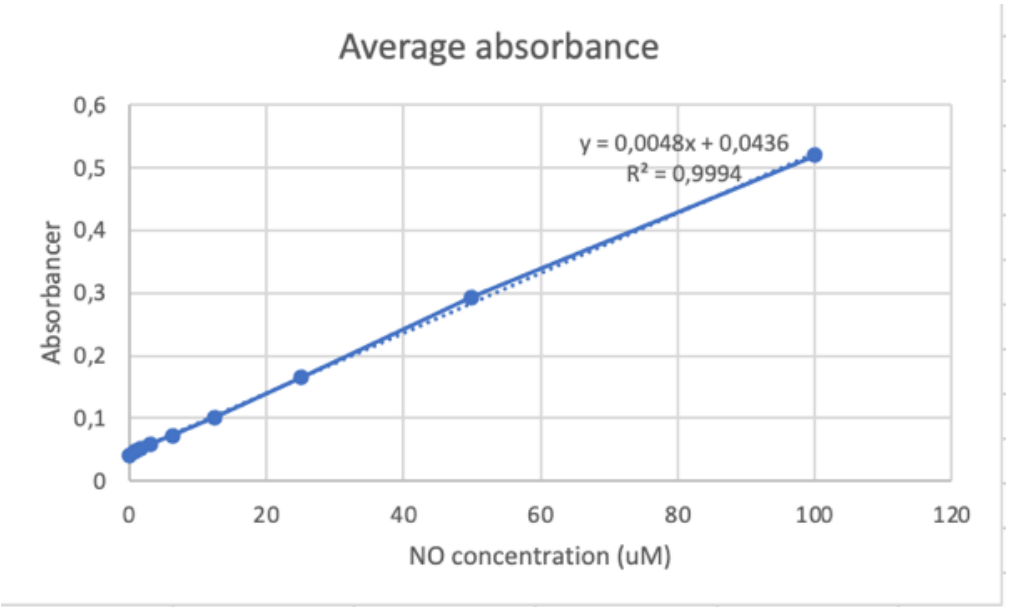


*Table 9. Absorbance and Concentration NO Assay sample 3 experiment 1.2 (**fig. 8**) t = 0, 2 and 24 hr respectively*

Absorbance t = 0 hr	Treatment			
	Control	P2	IFN-γ + LPS	IFN-γ + LPS + P2
1	0,045	0,046	0,092	0,113
2	0,045	0,045	0,106	0,126
3	0,048	0,045	0,11	0,109
Average	0,046	0,045	0,108	0,111
St Dev	0,001732	0,000577	0,00282	0,002828

Concentration t = 0 hr	Treatment			
	Control	P2	IFN-γ + LPS	IFN-γ + LPS + P2
[NO] (uM)	0,00	0,00	16,49	17,30
St Dev	0,00	0,00	0,43	0,44
[NO] (uM) ± Sd	0 ± 0	0 ± 0	16,49 ± 0,43	17,30 ± 0,44

Absorbance t = 2 hr	Treatment			
	Control	P2	IFN-γ + LPS	IFN-γ + LPS + P2
1	0,047	0,045	0,109	0,111
2	0,046	0,046	0,105	0,122
3	0,046	0,043	0,109	0,108
Average	0,046	0,045	0,108	0,110
St Dev	0,000577	0,001528	0,00231	0,002121

Concentration t = 2 hr	Treatment			
	Control	P2	IFN-γ + LPS	IFN-γ + LPS + P2
[NO] (uM)	0,00	0,00	16,40	16,90
St Dev	0,00	0,00	0,35	0,33
[NO] (uM) ± Sd	0 ± 0	0 ± 0	16,40 ± 0,35	16,90 ± 0,33

Absorbance t = 24 hr	Treatment			
	Control	P2	IFN-γ + LPS	IFN-γ + LPS + P2
1	0,05	0,046	0,195	0,174
2	0,054	0,045	0,166	0,157
3	0,056	0,047	0,154	0,146
Average	0,053	0,046	0,160	0,152
St Dev	0,003055	0,001	0,008485	0,007778

Concentration t = 24 hr	Treatment			
	Control	P2	IFN-γ + LPS	IFN-γ + LPS + P2
[NO] (uM)	1,84	0,00	30,44	28,16
St Dev	0,1052	0,00	1,61	1,45
[NO] (uM) ± Sd	1,84 ± 0,1052	0 ± 0	30,44 ± 1,61	28,16 ± 1,45

*Fig. 23. Calibration curve NO Assay sample 3 experiment 1.2 (**fig. 8**)*

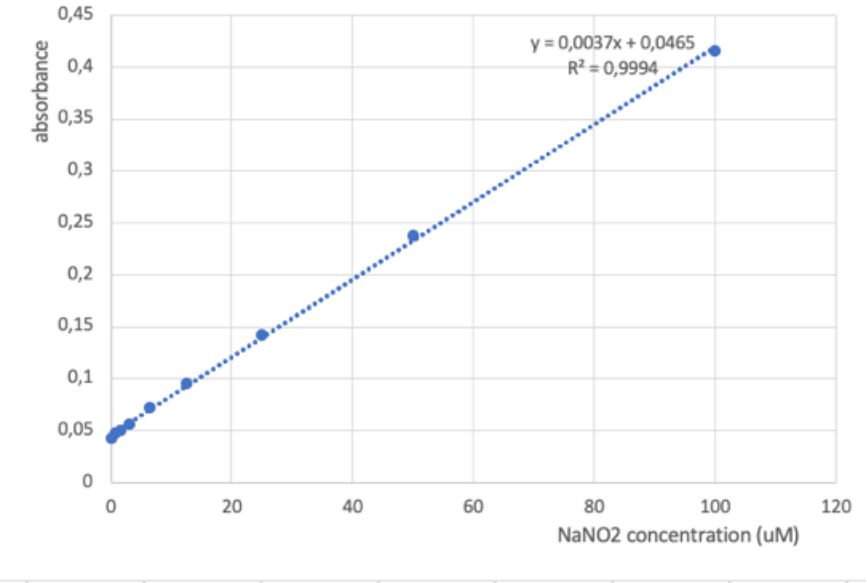


*Table 10. Absorbance and Cell Viability MTT Assay representative sample 1 experiment 1.2 (**fig. 9**)*

Absorbance	Treatment								
	Control	P1	P3	IL-10	IFN-γ + LPS	IFN-γ + LPS + IL-10	IFN-γ + LPS + P1	IFN-γ + LPS + P3	MTT control
1	0,241	0,174	0,271	0,246	0,183	0,207	0,177	0,249	0,065
2	0,22	0,311	0,277	0,263	0,252	0,26	0,24	0,267	0,065
3	0,352	0,285	0,275	0,302	0,307	0,285	0,266	0,217	0,069
Average	0,271	0,257	0,274	0,270	0,247	0,251	0,228	0,244	0,0663
St Dev	0,07093	0,0728	0,00306	0,0287	0,0621	0,0398	0,0458	0,0253	0,002309

	Treatment								
	Control	P1	P3	IL-10	IFN-γ + LPS	IFN-γ + LPS + IL-10	IFN-γ + LPS + P1	IFN-γ + LPS + P3	MTT control
% Cell viability	100	92,99	101,63	99,67	88,44	90,07	78,83	86,97	0
St Dev	0	26,36	1,13	10,59	22,22	14,31	15,84	9,01	0
% Viability ± Sd	100 ± 0	92,99 ± 26,36	101,63 ± 1,13	99,67 ± 10,59	88,44± 22,22	90,07 ± 14,31	78,83 ± 15,84	86,97 ± 9,01	0 ± 0

Appendix C: Raw Data NO Assay Experiment 1.3

*Table 11. Absorbance and Concentration NO Assay sample 1 experiment 1.3 (**fig. 10, 11 and 12**) t = 24 and 26 hr respectively*

Absorbance t = 24 hr	Treatment					
	Control	LPS	IL-10	Pre-stim. LPS	Pre-stim. IL-10	Co-stim. IL-10 + LPS
1	0,048	0,178	0,047	0,193	0,158	0,161
2	0,046	0,189	0,052	0,176	0,155	0,153
3	0,047	0,176	0,049	0,161	0,154	0,161
Average	0,047	0,181	0,0493	0,1767	0,1556	0,1583
St Dev	0,001	0,007	0,002517	0,016010	0,002082	0,004619

Concentration t = 24 hr	Treatment					
	Control	LPS	IL-10	Pre-stim. LPS	Pre-stim. IL-10	Co-stim. IL-10 + LPS
[NO] (uM)	0	23,94	0	23,14	19,25	19,75
St Dev	0	0,926	0	2,097	0,257	0,576
[NO] (uM) ± Sd	0 ± 0	23,94 ± 0,926	0 ± 0	23,14 ± 2,097	19,25 ± 0,257	19,75 ± 0,576

Absorbance t = 26 hr	Treatment					
	Control	LPS	IL-10	Pre-stim. LPS	Pre-stim. IL-10	Co-stim. IL-10 + LPS
1	0,063	0,198	0,087	0,294	0,224	0,201
2	0,054	0,211	0,055	0,189	0,243	0,332
3	0,053	0,277	0,052	0,173	0,317	0,195
Average	0,0567	0,229	0,0647	0,219	0,261	0,243
St Dev	0,00551	0,04236	0,0194	0,06573	0,04914	0,077423

Concentration t = 26 hr	Treatment					
	Control	LPS	IL-10	Pre-stim. LPS	Pre-stim. IL-10	Co-stim. IL-10 + LPS
[NO] (uM)	0,9197	32,77	2,40	30,92	38,82	35,36
St Dev	0,0893	6,071	0,7203	9,294	7,299	11,283
[NO] (uM) ± Sd	0,9197 ± 0,0893	32,77 ± 6,071	2,40 ± 0,7203	30,92 ± 9,294	38,82 ± 7,299	35,36 ± 11,283

*Figure 24. Calibration curve NO Assay sample 1 experiment 1.3 (**fig. 10, 11 and 12**)*

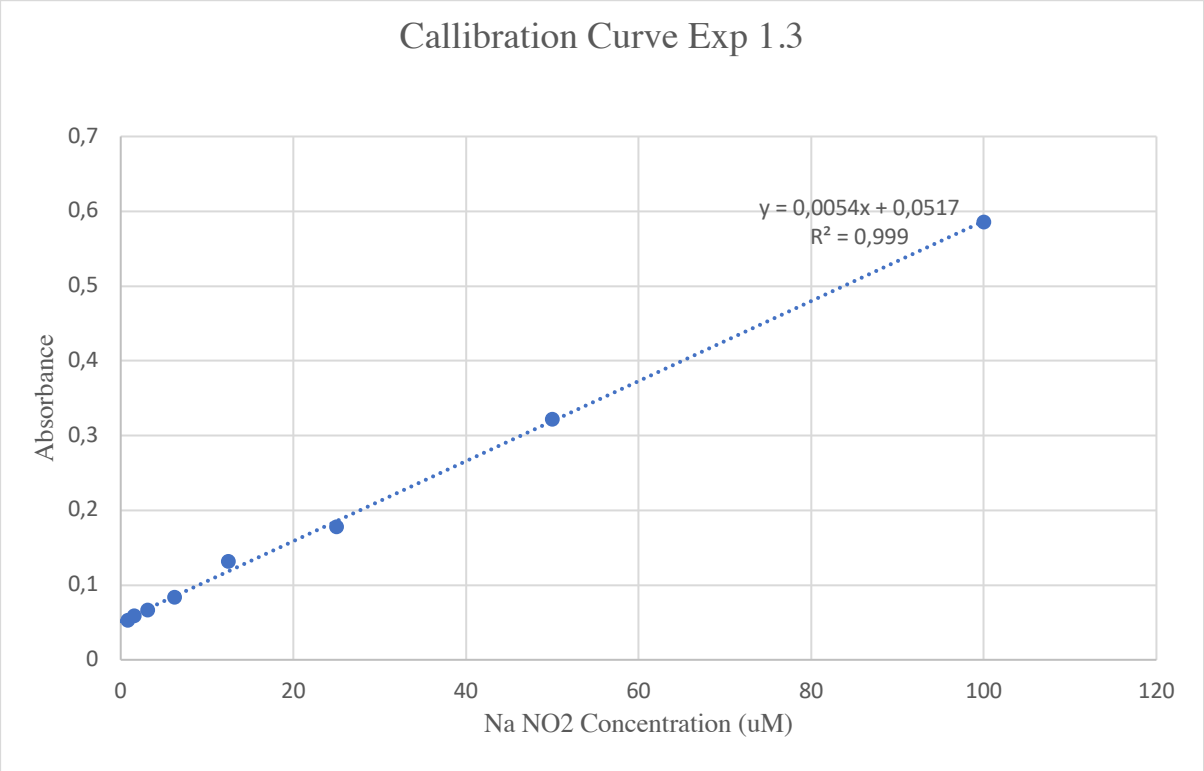


*Table 12. Absorbance and Concentration NO Assay sample 2 experiment 1.3 (**fig. 10, 11 and 12**) t = 24 and 26 hr respectively*

Absorbance t = 24 hr	Treatment					
	Control	LPS	IL-10	Pre-stim. LPS	Pre-stim. IL-10	Co-stim. IL-10 + LPS
1	0,048	0,178	0,047	0,193	0,158	0,161
2	0,046	0,189	0,052	0,176	0,155	0,153
3	0,047	0,176	0,049	0,161	0,154	0,161
Average	0,047	0,177	0,0493	0,1767	0,1557	0,1583
St Dev	0,001	0,00141	0,00252	0,01601	0,00208	0,00462

Concentration t = 24 hr	Treatment					
	Control	LPS	IL-10	Pre-stim. LPS	Pre-stim. IL-10	Co-stim. IL-10 + LPS
[NO] (uM)	0	22,45	0	22,39	18,43	18,93
St Dev	0	0,179	0	2,029	0,246	0,552
[NO] (uM) ± Sd	0 ± 0	22,45± 0,179	0 ± 0	22,39 ± 2,029	18,43± 0,246	18,93± 0,552

Absorbance t = 26 hr	Treatment					
	Control	LPS	IL-10	Pre-stim. LPS	Pre-stim. IL-10	Co-stim. IL-10 + LPS
1	0,063	0,198	0,087	0,294	0,224	0,201
2	0,054	0,211	0,055	0,189	0,243	0,332
3	0,053	0,277	0,052	0,173	0,317	0,195
Average	0,0567	0,2045	0,0647	0,181	0,2335	0,198
St Dev	0,005507	0,00919	0,0194	0,0113	0,013435	0,00424

Concentration t = 26 hr	Treatment					
	Control	LPS	IL-10	Pre-stim. LPS	Pre-stim. IL-10	Co-stim. IL-10 + LPS
[NO] (uM)	0	32,20	0	30,31	38,36	34,84
St Dev	0	1,24	0	1,45	1,9052	0,566
[NO] (uM) ± Sd	0 ± 0	32,20 ± 1,24	0 ± 0	30,31 ± 1,45	38,36 ± 1,9052	34,84 ± 0,566

*Figure 25. Calibration curve NO Assay sample 2 experiment 1.3 (**fig. 10, 11 and 12**)*

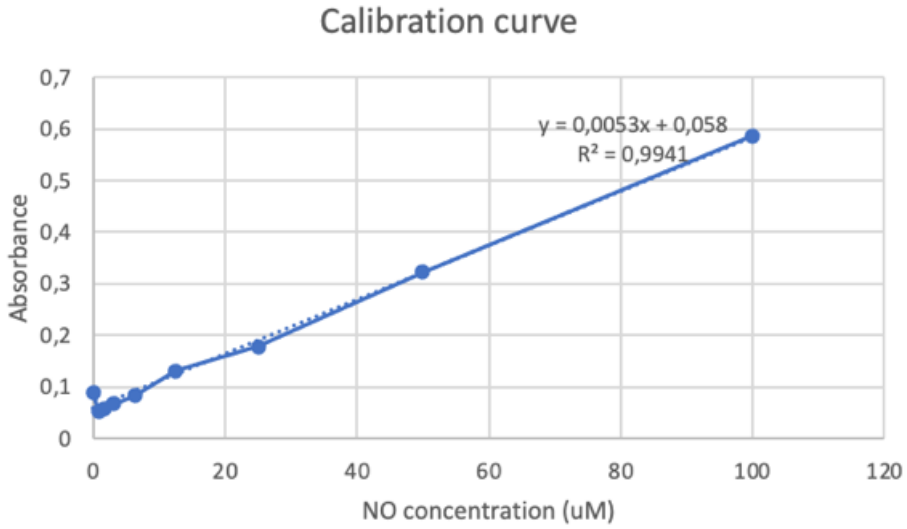


*Table 13. Absorbance and Concentration NO Assay sample 3 experiment 1.3 (**fig. 10, 11 and 12**) t = 24 and 26 hr respectively*

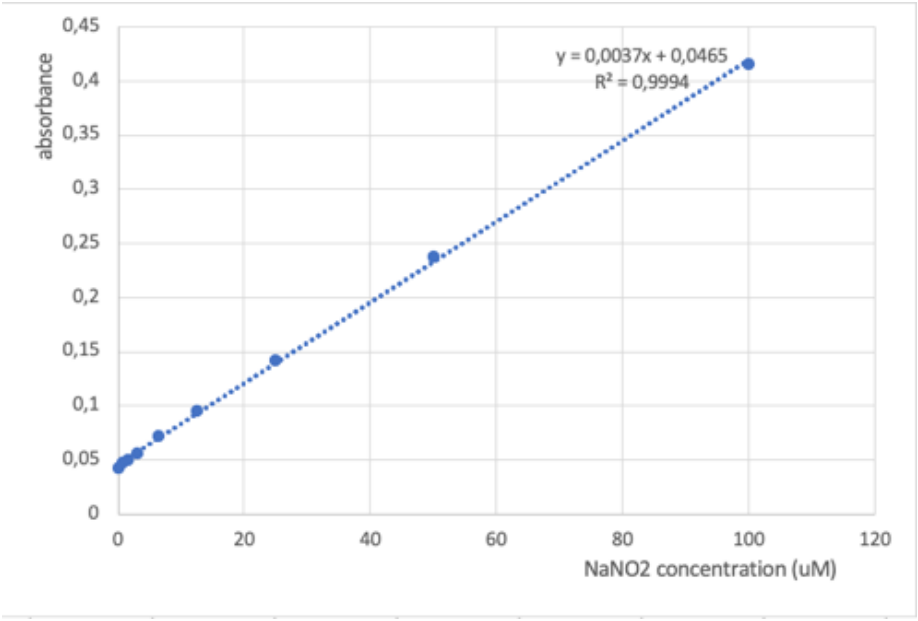
Absorbance t = 24 hr	Treatment					
	Control	LPS	IL-10	Pre-stim. IL-10	Pre-stim. LPS	Co-stim. IL-10 + LPS
1	0,050	0,128	0,050	0,130	0,115	0,118
2	0,045	0,126	0,048	0,126	0,112	0,113
3	0,046	0,121	0,049	0,160	0,109	0,110
Average	0,046	0,127	0,049	0,128	0,112	0,114
St Dev	0,00071	0,00141	0,00100	0,00283	0,00300	0,00404

Concentration t = 24 hr	Treatment					
	Control	LPS	IL-10	Pre-stim. IL-10	Pre-stim. LPS	Co-stim. IL-10 + LPS
[NO] (uM)	0,58	21,20	1,47	17,41	21,46	17,83
St Dev	0,00904	0,236	0,0299	0,466	0,474	0,634
[NO] (uM) ± Sd	0,58 ± 0,00904	21,20 ± 0,236	1,47 ± 0,0299	17,41± 0,466	21,46 ± 0,474	17,83 ± 0,634

Absorbance t = 26 hr	Treatment					
	Control	LPS	IL-10	Pre-stim. IL-10	Pre-stim. LPS	Co-stim. IL-10 + LPS
1	0,05	0,126	0,046	0,121	0,112	0,117
2	0,046	0,123	0,045	0,121	0,112	0,113
3	0,054	0,119	0,047	0,120	0,106	0,107
Average	0,050	0,123	0,046	0,110	0,121	0,112
St Dev	0,00400	0,00351	0,00100	0,00346	0,00058	0,00503

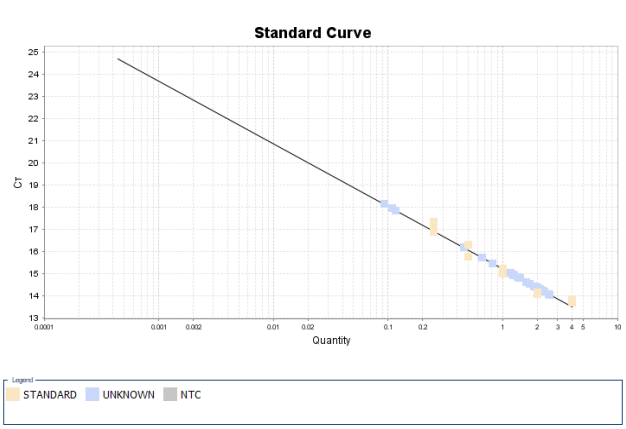
Concentration t = 26 hr	Treatment					
	Control	LPS	IL-10	Pre-stim. IL-10	Pre-stim. LPS	Co-stim. IL-10 + LPS
[NO] (uM)	1,72	20,11	0,71	16,90	19,60	17,49
St Dev	0,138	0,576	0,0154	0,532	0,0938	0,784
[NO] (uM) ± Sd	1,72 ± 0,138	20,11± 0,576	0,71± 0,0154	16,90 ± 0,532	19,60 ± 0,0938	17,49 ± 0,784

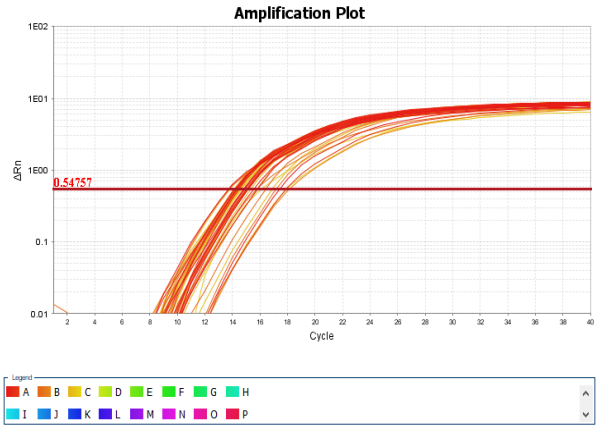
*Figure 26. Calibration curve NO Assay sample 3 experiment 1.3 (**fig. 10, 11 and 12**)*



Appendix D: Raw Data qPCR Experiment 2.1

Figure 27. Standard curve, amplification curve and melting curve Beta-actin qPCR corresponding to sample 1 (**fig. 13**)





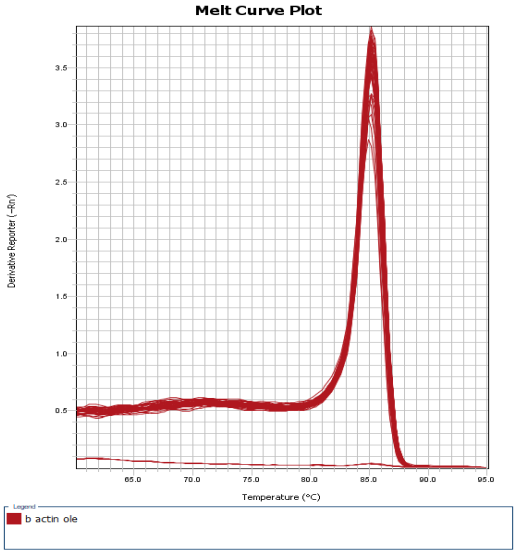


Table 14. Beta-actin Ct, Quantity and Melting Temp. sample 1 experiment 2.1 (**fig. 13**)

Sample Name	CT	Quantity	Tm1	Tm2
STD4	16,486	4,000	85,912	
STD4	16,981	4,000	85,912	
STD4	17,147	4,000	85,912	
STD2	16,739	2,000	85,912	
STD2	17,499	2,000	86,044	
STD2	17,064	2,000	85,912	
STD1	17,864	1,000	85,912	
STD1	18,489	1,000	86,044	
STD1	18,443	1,000	86,044	

STD0.5	19,558	0,500	85,912	
STD0.5	19,307	0,500	85,912	
STD0.5	19,213	0,500	85,912	
STD0.25	20,805	0,250	85,912	
STD0.25	21,215	0,250	86,044	
STD0.25	21,251	0,250	86,044	
Control 1	17,973	1,441	85,912	
Control 1	18,352	1,127	86,044	
Control 1	18,296	1,170	86,044	
Control 2	17,791	1,622	85,912	
Control 2	18,226	1,224	86,044	
Control 2	17,870	1,542	86,044	
IL-10 1	17,730	1,687	85,912	
IL-10 1	17,781	1,632	86,044	
IL-10 1	17,984	1,431	86,044	
IL-10 2	17,332	2,184	86,044	
IL-10 2	18,111	1,318	86,044	
IL-10 2	17,741	1,675	86,044	
LPS 1	17,399	2,091	86,044	
LPS 1	17,843	1,568	86,176	
LPS 1	17,801	1,612	86,044	
LPS 2	17,600	1,835	86,044	
LPS 2	18,219	1,229	86,176	
LPS 2	18,336	1,140	86,176	
IL-10 pre	18,222	1,227	86,044	
IL-10 pre	17,901	1,510	86,044	
IL-10 pre	18,030	1,389	86,044	
IL-10 pre 2	17,596	1,840	86,044	
IL-10 pre 2	18,178	1,262	86,176	
IL-10 pre 2	17,551	1,895	86,044	
Costim. 1	17,919	1,493	86,044	
Costim. 1	18,112	1,317	86,176	
Costim. 1	18,107	1,322	86,044	
Costim. 2	18,992	0,745	86,044	
Costim. 2	18,187	1,255	86,176	
Costim. 2	17,809	1,603	86,044	
NC 2	Undetermined		85,780	61,371
NC 2	Undetermined		85,780	61,239
NC 2	Undetermined		85,912	61,239
PC 1	18,096	1,331	85,648	
PC 1	17,991	1,425	85,780	
PC 1	18,046	1,375	85,780	

Table 15. Beta-actin average quantity and corresponding St Dev sample 1 experiment 2.1 (**fig. 13**)

Treatment	Average Beta-actin quantity	St Dev
Control 1	1,246083	0,17039705
Control 2	1,462347	0,21067231
Cost. 1	1,377079	0,10008503
Costi. 2	1,428832	0,2462503
IL-10 + LPS 1	1,375558	0,142217
IL-10 + LPS 2	1,867315	0,03855021
IL-10 1	1,583540	0,13475189
IL-10 2	1,496513	0,25266024
LPS 1	1,589977	0,03059625
LPS 2	1,184494	0,06351867

Figure 28: Standard curve, amplification curve and melting curve TNF-α qPCR sample 1 experiment 2.1(**fig. 13**)

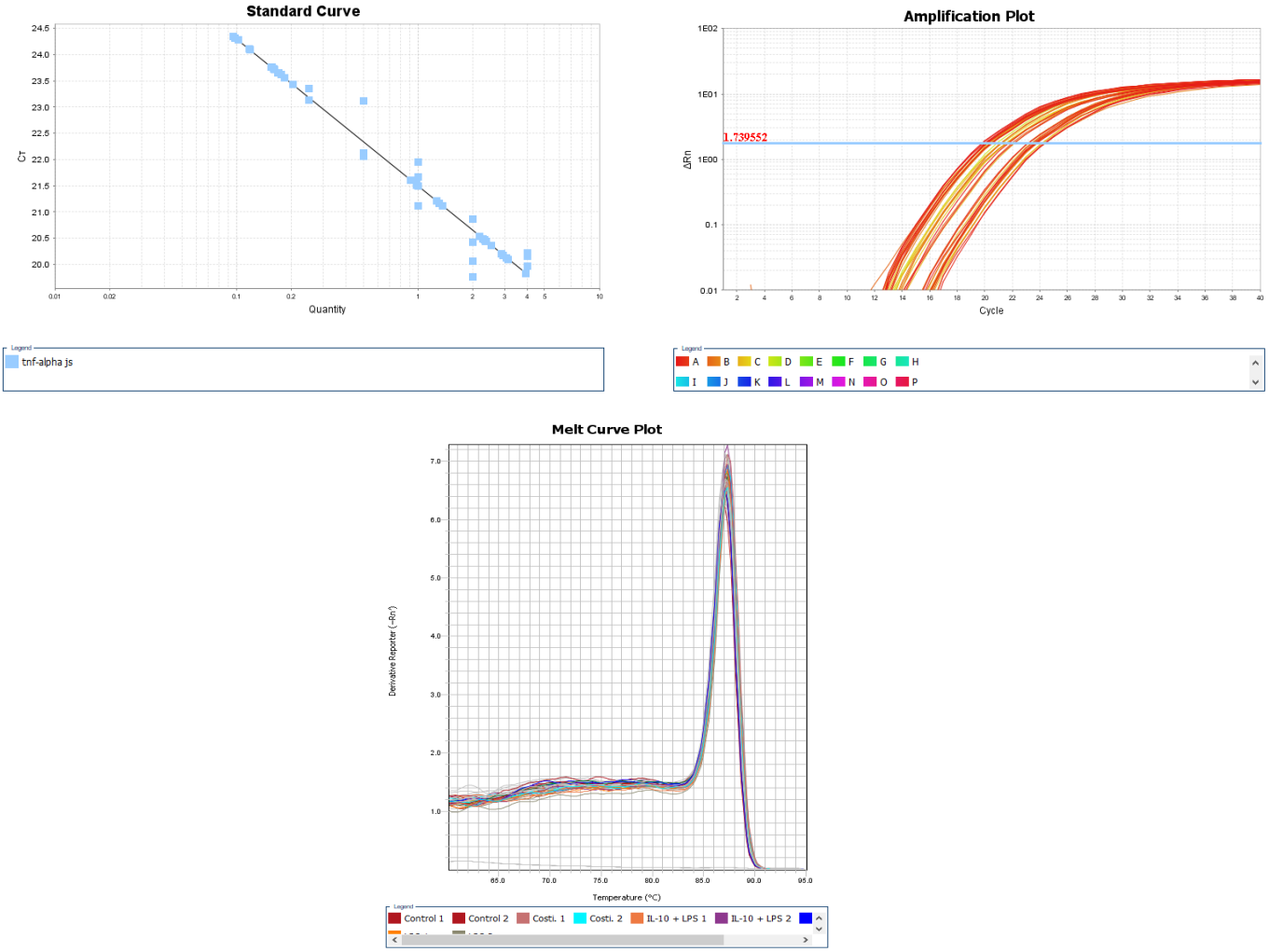


Table 16. TNF-α Ct, Quantity and Melting Temp. sample 1 experiment 2.1 **(fig. 13)**

Sample Name	CT	Quantity	Tm1	Tm2	Tm3
STD 4	19,974	4,000	87,081		
STD 4	20,216	4,000	87,213		
STD 4	20,161	4,000	87,213		
STD 2	19,765	2,000	86,949		
STD 2	20,066	2,000	87,081		
STD 2	20,419	2,000	87,081		
STD 1	21,665	1,000	87,081		
STD 1	21,943	1,000	87,213		
STD 1	21,109	1,000	87,081		
STD 0.5	22,065	0,500	87,081		
STD 0.5	23,111	0,500	87,213		
STD 0.5	22,119	0,500	87,213		
STD 0.25	23,126	0,250	87,081		
STD 0.25	23,135	0,250	87,213		
STD 0.25	23,358	0,250	87,081		
Control 1	23,759	0,157	87,081		
Control 1	23,560	0,185	87,213		
Control 1	23,765	0,156	87,081		
Control 2	23,613	0,177	87,081		
Control 2	23,653	0,171	87,213		
Control 2	24,095	0,120	87,081		
IL-10 1	24,348	0,097	87,213		
IL-10 1	23,732	0,161	87,213		
IL-10 1	24,107	0,118	87,081		
IL-10 2	24,273	0,103	87,213		
IL-10 2	23,713	0,163	87,213		
IL-10 2	23,658	0,171	87,213		
LPS 1	20,126	3,011	87,213		
LPS 1	20,463	2,290	87,345		
LPS 1	20,435	2,342	87,213		
LPS 2	20,096	3,086	87,213		
LPS 2	21,170	1,289	87,476		
LPS 2	21,603	0,906	87,345		
IL-10 + LPS 1	20,444	2,325	87,213		
IL-10 + LPS 1	21,520	0,970	87,345		
IL-10 + LPS 1	21,511	0,977	87,345		
IL-10 + LPS 2	19,821	3,858	87,345		
IL-10 + LPS 2	20,202	2,831	87,345		
IL-10 + LPS 2	20,356	2,498	87,345		

Costi. 1	20,176	2,891	87,345		
Costi. 1	21,118	1,345	87,345		
Costi. 1	21,496	0,989	87,345		
Costi. 2	20,488	2,243	87,213		
Costi. 2	20,538	2,153	87,345		
Costi. 2	21,204	1,253	87,213		
NC	Undetermined		61,369	87,081	82,466
NC	Undetermined		61,369	87,081	
PC	23,429	0,205	87,213		
PC	24,320	0,100	87,213		

Table 17: *TNF-α average quantity and corresponding St Dev sample 1 experiment 2.1* (**fig. 13**)

Treatment	Average TNF-α quantity	St Dev
Control 1	0,157	0,0005508
Control 2	0,174	0,00396493
Cost. 1	1,167	0,25149113
Costi. 2	2,198	0,06365304
IL-10 + LPS 1	0,973	0,00503212
IL-10 + LPS 2	2,664	0,23602825
IL-10 1	0,125	0,03222393
IL-10 2	0,167	0,00528722
LPS 1	2,316	0,03704877
LPS 2	1,097	0,27073985

*Table 18: Relative TNF- α expression and average relative TNF- α expression sample 1 experiment 2.1 (***fig. 13**)

	Relative	Average relative expression	St Dev Average relative expression
Control 1	0,12580186	0,12240311	0,00480657
Control 2	0,11900435		
Cost. 1	0,84737443	1,19300389	0,48879387
Costi. 2	1,53863335		
IL-10 + LPS 1	0,70757555	1,06723788	0,50863935
IL-10 + LPS 2	1,42690022		
IL-10 1	0,0792021	0,09534673	0,02283196
IL-10 2	0,11149136		
LPS 1	1,4567576	1,19161692	0,37496554
LPS 2	0,92647624		

Figure 29. Standard curve, amplification curve and melting curve TNF- α sample 2 experiment 2.1 (**fig. 13**)

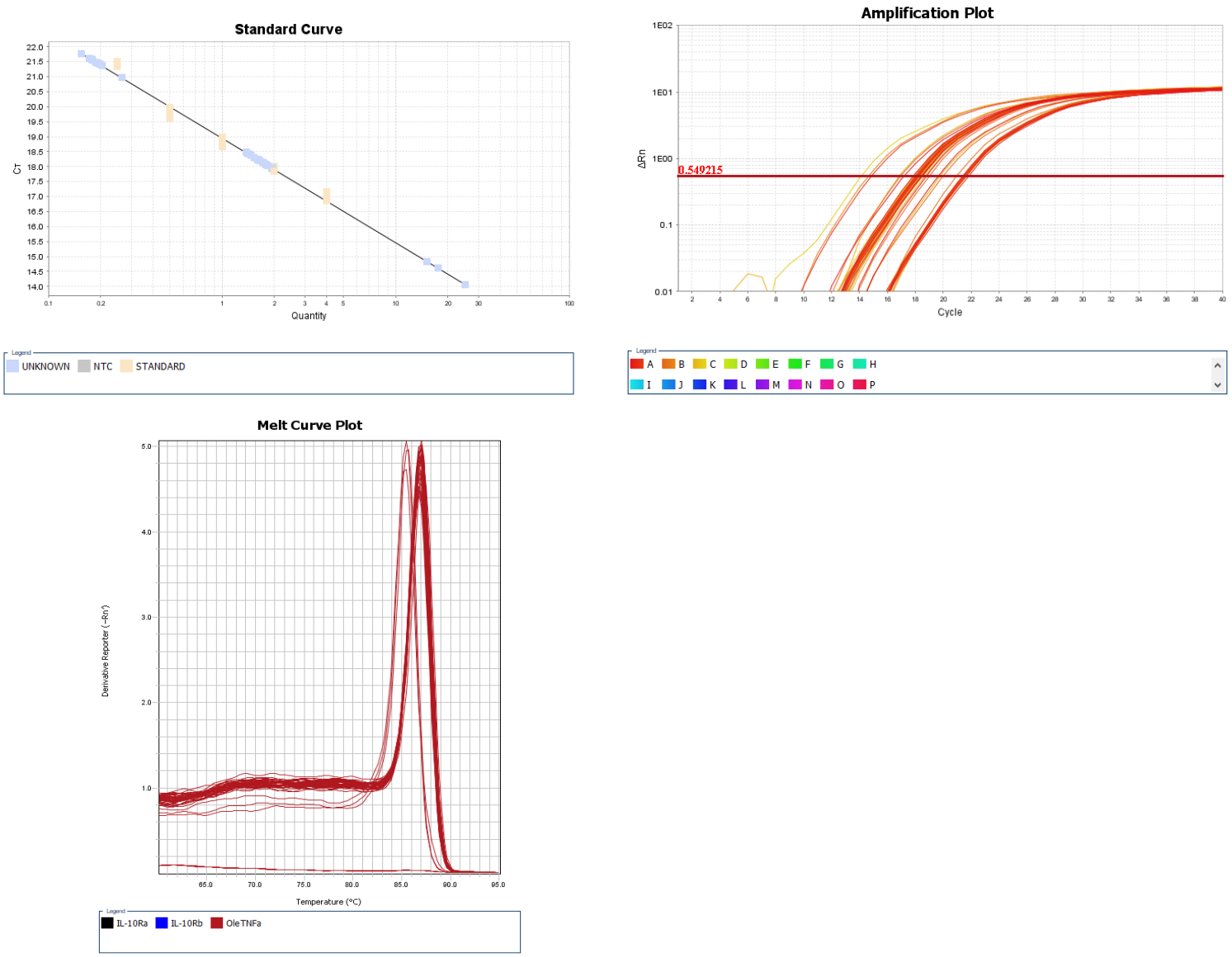


Table 19. TNF-α Ct, Quantity and Melting Temp. sample 2 experiment 2.1 (**fig 13**)

Sample Name	CT	Quantity	Tm1	Tm2	Tm3
STD4	17,165	4,000	86,763		
STD4	16,966	4,000	86,895		
STD4	16,857	4,000	86,895		
STD2	17,847	2,000	86,763		
STD2	17,995	2,000	86,763		
STD2	17,847	2,000	86,895		
STD1	18,785	1,000	86,763		
STD1	18,977	1,000	86,895		
STD1	18,681	1,000	86,895		

STD0,5	19,630	0,500	86,763		
STD0,5	19,961	0,500	86,895		
STD0,5	19,778	0,500	86,895		
STD0,25	21,337	0,250	86,763		
STD0,25	21,502	0,250	86,763		
STD0,25	22,193	0,250	86,763		
Control 1	21,567	0,210	86,763		
Control 1	21,491	0,220	86,895		
Control 1	23,292	0,073	86,500		
Control 2	21,778	0,185	86,763		
Control 2	20,966	0,304	87,158		
Control 2	21,371	0,237	86,895		
IL-10 1	21,580	0,209	86,895		
IL-10 1	21,451	0,226	86,895		
IL-10 1	21,423	0,230	86,895		
IL-10 2	21,455	0,225	86,895		
IL-10 2	21,491	0,220	86,895		
IL-10 2	21,622	0,203	86,763		
LPS 1	18,195	1,677	86,895		
LPS 1	18,311	1,561	86,895		
LPS 1	18,217	1,655	86,895		
LPS 2	18,138	1,737	86,895		
LPS 2	18,197	1,675	87,026		
LPS 2	18,229	1,643	86,895		
IL-10 pre	18,426	1,455	87,026		
IL-10 pre	18,474	1,413	87,026		
IL-10 pre	18,380	1,496	87,026		
IL-10 pre 2	17,949	1,951	87,026		
IL-10 pre 2	18,069	1,812	87,026		
IL-10 pre 2	17,940	1,962	87,026		
Costim. 1	18,046	1,838	87,026		
Costim. 1	18,084	1,795	87,026		
Costim. 1	17,963	1,935	87,026		
Costim. 2	18,376	1,500	86,895		
Costim. 2	18,438	1,444	87,026		
Costim. 2	18,255	1,617	86,895		
NC	Undetermined		61,237	92,290	85,579
NC	Undetermined		85,579	61,368	92,290
NC	Undetermined		62,158	85,974	83,737
PC	14,832	13,307	85,316		
PC	14,620	15,161	85,447		
PC	14,065	21,335	85,579		

Table 20. TNF-α average quantity and corresponding St Dev sample 2 experiment 2.1 (**fig. 13**)

Treatment	Average Quantity	St Dev
Control 1	0,16774548	0,08250649
Control 2	0,24209711	0,06005621
IL-10 1	0,22138794	0,01121699
IL-10 2	0,21626469	0,01277375
LPS 1	1,63106855	0,06146058
LPS 2	1,68473502	0,04795099
IL-10 pre	1,45455253	0,04167659
IL-10 pre 2	1,90843999	0,08365708
CO 1	1,85622493	0,03019426
CO 2	1,52027845	0,08813179

*Table 21. Relative TNF- α expression and average relative TNF- α expression sample 2 experiment 2.1 (***fig. 13**)

Treatment type	Relative TNF-a expression	Average relative expression	St Dev average relative expression
Control 1	0,13461822	0,15008599	0,02187472
Control 2	0,16555375		
IL-10 1	0,13980572	0,14215905	0,0033281
IL-10 2	0,14451237		
LPS 1	1,02584394	1,22408443	0,28035439
LPS 2	1,42232492		
IL-10 pre	1,05742713	1,03972545	0,02503396
IL-10 pre 2	1,02202376		
Costim, 1	1,34794351	1,20597236	0,20077753
Costim, 2	1,0640012		

Appendix E: Raw Data qPCR Experiment 2.2

Figure 30: Standard curve, amplification curve and melting curve Beta-actin qPCR sample 1 experiment 2.2 (**fig. 14 and 15**)

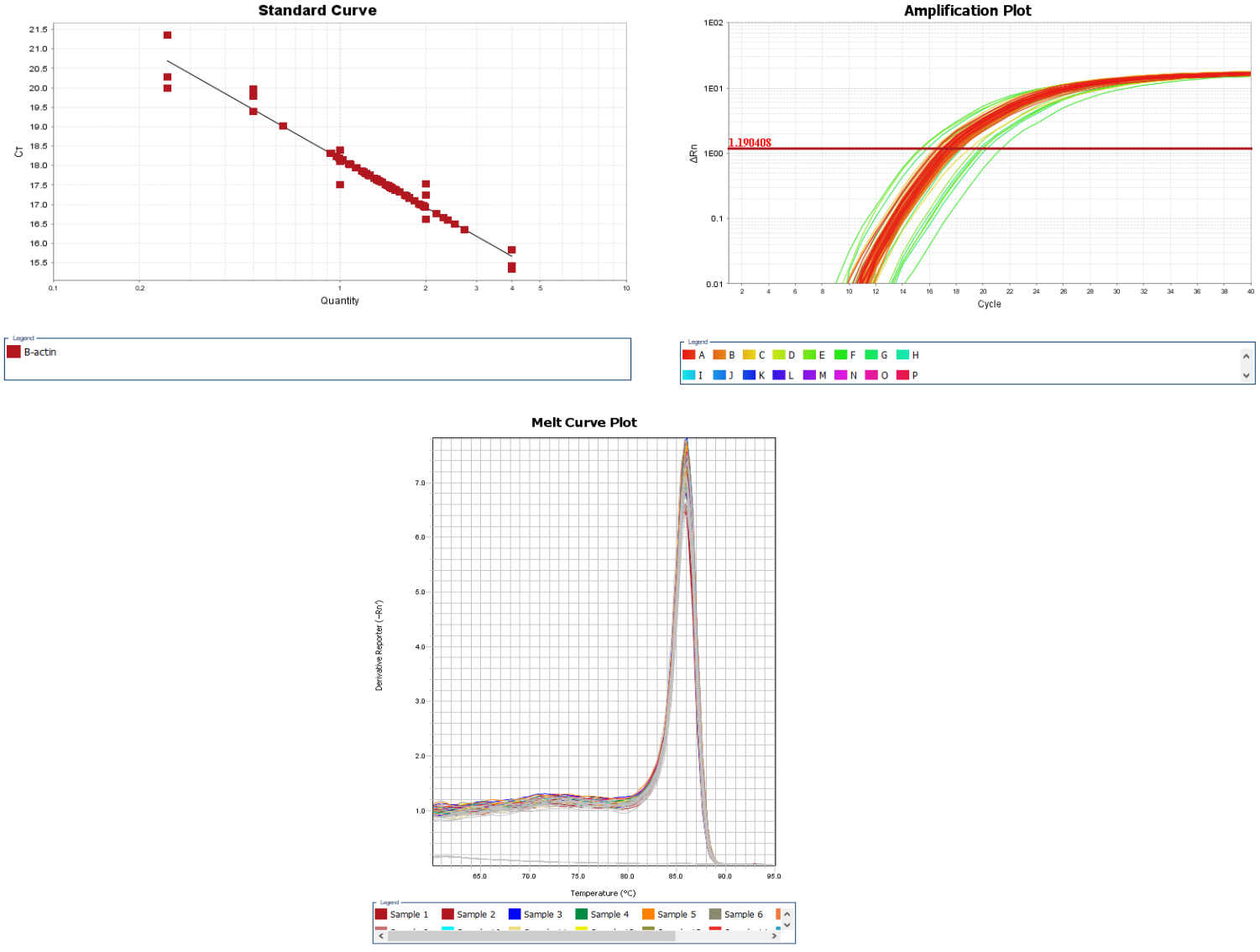


Table 22. Beta-actin Ct, Quantity and Melting Temp. sample 1 experiment 2.2 (**fig. 14 and 15**)

Target Name	CT	Quantity	Tm1
STD 4	15,423	4,000	85,929
STD 4	15,339	4,000	86,061
STD 4	15,838	4,000	86,061
STD 2	17,531	2,000	85,929
STD 2	16,609	2,000	85,929
STD 2	17,247	2,000	86,061

STD 1	17,506	1,000	85,929
STD 1	18,391	1,000	85,929
STD 1	18,102	1,000	85,929
STD 0.5	19,392	0,500	85,797
STD 0.5	19,962	0,500	85,929
STD 0.5	19,778	0,500	86,061
STD 0.25	19,991	0,250	85,797
STD 0.25	21,362	0,250	85,929
STD 0.25	20,273	0,250	85,929
Control 1	16,963	1,957	85,797
Control 1	17,210	1,708	85,797
Control 1	17,441	1,504	85,797
Control 2	17,745	1,272	85,797
Control 2	17,640	1,348	85,797
Control 2	17,950	1,137	85,929
IL-10 1	17,373	1,561	85,797
IL-10 1	17,798	1,235	85,929
IL-10 1	17,411	1,529	85,929
IL-10 2	17,430	1,513	85,797
IL-10 2	17,620	1,363	85,929
IL-10 2	17,787	1,243	85,929
LPS10 1	16,997	1,920	85,797
LPS10 1	18,039	1,082	85,929
LPS10 1	17,568	1,402	85,929
LPS10 2	17,020	1,896	85,797
LPS10 2	17,372	1,562	85,929
LPS10 2	17,427	1,515	85,929
LPS30 1	17,461	1,488	85,797
LPS30 1	18,317	0,929	85,929
LPS30 1	17,449	1,497	85,929
LPS30 2	17,800	1,235	85,929
LPS30 2	18,028	1,089	85,929
LPS30 2	18,050	1,076	85,929
LPS100 1	17,235	1,685	85,929
LPS100 1	17,168	1,748	85,929
LPS100 1	16,773	2,172	85,929
LPS100 2	16,596	2,394	85,929
LPS100 2	16,935	1,987	86,061
LPS100 2	17,450	1,496	85,929
P1 1	17,313	1,614	86,061
P1 1	17,835	1,211	85,929
P1 1	18,033	1,086	86,061

P1 2	17,089	1,825	85,929
P1 2	18,187	0,998	86,061
P1 2	17,516	1,443	86,061
IL-10 + LPS10 1	17,419	1,523	86,061
IL-10 + LPS10 1	18,133	1,028	86,061
IL-10 + LPS10 1	18,139	1,024	86,061
IL-10 + LPS10 2	17,645	1,344	86,061
IL-10 + LPS10 2	17,609	1,371	86,061
IL-10 + LPS10 2	17,567	1,403	86,061
IL-10 + LPS30 1	17,456	1,492	86,061
IL-10 + LPS30 1	16,663	2,308	86,061
IL-10 + LPS30 1	16,354	2,735	86,061
IL-10 + LPS30 2	16,953	1,968	85,929
IL-10 + LPS30 2	17,165	1,751	86,061
IL-10 + LPS30 2	17,681	1,318	86,061
IL-10 + LPS100 1	16,494	2,533	85,929
IL-10 + LPS100 1	17,764	1,259	85,929
IL-10 + LPS100 1	17,860	1,195	85,929
IL-10 + LPS100 2	16,997	1,920	85,929
IL-10 + LPS100 2	17,487	1,466	85,929
IL-10 + LPS100 2	17,357	1,575	85,929
P1 + LPS 100 1	18,225	0,977	85,929
P1 + LPS 100 1	17,680	1,319	85,929
P1 + LPS 100 1	19,010	0,634	85,929
P1 + LPS 100 2	17,933	1,147	85,797
P1 + LPS 100 2	17,584	1,390	85,929
P1 + LPS 100 2	17,736	1,279	85,929
NC	Undetermined		61,369
NC	Undetermined		61,237
NC	Undetermined		61,237
PC	18,308	0,933	85,929
PC	17,476	1,475	85,665
PC	17,491	1,463	85,929

Table 23. Beta-actin average quantity and corresponding St Dev sample 1 experiment 2.2 (**fig. 14 and 15**)

Treatment	Average Quantity	St. dev
Control 1	1,723	0,22663031
Control 2	1,252	0,10685477
IL-10 1	1,442	0,02250675

IL-10 2	1,373	0,13548675
LPS10 1	1,468	0,42294663
LPS10 2	1,658	0,20760038
LPS30 1	1,492	0,0065819
LP30 2	1,082	0,00920361
LPS100 1	1,716	0,04445616
LPS100 2	1,959	0,44937552
P1 1	1,148	0,08826831
P1 2	1,422	0,41405832
IL-10 + LPS10 1	1,026	0,00228301
IL-10 + LPS10 2	1,373	0,02952994
IL-10 + LPS30 1	2,521	0,30179824
IL-10 + LPS30 2	1,859	0,153178
IL-10 + LPS100 1	1,227	0,04548252
IL-10 + LPS100 2	1,520	0,07692862
P1 + LPS100 1	0,977	0,34209888
P1 + LPS100 2	1,272	0,12164316

Figure 31. Standard curve, amplification curve and melting curve TNF- α sample 1 experiment 2.2 (**fig. 14 and 15**)

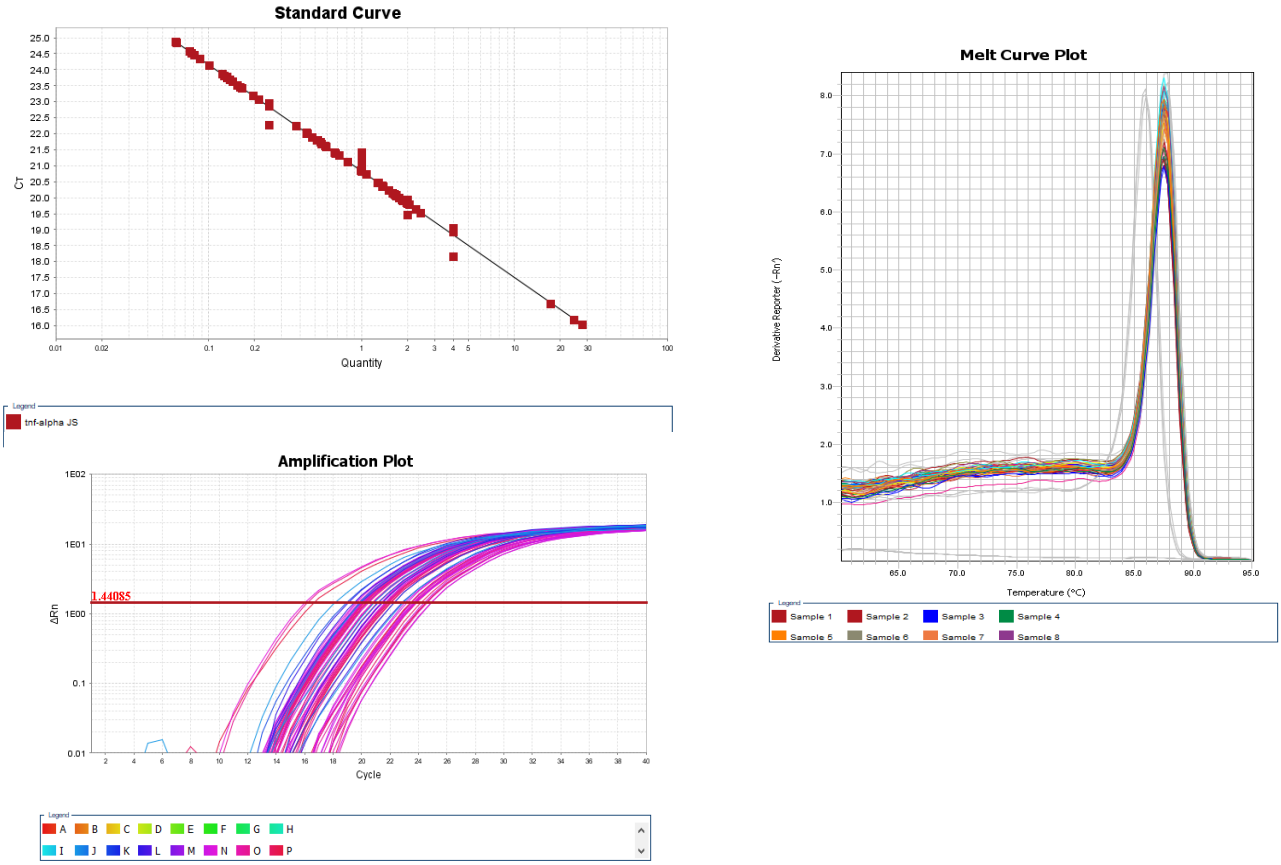


Table 24. TNF- α Ct, Quantity and Melting Temp. sample 1 experiment 2.2 (**fig. 14 and 15)**

Sample Name	CT	Quantity	Tm1
STD 4	18,141	4,000	87,688
STD 4	18,926	4,000	87,819
STD 4	19,055	4,000	87,688
STD 2	19,877	2,000	87,688
STD 2	19,920	2,000	87,688
STD 2	19,468	2,000	87,688
STD 1	20,880	1,000	87,688
STD 1	21,136	1,000	87,688
STD 1	21,410	1,000	87,688
Control 1	23,759	0,131	87,425
Control 1	23,696	0,137	87,425
Control 1	24,137	0,101	87,425
Control 2	23,522	0,155	87,425
Control 2	23,855	0,123	87,556
Control 2	23,804	0,127	87,425
IL-10 1	24,346	0,088	87,556
IL-10 1	24,842	0,062	87,556
IL-10 1	24,463	0,081	87,556
IL-10 2	24,865	0,061	87,556
IL-10 2	24,574	0,075	87,556
IL-10 2	24,517	0,078	87,425
LPS10 1	19,907	1,884	87,425
LPS10 1	20,065	1,690	87,556
LPS10 1	20,093	1,657	87,556
LPS10 2	19,778	2,059	87,556
LPS10 2	20,075	1,678	87,556
LPS10 2	19,833	1,982	87,556
LPS30 1	20,451	1,294	87,425
LPS30 1	20,717	1,076	87,556
LPS30 1	20,357	1,381	87,425
LPS30 2	20,001	1,765	87,556
LPS30 2	20,087	1,664	87,556
LPS30 2	20,144	1,599	87,556
LPS100 1	19,812	2,011	87,556
LPS 100 1	20,135	1,610	87,556
LPS 100 1	20,475	1,272	87,556
LPS100 2	19,908	1,883	87,556
LPS100 2	19,938	1,844	87,556
LPS100 2	20,056	1,699	87,556
P1 1	23,750	0,132	87,556
P1 1	23,618	0,145	87,556

P1 1	23,417	0,167	87,556
P1 2	23,058	0,213	87,556
P1 2	23,757	0,132	87,688
P1 2	23,455	0,162	87,688
IL10 + LPS10 1	21,381	0,680	87,556
IL10 + LPS10 1	21,790	0,513	87,688
IL10 + LPS10 1	21,886	0,480	87,688
IL10 + LPS10 2	21,310	0,714	87,556
IL10 + LPS10 2	21,720	0,538	87,688
IL10 + LPS10 2	21,778	0,517	87,688
IL10 + LPS30 1	20,814	1,007	87,556
IL10 + LPS30 1	21,122	0,814	87,688
IL10 + LPS30 1	20,835	0,992	87,688
IL10 + LPS30 2	21,614	0,579	87,425
IL10 + LPS30 2	22,249	0,373	87,556
IL10 + LPS30 2	22,024	0,436	87,688
IL10 + LPS100 1	23,171	0,197	87,425
IL10 + LPS100 1	22,010	0,440	87,556
IL10 + LPS100 1	21,989	0,447	87,556
IL10 + LPS100 2	21,594	0,587	87,556
IL10 + LPS100 2	21,401	0,671	87,556
IL10 + LPS100 2	21,686	0,551	87,556
P1 + LPS 100 1	19,526	2,451	87,425
P1 + LPS 100 1	20,223	1,514	87,556
P1 + LPS 100 1	20,223	1,515	87,556
P1 + LPS 100 2	19,641	2,265	87,425
P1 + LPS 100 2	20,367	1,371	87,556
P1 + LPS 100 2	20,335	1,401	87,556

Table 25. TNF-α average quantity and corresponding St Dev sample 1 experiment 2.2 (**fig. 14 and 15**)

Treatment	Average Quantity	St. dev
Control 1	0,134	0,00413006
Control 2	0,125	0,00313835
IL-10 1	0,084	0,00479961
IL-10 2	0,076	0,00213393
LPS10 1	1,673	0,02318894
LPS10 2	2,021	0,05473046
LPS30 1	1,337	0,06137272
LP30 2	1,676	0,08364326
LPS100 1	1,631	0,3700889
LPS100 2	1,864	0,02728663
P1 1	0,139	0,00893834
P1 2	0,147	0,02163957

IL-10 + LPS10 1	0,496	0,02343974
IL-10 + LPS10 2	0,528	0,01491703
IL-10 + LPS30 1	0,999	0,0103747
IL-10 + LPS30 2	0,405	0,04432101
IL-10 + LPS100 1	0,444	0,004522
IL-10 + LPS100 2	0,569	0,02552173
P1 + LPS100 1	1,514	0,00035572
P1 + LPS100 2	1,386	0,0214138

Table 26. Relative TNF- α expression and average relative TNF- α expression sample 1 experiment 2.2 (**fig. 14 and 15**)

Treatment	Relative expression	Average relative expression	St Dev
Control 1	0,07801534	0,0890239	0,01556846
Control 2	0,10003246		
IL-10 1	0,0584362	0,05703271	0,00198483
IL-10 2	0,05562923		
LPS10 1	1,13948458	1,17927541	0,05627274
LPS10 2	1,21906625		
LPS30 1	0,89610867	1,2222279	0,250152
LP30 2	1,54834712		
LPS100 1	0,95042868	0,95083137	0,00056949
LPS100 2	0,95123406		
P1 1	0,12076688	0,11205462	0,012321
P1 2	0,10334236		
IL-10 + LPS10 1	0,48374787	0,43395707	0,07041483
IL-10 + LPS10 2	0,38416627		
IL-10 + LPS30 1	0,39636134	0,30703519	0,12632625
IL-10 + LPS30 2	0,21770904		
IL-10 + LPS100 1	0,36165162	0,36796534	0,00892895
IL-10 + LPS100 2	0,37427906		
P1 + LPS100 1	1,55056357	1,31999807	0,32606886
P1 + LPS100 2	1,08943257		

Figure 32, Standard curve, amplification curve and melting curve TNF- α sample 2 experiment 2.2 (**fig. 14 and 15**)

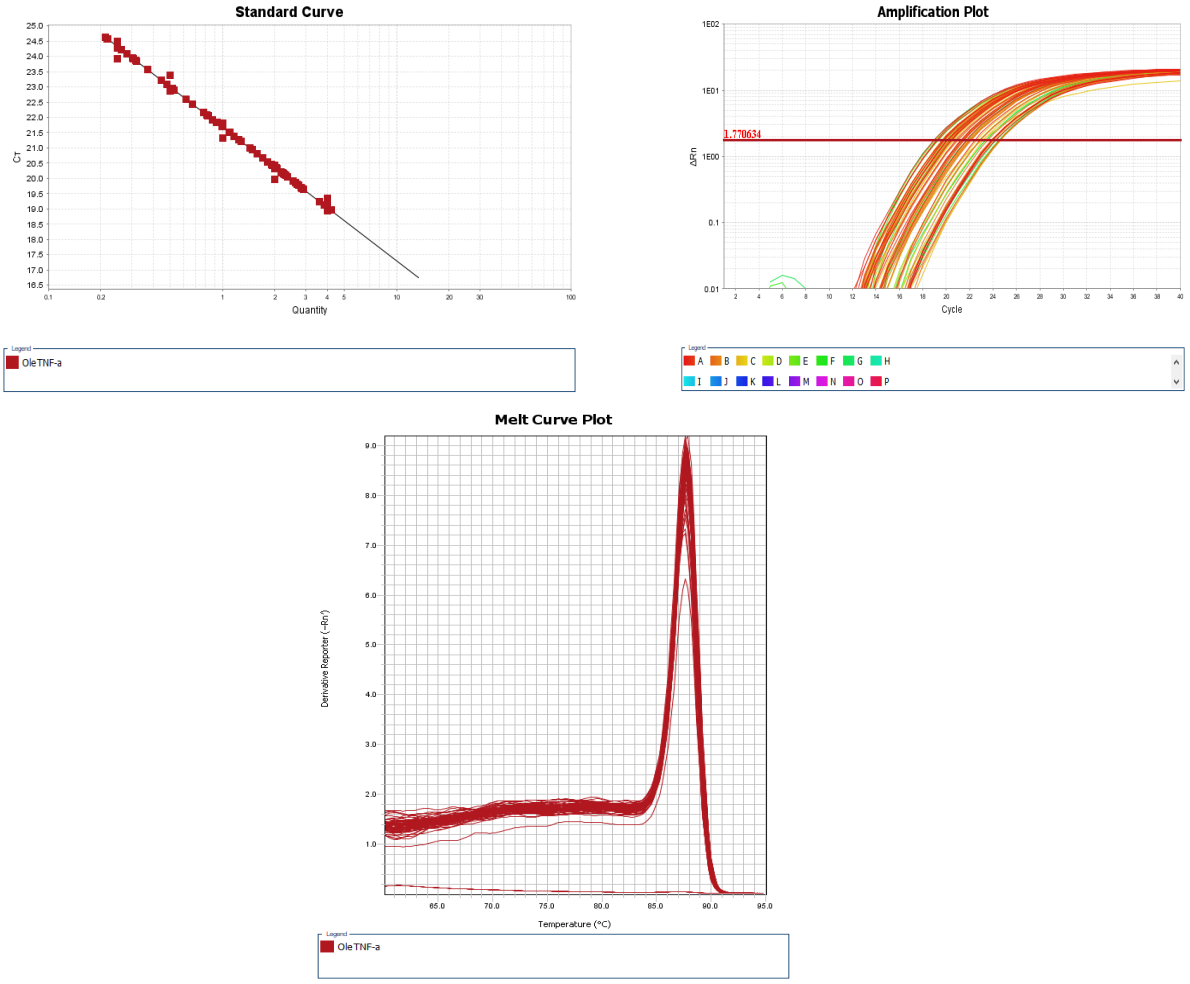


Table 27. TNF- α Ct, Quantity and Melting Temp. sample 2 experiment 2.2 (**fig. 14 and 15**)

Sample Name	CT	Quantity	Tm1
STD 4	19,111	4,000	87,628
STD 4	19,345	4,000	87,760
STD 4	18,948	4,000	87,760
STD 2	19,962	2,000	87,760
STD 2	20,419	2,000	87,760
STD 2	20,322	2,000	87,760
STD 1	21,333	1,000	87,628
STD 1	21,797	1,000	87,760
STD 1	21,734	1,000	87,760
STD 0,5	22,852	0,500	87,760
STD 0,5	23,392	0,500	87,760
STD 0,5	23,389	0,500	87,760

STD 0,25	23,909	0,250	87,760
STD 0,25	24,491	0,250	87,760
STD 0,25	24,280	0,250	87,760
Control 1	24,230	0,262	87,496
Control 1	23,847	0,320	87,496
Control 1	24,087	0,282	87,628
Control 2	23,927	0,307	87,496
Control 2	23,567	0,371	87,628
Control 2	23,870	0,317	87,628
IL-10 1	23,933	0,306	87,496
IL-10 1	24,581	0,218	87,628
IL-10 1	24,622	0,213	87,760
IL-10 2	23,950	0,303	87,628
IL-10 2	24,568	0,219	87,628
IL-10 2	24,633	0,212	87,628
LPS 10 1	19,683	2,871	87,628
LPS 10 1	19,778	2,731	87,760
LPS 10 1	19,641	2,935	87,628
LPS 10 2	20,174	2,216	87,628
LPS 10 2	20,135	2,263	87,760
LPS 10 2	20,191	2,197	87,628
LPS 30 1	20,439	1,928	87,628
LPS 30 1	20,991	1,442	87,760
LPS 30 1	20,550	1,819	87,628
LPS 30 2	19,810	2,685	87,628
LPS 30 2	20,310	2,064	87,760
LPS 30 2	20,342	2,029	87,760
LPS 100 1	18,957	4,207	87,760
LPS 100 1	20,101	2,304	87,760
LPS 100 1	19,711	2,829	87,760
LPS 100 2	19,138	3,826	87,628
LPS 100 2	19,852	2,627	87,760
LPS 100 2	19,710	2,831	87,628
P1 1	22,446	0,670	87,760
P1 1	22,943	0,516	87,760
P1 1	23,216	0,447	87,760
P1 2	21,706	0,989	87,760
P1 2	22,898	0,528	87,892
P1 2	22,599	0,618	87,760
Il-10 + LPS 10 1	21,272	1,243	87,760
Il-10 + LPS 10 1	21,827	0,928	87,760
Il-10 + LPS 10 1	23,082	0,479	87,628

IL-10 + LPS 10 2	21,216	1,280	87,760
IL-10 + LPS 10 2	21,929	0,879	87,760
IL-10 + LPS 10 2	21,510	1,097	87,760
IL-10 + LPS 30 1	20,462	1,905	87,628
IL-10 + LPS 30 1	20,942	1,479	87,760
IL-10 + LPS 30 1	20,668	1,709	87,628
IL-10 + LPS 30 2	21,390	1,168	87,628
IL-10 + LPS 30 2	22,153	0,782	87,760
IL-10 + LPS 30 2	21,389	1,169	87,628
IL-10 + LPS 100 1	21,501	1,102	87,628
IL-10 + LPS 100 1	22,078	0,813	87,760
IL-10 + LPS 100 1	22,053	0,824	87,760
IL-10 + LPS 100 2	20,821	1,576	87,628
IL-10 + LPS 100 2	22,044	0,828	87,760
IL-10 + LPS 100 2	21,826	0,929	87,760
P1 + LPS 100 1	19,249	3,608	87,496
P1 + LPS 100 1	19,904	2,556	87,628
P1 + LPS 100 1	20,215	2,169	87,628
P1 + LPS 100 2	20,058	2,356	87,496
P1 + LPS 100 2	20,374	1,996	87,628
P1 + LPS 100 2	20,150	2,245	87,628
NC	Undetermined		61,109
NC	Undetermined		61,373
NC	Undetermined		61,241
PC	16,754	13,426	85,913
PC	17,563	8,770	86,045
PC	17,346	9,833	86,177

Table 28. TNF-α average quantity and corresponding St Dev sample 2 experiment 2.2 (**fig. 14 and 15**)

Treatment	Average quantity tnf-alpha	St Dev
Control 1	0,28816667	0,02969512
Control 2	0,33166778	0,03463817
IL-10 1	0,24561885	0,05245897
IL-10 2	0,24478932	0,05096132
LPS 10 1	2,84573189	0,10424008
LPS 10 2	2,22542596	0,03417419
LPS 30 1	1,72959423	0,25500893
LPS 30 2	2,25957187	0,36925209
LPS 100 1	3,113554	0,98301405
LPS 100 2	3,09468015	0,64178442
P1 1	0,54420251	0,11441224
P1 2	0,71186558	0,24448991

Il-10 + LPS 10 1	0,88363145	0,38396269
IL-10 + LPS 10 2	1,08547368	0,20062018
IL-10 + LPS 30 1	1,69768349	0,21311856
IL-10 + LPS 30 2	1,03967615	0,22348385
IL-10 + LPS 100 1	0,91322186	0,16355245
IL-10 + LPS 100 2	1,11115557	0,40611621
P1 + LPS 100 1	2,77769407	0,74445953
P1 + LPS 100 2	2,19899825	0,18464256

Table 29. Relative TNF- α expression and average relative TNF- α expression sample 2 experiment 2.2 (**fig. 14 and 15**)

Treatment	Relative TNF-a expression	Av relative expression	St dev av. Relative expression
Control 1	0,16725652	0,21604663	0,06899964
Control 2	0,26483675		
IL-10 1	0,17034791	0,17431316	0,00560772
IL-10 2	0,17827841		
LPS 10 1	1,93807848	1,6402987	0,4211242
LPS 10 2	1,34251893		
LPS 30 1	1,32576359	1,65991111	0,47255597
LPS 30 2	1,99405864		
LPS 100 1	1,66659354	1,62313079	0,06146561
LPS 100 2	1,57966804		
P1 1	0,41750293	0,45904203	0,05874515
P1 2	0,50058113		
Il-10 + LPS 10 1	0,74158049	0,76604687	0,0346007
IL-10 + LPS 10 2	0,79051326		
IL-10 + LPS 30 1	0,6734167	0,64633892	0,03829376
IL-10 + LPS 30 2	0,61926114		
IL-10 + LPS 100 1	0,54947352	0,61070863	0,08659952
IL-10 + LPS 100 2	0,67194374		
P1 + LPS 100 1	2,8439073	2,28620917	0,78870427
P1 + LPS 100 2	1,72851103		

Appendix F: Raw Data qPCR Experiment 2.3

Figure 33. Standard curve, amplification curve and melting curve beta-actin experiment 2.3 (**fig. 16**)

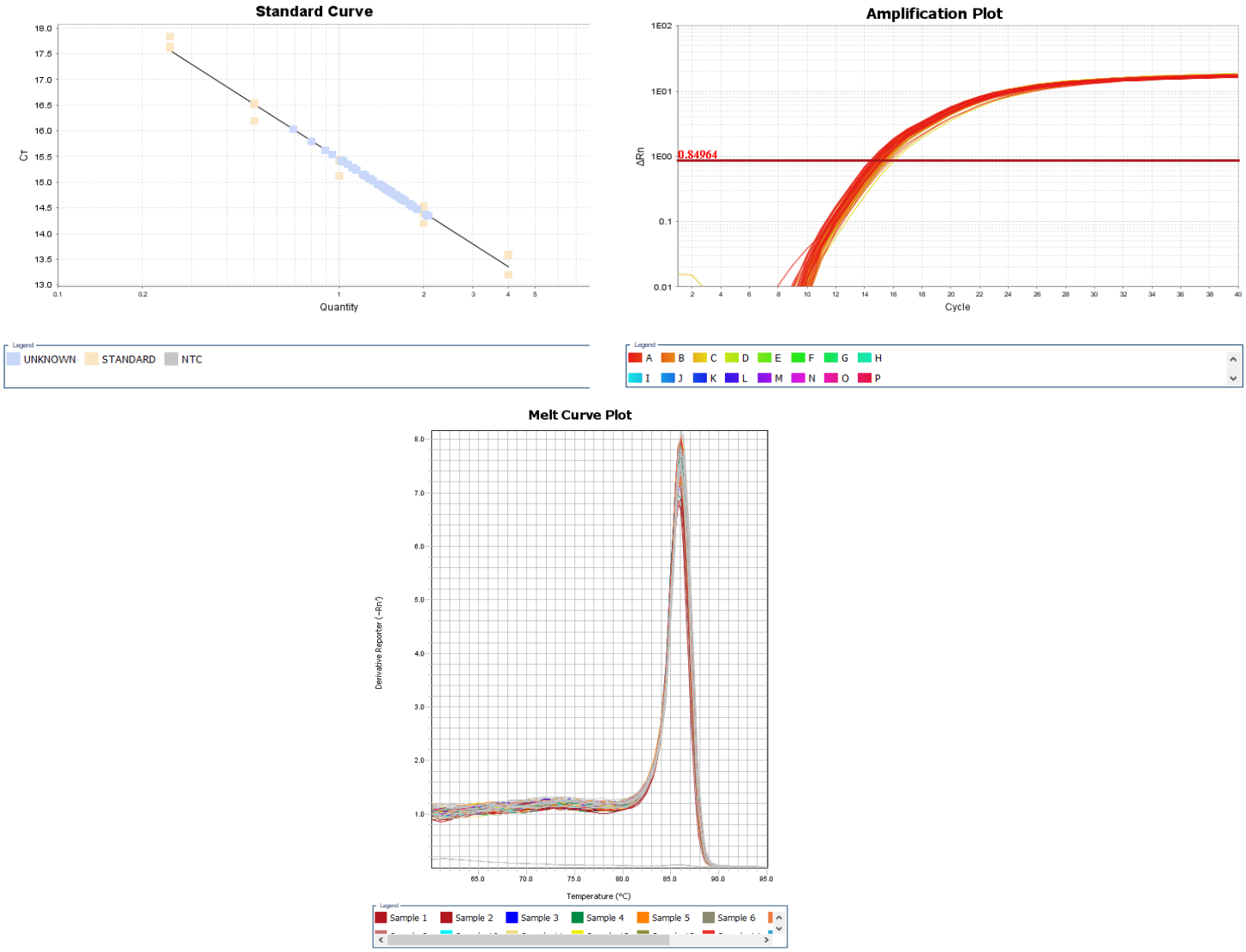


Table 30. Beta-actin Ct, Quantity and Melting Temp. sample 1 experiment 2.3 (**fig. 16**)

Sample Name	CT	Quantity	Tm1
STD 4	13,198	4,000	86,042
STD 4	13,588	4,000	86,174
STD 4	13,574	4,000	86,174
STD 2	14,195	2,000	86,042
STD 2	14,540	2,000	86,174
STD 2	14,392	2,000	86,174
STD 1	15,119	1,000	86,042
STD 1	15,418	1,000	86,174
STD 1	15,436	1,000	86,174
STD 0.5	16,193	0,500	86,042
STD 0.5	16,519	0,500	86,174
STD 0.5	16,550	0,500	86,174

STD 0.25	17,618	0,250	86,042
STD 0.25	17,644	0,250	86,042
STD 0.25	17,841	0,250	86,174
Control 1	15,154	1,219	85,778
Control 1	15,394	1,041	85,910
Control 1	15,287	1,117	85,910
Control 2	15,076	1,283	85,778
Control 2	14,960	1,385	85,910
Control 2	15,428	1,018	85,910
IL-10 1	14,864	1,475	85,778
IL-10 1	15,064	1,293	85,910
IL-10 1	14,944	1,400	85,910
IL-10 2	14,689	1,655	85,910
IL-10 2	14,785	1,553	85,910
IL-10 2	14,752	1,588	85,910
P1 1	14,542	1,823	85,778
P1 1	14,815	1,523	85,910
P1 1	14,806	1,532	85,910
P1 2	14,341	2,081	85,910
P1 2	14,696	1,648	85,910
P1 2	14,561	1,800	85,910
P2 1	14,673	1,672	85,910
P2 1	15,083	1,277	86,042
P2 1	14,836	1,502	85,910
P2 2	14,476	1,903	85,910
P2 2	14,754	1,586	86,042
P2 2	14,859	1,480	85,910
P3 1	14,519	1,851	85,910
P3 1	15,025	1,327	86,042
P3 1	14,846	1,492	86,042
P3 2	14,575	1,784	86,042
P3 2	14,885	1,454	86,042
P3 2	14,901	1,440	86,042
LPS 30 1	14,372	2,038	86,042
LPS 30 1	14,656	1,691	86,042
LPS 30 1	14,555	1,807	86,042
LPS30 2	14,857	1,482	86,042
LPS30 2	15,241	1,151	86,042
LPS30 2	14,959	1,386	86,042
LPS30 + IL-10 1	14,846	1,492	86,042
LPS30 + IL-10 1	15,411	1,029	86,174
LPS30 + IL-10 1	15,342	1,077	86,042
LPS30 + IL-10 2	15,126	1,242	86,042
LPS30 + IL-10 2	15,235	1,156	86,174
LPS30 + IL-10 2	15,534	0,949	86,042
LPS30 + P1 1	14,346	2,073	86,042
LPS30 + P1 1	14,948	1,395	86,174
LPS30 + P1 1	14,644	1,705	86,042
LPS30 + P1 2	14,827	1,511	86,042
LPS30 + P1 2	15,056	1,300	86,042
LPS30 + P1 2	14,960	1,385	86,042

LPS30 + P2 1	14,680	1,665	85,910
LPS30 + P2 1	14,818	1,520	86,042
LPS30 + P2 1	14,738	1,602	86,042
LPS30 + P2 2	14,626	1,724	85,910
LPS30 + P2 2	14,970	1,376	86,042
LPS30 + P2 2	14,925	1,417	86,042
LPS30 + P3 1	14,962	1,383	85,910
LPS30 + P3 1	15,277	1,124	86,042
LPS30 + P3 1	15,138	1,232	86,042
LPS30 + P3 2	14,802	1,536	85,910
LPS30 + P3 2	14,705	1,637	85,910
LPS30 + P3 2	15,156	1,217	86,042
NC	Undetermined		61,238
NC	Undetermined		85,910
NC	Undetermined		85,778
PC	15,621	0,896	85,778
PC	15,800	0,797	85,910
PC	16,028	0,686	85,910

Table 31. Beta-actin average quantity and corresponding St Dev experiment 2.3 (**fig. 16**)

Sample	Av Quantity	St Dev
Control 1	1,125	0,08959991
Control 2	1,334	0,07213433
IL-10 1	1,389	0,091333
IL-10 2	1,570	0,02428451
P1 1	1,528	0,00655097
P1 2	1,724	0,10792704
P2 1	1,484	0,19804508
P2 2	1,533	0,07482844
P3 1	1,410	0,11694065
P3 2	1,447	0,01035986
LPS30 1	1,749	0,08226492
LPS30 2	1,434	0,06830765
LPS30 + IL-10 1	1,053	0,03399152
LPS30 + IL-10 2	1,199	0,06075949
LPS30 + P1 1	1,724	0,33927007
LPS30 + P1 2	1,399	0,10627138
LPS30 + P2 1	1,596	0,07238338
LPS30 + P2 2	1,396	0,02888585
LPS30 + P3 1	1,246	0,13023292
LPS30 + P3 2	1,587	0,07157749

Figure 34. Standard curve, amplification curve and melting curve TNF- α experiment 2.3 (**fig. 16**)

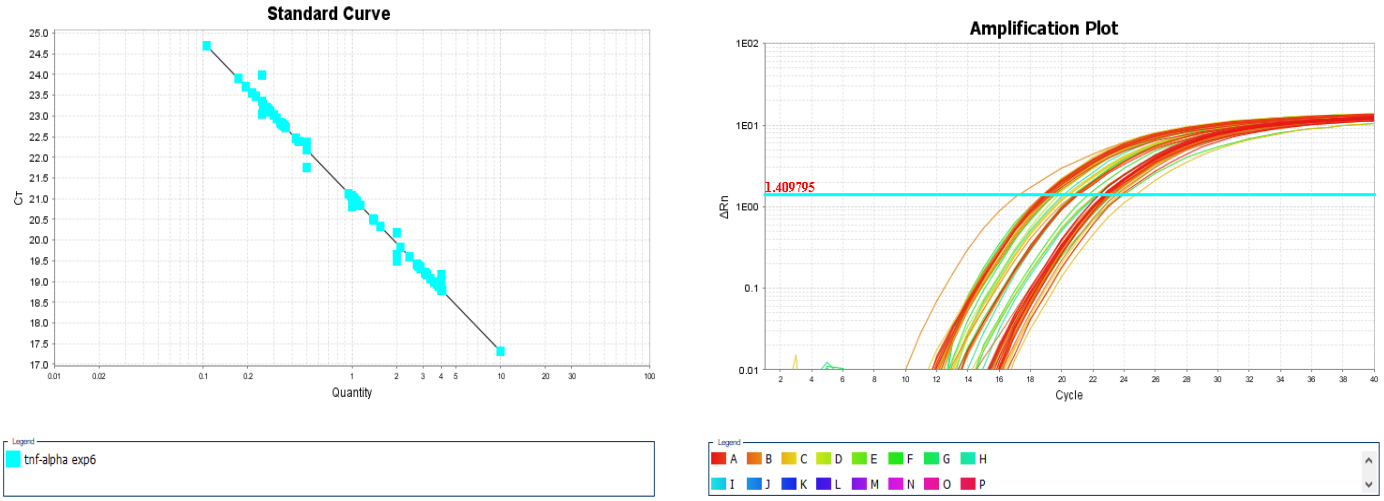


Table 32. TNF- α Ct, Quantity and Melting Temp. experiment 2.3 (**fig. 16**)

Sample Name	CT	Quantity	Tm1
STD 4	18,877	4,000	87,165
STD 4	19,155	4,000	87,297
STD 4	18,988	4,000	87,297
STD 2	19,650	2,000	87,033
STD 2	19,488	2,000	87,297
STD 2	20,173	2,000	87,033
STD 1	20,819	1,000	87,033
STD 1	20,873	1,000	87,165
STD 1	21,077	1,000	87,165

STD 0.5	21,748	0,500	87,033
STD 0.5	22,283	0,500	87,165
STD 0.5	22,355	0,500	87,165
STD 0.25	23,976	0,250	86,902
STD 0.25	23,045	0,250	87,033
STD 0.25	23,357	0,250	87,033
Control 1	22,455	0,423	86,770
Control 1	22,800	0,342	86,902
Control 1	23,719	0,194	87,033
Control 2	22,388	0,441	86,902
Control 2	22,715	0,360	86,902
Control 2	22,801	0,342	86,902
IL-10 1	22,950	0,312	86,770
IL-10 1	23,562	0,214	86,902
IL-10 1	23,101	0,284	87,033
IL-10 2	22,832	0,336	86,902
IL-10 2	23,285	0,254	86,902
IL-10 2	22,857	0,330	87,033
P1 1	22,817	0,339	86,902
P1 1	23,146	0,277	87,033
P1 1	22,808	0,340	87,033
P1 2	22,935	0,315	86,902
P1 2	22,723	0,359	87,033
P1 2	24,704	0,106	87,033
P2 1	22,742	0,355	86,902
P2 1	23,188	0,269	87,033
P2 1	23,010	0,301	87,033
P2 2	23,911	0,173	87,033
P2 2	22,846	0,333	87,033
P2 2	22,723	0,359	87,033
P3 1	22,942	0,314	87,033
P3 1	22,723	0,359	87,033
P3 1	22,787	0,345	86,902
P3 2	22,760	0,351	87,033
P3 2	23,177	0,271	87,165
P3 2	23,121	0,281	87,033
LPS30 1	18,793	4,029	87,033
LPS30 1	20,333	1,562	87,165
LPS30 1	20,489	1,419	87,033
LPS30 2	19,172	3,192	87,033
LPS30 2	19,422	2,737	87,165
LPS30 2	19,830	2,129	87,165
LPS30 + IL-10 1	20,937	1,077	87,165
LPS30 + IL-10 1	21,009	1,030	87,165
LPS30 + IL-10 1	20,970	1,055	87,033
LPS30 + IL-10 2	21,132	0,955	87,033
LPS30 + IL-10 2	21,079	0,987	87,165

LPS30 + IL-10 2	22,187	0,499	87,165
LPS30 + P1 1	19,388	2,794	87,033
LPS30 + P1 1	18,993	3,563	87,165
LPS30 + P1 1	18,786	4,047	87,033
LPS30 + P1 2	19,189	3,158	87,033
LPS30 + P1 2	18,926	3,712	87,165
LPS30 + P1 2	20,524	1,389	87,033
LPS30 + P2 1	19,071	3,395	87,033
LPS30 + P2 1	18,994	3,561	87,033
LPS30 + P2 1	19,205	3,126	87,033
LPS30 + P2 2	18,883	3,814	86,902
LPS30 + P2 2	19,329	2,897	87,033
LPS30 + P2 2	19,207	3,123	87,033
LPS30 + P3 1	19,077	3,384	86,902
LPS30 + P3 1	19,602	2,449	87,033
LPS30 + P3 1	19,377	2,813	86,902
LPS30 + P3 2	18,977	3,597	86,902
LPS30 + P3 2	20,851	1,135	87,033
LPS30 + P3 2	19,341	2,877	86,902
NC	Undetermined		86,770
NC	Undetermined		86,244
NC	Undetermined		61,365
PC	22,719	0,360	86,902
PC	17,320	9,975	85,585
PC	23,472	0,226	86,902

Table 33. TNF-α average quantity and corresponding St Dev experiment 2.3 (**fig. 16**)

Sample	Average Quantity	St dev
Control 1	0,383	0,05728273
Control 2	0,351	0,01314004
IL-10 1	0,270	0,05052383
IL-10 2	0,333	0,00358935
P1 1	0,340	0,00139413
P1 2	0,337	0,03110718
P2 1	0,285	0,02202877
P2 2	0,346	0,01845771
P3 1	0,352	0,00981558
P3 2	0,276	0,00679902
LPS30 1	2,337	1,46717585
LPS30 2	2,686	0,53342469
LPS30 + IL-10 1	1,054	0,02334593
LPS30 + IL-10 2	0,971	0,02222745
LPS30 + P1 1	3,468	0,63165717
LPS30 + P1 2	3,435	0,3923644

LPS30 + P2 1	3,361	0,21956119
LPS30 + P2 2	3,010	0,16001405
LPS30 + P3 1	2,882	0,47142057
LPS30 + P3 2	3,237	0,50944649

Table 29. Relative TNF- α expression and average relative TNF- α expression experiment 2.3 (**fig. 16**)

Sample	Relative expression	Average Relative Expression	St dev average relative expression
Control 1	0,340017313	0,301650227	0,05425925
Control 2	0,26328314		
IL-10 1	0,194405341	0,203217653	0,01246249
IL-10 2	0,212029964		
P1 1	0,222219181	0,208798598	0,01897957
P1 2	0,195378014		
P2 1	0,192110757	0,208794642	0,02359458
P2 2	0,225478528		
P3 1	0,249566966	0,220186409	0,04155038
P3 2	0,190805852		
LPS30 1	1,33583536	1,604468647	0,37990484
LPS30 2	1,873101934		
LPS30 + IL-10 1	1,000879139	0,905448791	0,13495889
LPS30 + IL-10 2	0,810018444		
LPS30 + P1 1	2,010992258	2,233343017	0,31445146
LPS30 + P1 2	2,455693776		
LPS30 + P2 1	2,106103211	2,13107916	0,03532133
LPS30 + P2 2	2,15605511		
LPS30 + P3 1	2,312582262	2,176454017	0,19251441
LPS30 + P3 2	2,040325772		

Appendix G: Raw Data NO Assay Experiment 3.1

*Table 30. Absorbance and Concentration NO Assay sample 1 experiment 3.1 (**fig. 17**)*

Absorbance	Treatment									
	Control	IL-10	P1	P2	P3	LPS	IL-10 + LPS	P1 + LPS	P2 + LPS	P3 + LPS
1	0,054	0,051	0,059	0,054	0,055	0,263	0,258	0,229	0,277	0,195
2	0,05	0,05	0,052	0,056	0,054	0,337	0,33	0,33	0,321	0,277
3	0,051	0,06	0,055	0,056	0,056	0,29	0,321	0,327	0,305	0,249
Average	0,0517	0,05367	0,0553	0,0553	0,055	0,2967	0,303	0,2953	0,301	0,2403
St Dev	0,00208	0,00551	0,00351	0,00115	0,001	0,0374	0,0392	0,0575	0,0223	0,0417

	Treatment									
	Control	IL-10	P1	P2	P3	LPS	IL-10 + LPS	P1 + LPS	P2 + LPS	P3 + LPS
[NO] (uM)	0	0,322	0,609	0,609	0,552	42,22	43,31	41,99	42,97	32,51
St Dev	0	0,033	0,039	0,013	0,010	5,33	5,61	8,17	3,18	5,64
[NO] (uM) ± Sd	0 ± 0	0,322 ± 0,033	0,60 ± 0,039	0,60 ± 0,013	0,55 ± 0,010	42,22 ± 5,33	43,31 ± 5,61	41,99 ± 8,17	42,97± 3,18	32,51 ± 5,64

*Fig. 35. Calibration curve NO Assay sample 1 experiment 3.1 (**fig. 17**)*

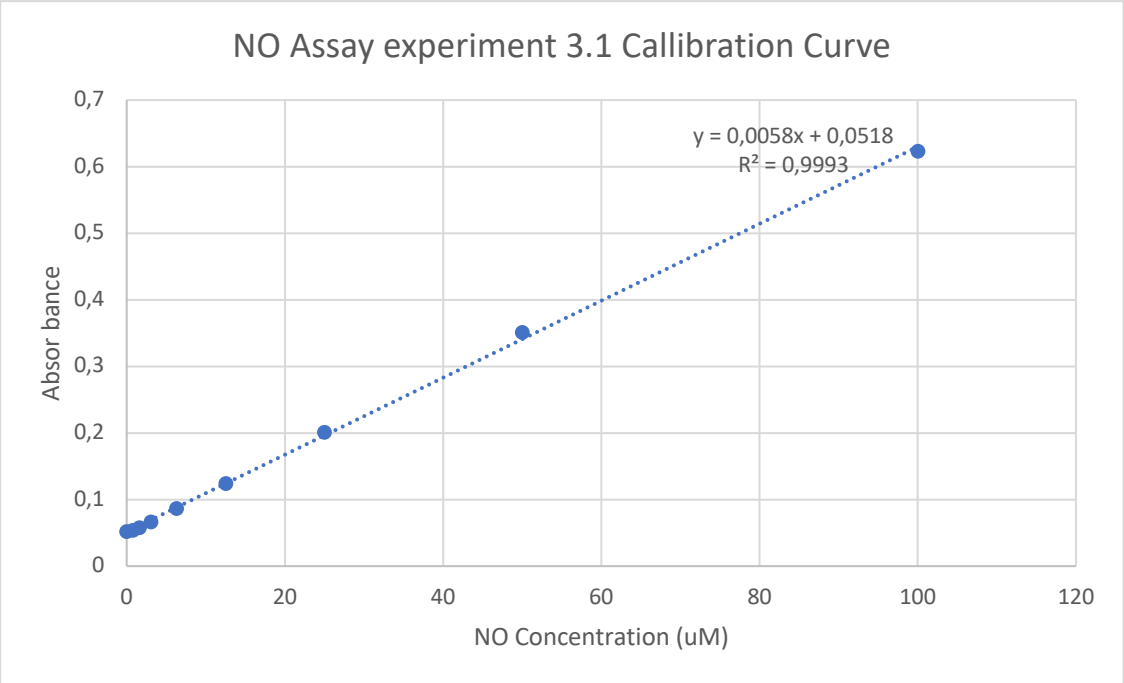
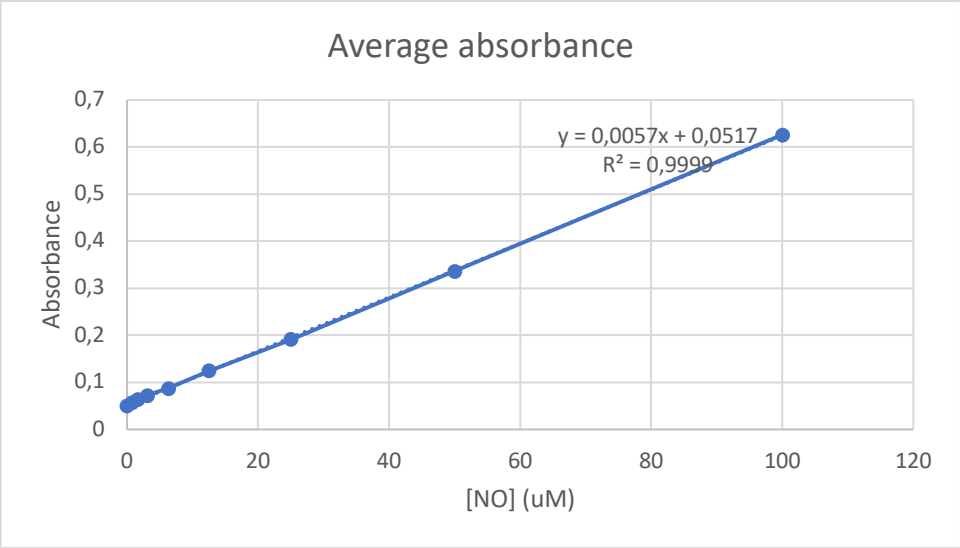


*Table 31. Absorbance and Concentration NO Assay sample 2 experiment 3.1 (**fig. 17**)*

Absorbance	Treatment									
	Control	IL-10	P1	P2	P3	LPS	IL-10 + LPS	P1 + LPS	P2 + LPS	P3 + LPS
1	0,053	0,052	0,051	0,05	0,05	0,317	0,307	0,294	0,28	0,229
2	0,05	0,051	0,051	0,051	0,052	0,341	0,297	0,323	0,311	0,276
3	0,052	0,048	0,051	0,051	0,05	0,301	0,305	0,316	0,275	0,252
Average	0,0517	0,0503	0,051	0,0507	0,0507	0,320	0,303	0,311	0,289	0,252
St Dev	0,00153	0,002082	0	0,000577	0,001155	0,020133	0,00529	0,01513	0,0195	0,0235

	Treatment									
	Control	IL-10	P1	P2	P3	LPS	IL-10 + LPS	P1 + LPS	P2 + LPS	P3 + LPS
[NO] (uM)	0	0	0	0	0	47,01	44,09	45,49	41,57	35,20
St Dev	0	0	0	0	0	2,96	0,77	2,21	2,81	3,28
[NO] (uM) ± Sd	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	47,01 ± 2,96	44,09 ± 0,77	45,49 ± 2,21	41,57 ± 2,81	35,20± 3,28

*Fig. 36. Calibration curve NO Assay sample 2 experiment 3.1 (**fig. 17**)*



Appendix H: NO Assay Protocol

Protocol NO assay

1. Seed RAW 264.7 cells in a 96-well flat bottom culture plate
   (100.000 cells in 200 µl/well) in DMEM supplemented with 10% FBS.
2. After 24h, remove supernatant. Add fresh medium containing the following treatment.
   a. Control
   b. LPS 25 ng/ml
   e. LPS 100 ng/ml
3. After 24h incubation, collect 100 ul of the supernatant to measure NO_2^-
   (one of the end products of NO synthesis)

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)
   (Store stock-solution in vials at -20°C)
2. Dilute stock-solution 100x in culture medium (= 1 mM solution).
   Pipet 100 ul 100 mM NaNO2 in 10 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

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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 bij 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 in100ml 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

Appendix I: MTT Assay Protocol

MTT Assay Protocol

1. Prepare the Tetrazolium (Sigma M5655; VMT1 4°C) solution 0,5 mg/mL in medium.
2. Soak the supernatant from well plate carefully with a pipet.
3. Add 200 μL MTT-solution (0,5 mg/mL in medium) and incubate for 1 hour. Check under the microscope whether cells are stained.
4. Remove MTT solution from the wells with a pipet.
5. Add 100 μL DMSO to each well and place on a shaker. Check under the microscope whether all the crystals/purple precipitate has dissolved.
6. Measure the absorbance at 550 nm.

Appendix J: RNA Isolation Protocol (Maxwell)

Maxwell® 16 LEV simplyRNA Cells Kit

INSTRUCTIONS FOR USE OF PRODUCTS AS1270 AND AS1280.

RNA isolation of cultured cells 12/ 6 well plate (area= 4 cm2):

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

	Cells/well	Cells/cm2	RNA yield (ng/ul)
3T3 12 well (4 cm2)	100.000	2.5 * 10^4	83 - 106
3T3 6 well (10 cm2)	250.000	2.5 * 10^4	113 - 179
RAW 24 well (2 cm2)	200.000	1* 10^5	74 – 95
RAW 12 well (4 cm2)	400.000	1* 10^5	166 - 180
AML-12 6 well (10 cm2)		1*10^5	

March 2022

RNA isolation Maxwell 16 LEV Simply RNA Cells.doc
M. Duin march 2022
x/2022/ protocols/RNA isolation cell culture with Maxwell 16 LEV simple RNA Cells Kit

Appendix K: cDNA conversion protocol

RNA conversion to cDNA

Use this protocol after RNA isolation with the Maxwell and on-column DNA digestion.

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

Make the RT mix according to the following quantities. The quantities given are for a single sample. Make a Master Mix (MM) by multiplying the quantities by x number of samples + 6 samples calibration curve.

RT mix:	uL per sample
RT buffer	2,0 ul
dNTP(=A,G,C,T)mix (10 mM)	0,1 ul
Rnasin	0,25 ul
Rev Transcriptase	0,5 ul
Random Hexamers	0,5 ul
RNA (0,5 ug in water)	5,0 ul
H_20	1,65 ul
	-------- +
Total volume	10 ul

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 PCR machine
Start the file ***MLVCDNA***

After the reaction is completed:

Spin the tubes (condensed water from the lids)
Store the samples at -20.

Appendix L: qPCR Protocol

Creating Standard Curve

1. Pool the undiluted cDNA of the samples that were assigned for the STD CURVE in the cDNA conversion.
2. Create the Standard Curve according to the tabel below (amounts given for the case of 10 standard curve samples):

STD (rel)	V (ul)	H2O (ul)
STD 4	100 ul of pooled cDNA	150 ul
STD 2	100 ul of STD 4	100
STD 1	100 ul of STD 2	100
STD 0.5	100 ul of STD 1	100
STD 0.25	100 ul of STD 0.5	100

Prepare the cDNA

1. Dilute the cDNA samples after the conversion 10 times:
   Add 90 ul RNase free H2O to the cDNA samples.

Prepare 10uM Primermx F+R

20 ul of 50 uM primer For
20 ul of 50 uM primer Rev
60 ul H2O

Design the 384 plate layout of the samples digital

Prepare the Taq MasterMix

	nr of samples (duplo!)	10	
Mix	**1***	**N**	
Sybr Green Mix	5	50	
primermix F+R (10µM)	0,3	3	
water	2,7	27	
Totaal	8	80	**→ 8 ul/well**
cDNA 10* verdund	2		**→ 2 ul/well**

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 the PCR

PCR protocol:

Stage 1:	10 min	95	activatie Taq
Stage 2:	15 sec	95	Amplificatie
	30 sec	60	
	40 cycli		
Stage 3:	15 sec	95	Melt curve
	1 min	60	
	Gradient van 0.05/sec naar 95		