INTERLEUKIN-10: EFFECTS ON 264.7 MURINE MACROPHAGES AND POTENTIAL AS NOVEL LIVER FIBROSIS THERAPEUTIC

Bachelor research project 2024

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Abstract

Liver fibrosis, the excessive production and accumulation of scar tissue in the liver, is a potentially fatal disease with no current pharmacotherapeutic cure, leaving liver transplantation as the only option. This study aimed to evaluate the potential of interleukin-10 (IL-10), a strong anti-inflammatory cytokine, in treating liver fibrosis by examining its effects on macrophages. Although IL-10 was found to have no significant effect on the expression of the IL-10 receptor α or NO production of the RAW cells, it was found to down-regulate the gene expression of TNF-α, a prominent inflammatory cytokine.

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Introduction

Liver fibrosis

Liver diseases, while not as infamous as cardiovascular or respiratory diseases, still account for two million deaths annually, and is the 11th leading cause of death worldwide [1]. Liver fibrosis, itself technically a liver disease, is also a consequence of damage caused by other chronic liver diseases. Liver fibrosis can be defined as the production and accumulation of excessive scar tissue in the liver [2-5]. Scar tissue formation, also known as wound healing, is an important part of our body's response to injury. When the wound healing becomes sustained over a longer period of time however, it may become irreversible, compromising normal liver function [3].

Although liver diseases which cause liver fibrosis may originate from several different origin, like hepatitis B and C viral infections, alcoholic and non-alcoholic steatohepatitis or cholestatic injury, the cellular processes behind fibrosis are generally similar for all of them. Hepatocyte death, chronic inflammation and disruption of the endothelium or epithelium of the liver all lead to activation of hepatic stellate cells (HSCs), the most prominent cell type in liver fibrosis. "Quiescent" HSCs are located in the space of Disse, where they store vitamin A. Upon activation however, they differentiate into myofibroblasts, migrate towards the site of injury and begin secreting extracellular matrix (ECM) proteins, like collagen types 1 and 3 and α-smooth muscle actin [3,4].

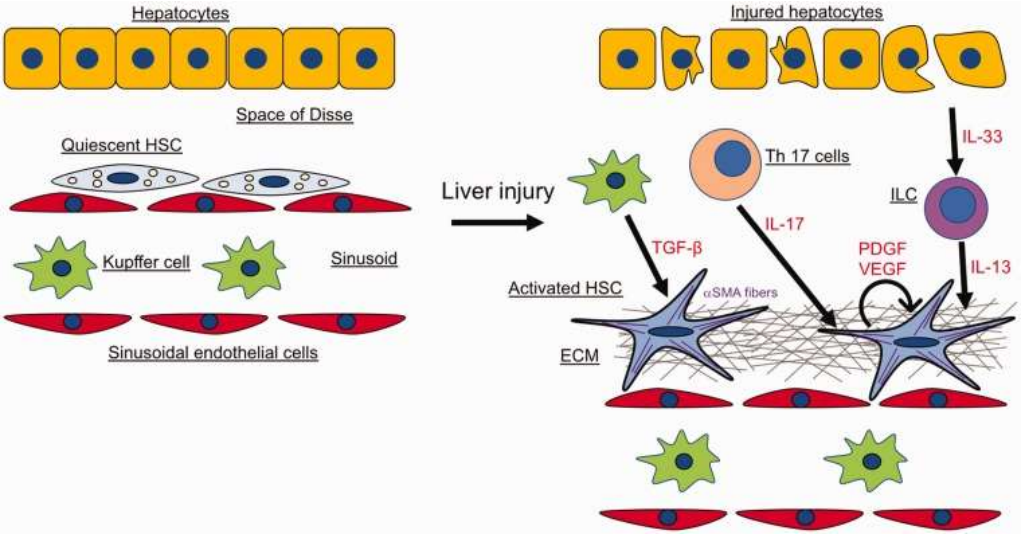


Figure 1: General schematic of the pathophysiology of liver fibrosis. Quiescent HSCs in the space of Disse are activated by injured hepatocytes or inflammation, leading to them producing ECM proteins [4].

Although the mechanism of liver fibrosis is fairly well understood, and many experimental therapeutics are in clinical trials, there are currently no approved anti-fibrotic drugs. The only current way to treat a patient with cirrhosis (the end stage of fibrosis) is with a liver transplantation [5,6]. A proposed strategy for targeting liver fibrosis is inhibiting the inflammatory reaction preceding the ECM producing process. For this reason, attention has fallen to interleukin-10 (IL-10), a potent anti-inflammatory cytokine, as a possible liver fibrosis therapeutic, and the topic of this research.

Interleukin-10

Interleukin-10 (IL-10) is a potent anti-inflammatory and immunosuppressive cytokine produced by almost all types of activated immune cells. Its effects are versatile, acting by inhibiting antigen presentation of dendritic cells and macrophages, inhibition of macrophage and HSC activation, and inhibition of pro-inflammatory cytokine release [7-9]. At a cellular level, IL-10 binds to the IL-10 receptor complex, which is comprised of two IL-10 receptor α (IL-10Rα) subunits responsible for ligand recognition and binding, and two IL-10 receptor β (IL-10Rβ) subunits, responsible for signal transduction. Upon binding of IL-10 to the IL-10Rα subunits, leads to oligomerization of the IL-10Rα with the IL-10Rβ subunits, causing phosphorylation and subsequent activation of the JAK/STAT3 signalling pathway (figure 2) [10,11].

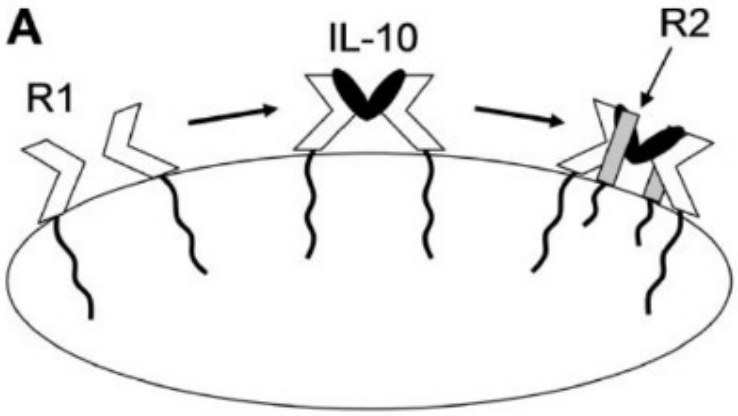


Figure 2: General schematic of the IL-10Rα – IL-10Rβ oligomerization [11].

As a potent anti-inflammatory cytokine, IL-10 has potential as an anti-fibrotic, as it can alleviate the chronic inflammation preceding the excessive inflammation, and it has seen success as such, demonstrating a cardioprotective effect in post-myocardial infarct cardiac remodelling [12]. However, IL-10 treatment is still in a juvenile state. Specific targeting of IL-10 to HSCs has shown a contradictory pro-fibrotic effect [13]. Additionally, administration of an immunosuppressive agent like IL-10 would be impossible for patients affected by hepatitis B and C. So although IL-10 has shown promise, its application is still plagued by several challenges. A possible solution for said challenges are IL-10 derived peptides. IL-10 derived peptides, as designed by Prof. Dr. K. Poelstra and named P1-P4, aim to retain the anti-fibrotic effects of IL-10 while leaving the immune system unaffected. A goal of this study, is examining the optimal analytical method for examining the biological activity of P1-P4 suitable for comparison with IL-10.

Aim

This study aims to evaluate the potential of interleukin-10, a potent anti-inflammatory cytokine, in treating liver fibrosis by examining its effects on macrophages. The NO production, IL-10 Receptorα expression and pro-/anti-inflammatory gene expression after IL-10 treatment of 264.7 RAW cells was examined.

Methods

Cell culturing

RAW 264.7 murine macrophages were cultured in T25 cell culture flasks in Dulbecco's Modified Eagle Medium (DMEM) (Gibco™, Cat: 31966-021) + 10% Foetal Bovine Serum (FBS) (Serana, Ref: S-FBS-SA-015) containing 11 µg/ml gentamicin (Gibco™, Cat: 15750-037). They were incubated at 37 °C with 5% CO_2. Cell culturing was continued by using cell scrapers to release the cells from the bottom of the culture flasks, transferral into a new T25 flask and subculturing in ratios of 1:2 for two days, 1:4 for three days and 1:6 for four days in fresh growth medium.

Polarization and stimulation

Experiments 24-ST-01

In experiment 1, 1 mL of RAW cells was seeded per well in a 12-well plate at $1,5*10^4$ cells/cm^2, and was left to recover for 24 hours after seeding. Native RAW cells were incubated with 40 ng/ml IFNγ (recombinant murine IFNγ, PeproTech, Cat: 315-05-100UG) for 2 hours to polarize the native RAW cells into an M1 phenotype. To polarize the native cells into an M2 phenotype, the cells were incubated with 20 ng/ml IL-4 (recombinant murine IL-4, PeproTech, Cat: 214-14-20UG) + 20 ng/ml IL-13 (recombinant murine IL-13, PeproTech, Cat: 210-13-10UG) for 2 hours. Afterwards, the medium containing the cytokines was removed and the cells were incubated with fresh medium or fresh medium + 10 ng/ml LPS (Sigma, E.Coli O55:B5, Cat: L2880-100MG) for 22 hours.

Experiment 02 and 03

In experiments 2 and 3, 1 mL of RAW cells was seeded per well in a 12-well plate at cell densities of $4*10^4$ and $2*10^4$ cells/cm^2 respectively, and they were left to recover for 24 hours. After 2 hours of polarization as performed in experiment 1, the medium was either removed, and the cells subsequently incubated with fresh medium ("Vm") or fresh medium + 100 ng/ml LPS for 22 hours ("LPS"), or 100 ng/ml LPS was added on-to the cytokine containing medium, which then resumed incubation for 22 hours ("Cyto+LPS"). Spiked samples were prepared which consisted of native RAW cells exposed to 100 ng/ml LPS for 2 hours.

Experiment 24-ST-04

In experiment 4, 2 mL of RAW cells was seeded per well in a 6-well plate at $4*10^4$ cells/cm^2, and cells were left to rest for 24 hours. Native RAW cells were incubated with 40 ng/ml IFNγ at T=24h (M1 -LPS), 20 ng/ml IL-4 + 20 ng/ml IL-13 at T=24h (M2), or 40 ng/ml IFNγ at T=24h + 100 ng/ml LPS added 2 hours later (M1 +LPS). Samples were collected at T=48 (figure 3).

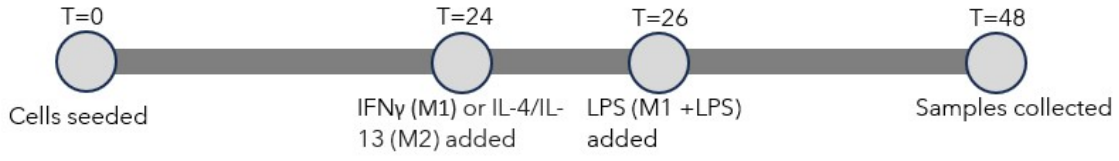


Figure 3: Timeline protocol of experiment 24-ST-04.

Experiment 24-ST-05

In experiment 5, 2mL of RAW cells was seeded per well in two 6-well plates at $1*10^4$ cells/cm^2 and left to rest for 24 hours. A 6-well plate of M1 cells and a 6-well plate of M2 cells were tested under 6 conditions each, these being (figure 4):

- (1) Without LPS or IL-10 (recombinant murine IL-10, Cat: 210-10-10UG).
- (2) 100 ng/ml LPS at T=26h without IL-10.
- (3) 30 ng/ml IL-10 at T=24h without LPS.
- (4) 30 ng/ml IL-10 at T=24h + 100 ng/ml LPS at T=26h.
- (5) 30 ng/ml IL-10 at T=26h without LPS.
- (6) 30 ng/ml IL-10 at T=26h + 100 ng/ml LPS at T=26h.

Cytokines were not removed during the incubation period. Samples were collected at T=48h. When the samples were run in the cytoflex, the different treatment conditions were all assigned a numerical value, detailed in table 1.

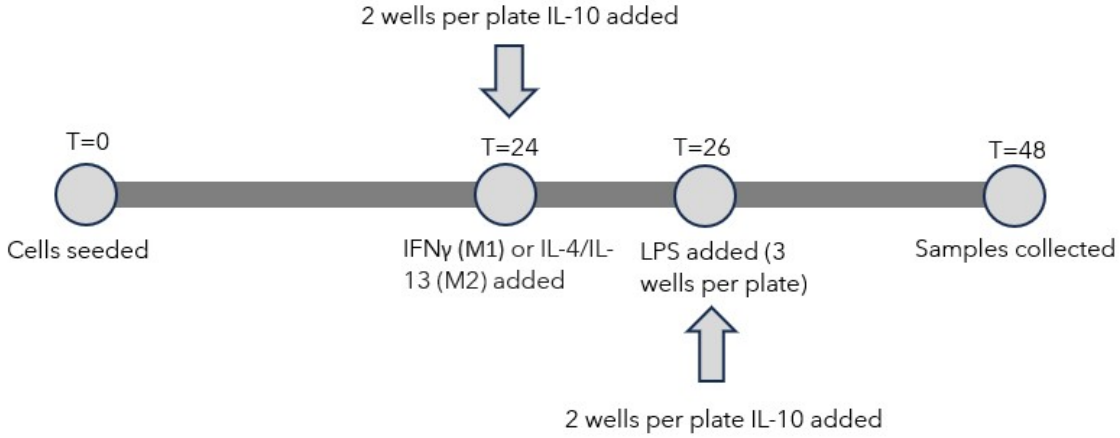


Figure 4: Timeline protocol of experiment 24-ST-05.

Table 1: Numerical codes of the treatments applied in experiment 24-ST-05.

Numerical code	Treatment
1	M1 -LPS -IL-10
2	M1 +LPS -IL-10
3	M1 -LPS +IL-10 at T=0
4	M1 +LPS +IL-10 at T=0
5	M1 -LPS +IL-10 at T=2
6	M1 +LPS +IL-10 at T=2
7	M2 -LPS -IL-10
8	M2 +LPS -IL-10
9	M2 -LPS +IL-10 at T=0
10	M2 +LPS +IL-10 at T=0
11	M2 -LPS +IL-10 at T=2
12	M2 +LPS +IL-10 at T=2
13	M2 -LPS +IL-10 at T=0 cells in medium
14	M2 -LPS +IL-10 at T=2 cells in medium

Experiment 24-ST-07

In experiment 7, 1 mL of RAW cells was seeded per well in a 12-well plate at $1*10^5$ cells/cm^2 and left to recover for 24 hours. Native RAW cells were then incubated at 6 different conditions (figure 5):

- (1) 10 ng/ml LPS at T=-24h.
- (2) 10 ng/ml LPS at T=-24h + 30 ng/ml IL-10 at T=-18h + 10 ng/ml IFNγ at T=0h.
- (3) 10 ng/ml LPS at T=-24h + 30 ng/ml IL-10 at T=0h + 10 ng/ml IFNγ at T=0h.
- (4) 10 ng/ml LPS at T=-24h + 10 ng/ml IFNγ at T=0h + 30 ng/ml IL-10 at T=6h.
- (5) 10 ng/ml LPS at T=-24h + 30 ng/ml IL-10 at T=-18h.
- (6) 10 ng/ml LPS at T=-24h + 10 ng/ml IFNγ at T=0h.

LPS or cytokines were not removed during the incubation period. Samples were collected at T=22h.

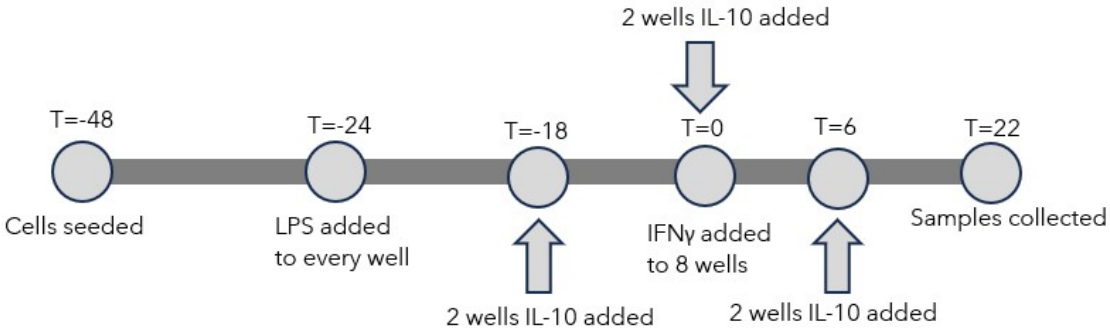


Figure 5: Timeline protocol of experiment 24-ST-07.

Experiment 24-ST-08

In experiment 8, 1 mL of RAW cells was seeded per well in 2 12-well plates, one for qPCR samples and the other for NO samples, at $1*10^5$ cells/cm^2 and left to recover for 24 hours. Native RAW cells were incubated under 5 different conditions (figure 6):

- (1) Native RAW cells.
- (2) 10 ng/ml LPS at T=-23h + 10 ng/ml IFNγ at T=0h.
- (3) 100 ng/ml IL-10 at T=-24h + 10 ng/ml LPS at T=-23h + 10 ng/ml IFNγ at T=0h.
- (4) 100 ng/ml IL-10 at T=-24h + 10 ng/ml LPS at T=-23h + 100 ng/ml IL-10 at T=-18h + 10 ng/ml IFNγ at T=0h.
- (5) 10 ng/ml LPS at T=-23h + 100 ng/ml IL-10 at T=-18h + 10 ng/ml IFNγ at T=0.

LPS or cytokines were not removed during incubation period. NO samples were taken at T=1h and T=22h. qPCR samples were collected at T=1h.

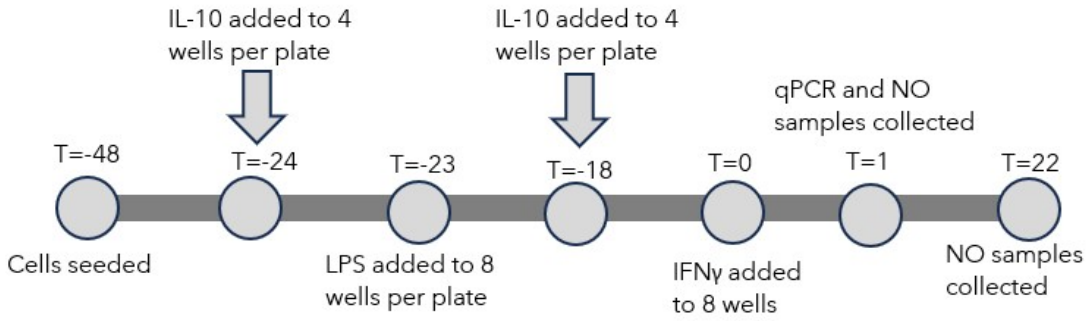


Figure 6: Timeline protocol of experiment 24-ST-08.

NO assay

NO assays were performed according to the Griess method [14] as stated in appendix A8. In short, sample supernatant was mixed with Griess reagent, allowing for absorbances to be measured in a Biotek Synergy H1 multimode reader (Agilent) at an optimum wavelength of 550 nm.

Flow Cytometry

Flow cytometry was performed according to the FACS protocol as stated in appendix A9. In short, 200 µL of polarized and stimulated cells were seeded at $25*10^4$ cells/cm^2 in a round bottom 96-well plate. The cells were stained with 1:50 IL-10Rα primary antibody (Santa Cruz., M20, Cat: sc-985) and 1:200 Goat anti-Rabbit polyclonal (GARPO) secondary antibody labelled with Alexa Fluor 488 (ThermoFisher, Cat: A11034). Samples were run on a Cytoflex S (Beckman Coulter, Serial No.: BA18059). The results were analysed and processed in FlowJo v10.8.1.

Quantitative Polymerase Chain Reaction

qPCR was performed according to the manufacturers protocol as stated in appendix A10-A12. In short, a Maxwell 16 LEV simplyRNA Cells kit (Promega, Cat: AS1340) was used to isolate cell RNA, which was done in a Maxwell 16 MDx (Promega, Ref: AS3000). A Nanodrop One Microvolume UV-Vis Spectrophotometer (Thermoscientific) was used to determine RNA concentration post conversion, and a T100 Thermal Cycler (Bio-Rad, Serial No.: 621BR81387) was used for cDNA conversion. The cDNA samples were run in a Quantstudio 7 Real-Time PCR machine (ThermoFisher), and the Quantstudio v1.3 software was used to analyse the results. A list of primers used can be found in table 2.

Table 2: Table of primers used in qPCR.

Primer	Forward sequence	Reverse sequence	Number
β-actin	ATCGTGCGTGACATCAAAGA	ATGCCACAGGATTCCATACC	35(F) & 36(R)
TNF-α	CATCTTCTCAAAATTCGAGTGACAA	GAGTAGACAAGGTACAACCC	23(F) & 24(R)
TGF-β	AGGGCTACCATGCCAACTTC	GTTGGACAACTGCTCCACCT	33(F) & 34(R)
IL-10Rα	CCAAACCAGTCTGAGAGCACCT	CAGGACAATGCCTGAGCCTTTC	65(F) & 66(R)

Microscopy

The Olympus IX50 Inverted Phase Contrast Microscope was used to examine cell morphology, of which photographs were taken using the cellSens Entry software.

Excel

Microsoft Excel was used for NO assay data analysis, as well as data presentation.

Results

24-ST-01: Effects of polarization of native RAW cells into classically activated (M1) or alternatively activated (M2) macrophages on NO production (n=2)

In order to study the differences between native, M1 and M2 RAW cells, an NO assay was performed (figure 7). Native RAW cells were polarized into an M1 phenotype by incubation with IFNγ for 2 hours, and incubation with IL-4 and IL-13 for 2 hours led to polarization into an M2 phenotype. Additionally, the effect of LPS on NO production was also examined, by incubating half of the native, M1 and M2 RAW cells with LPS.

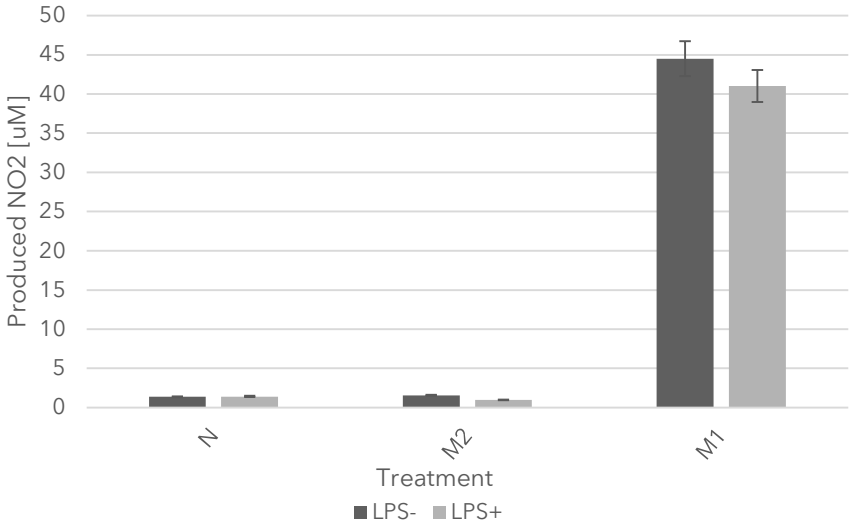


Figure 7: NO assay of native, M1 and M2 RAW cells treated with or without LPS.

As demonstrated in figure 7, while native and M2 cells show no significant NO production (<2 µM), M1 cell medium showed NO concentrations of 41 to 44,5 µM. LPS had little effect on NO production, causing insignificant decreases in NO concentrations in M1 and M2 cells.

As NO is produced by the immune system as toxic agent or immunoregulator [15], it follows logically that the pro-inflammatory M1 cells produce high concentrations of NO, while the anti-inflammatory M2 cells produce low concentrations. The native RAW cells also show little NO production, comparable to the M2 cells. The LPS treatment had little effect, which is surprising, considering the morphological changes the cells undergo when subjected to LPS treatment (demonstrated in experiments 2 and 3). M1 without LPS treatment (2 hour IFNγ) showing the highest NO production is especially curious, as IFNγ is more known to sustain the NO production when administered in combination with LPS, and not show NO concentration increases on its own.

24-ST-02 and -03: Differences between 2 and 24 hour exposure of RAW cells to the polarizing IFNγ or IL-4 + IL-13, and effect on NO production and cell morphology (n=2)

In experiments 2 and 3, two different polarization protocols were tested: one where the native RAW cells were exposed to the polarizing cytokine(s) for 2 hours followed by 22 hours solo LPS exposure, and one where the cytokines were not removed after 2 hours, and the LPS was added to the medium. Spiked samples consisting of native RAW cells exposed to 100 ng/ml LPS for 2 hours were also tested, in order to evaluate whether 2 hours LPS showed a similar NO response as 2 hours IFNγ, as was seen in experiment 1.

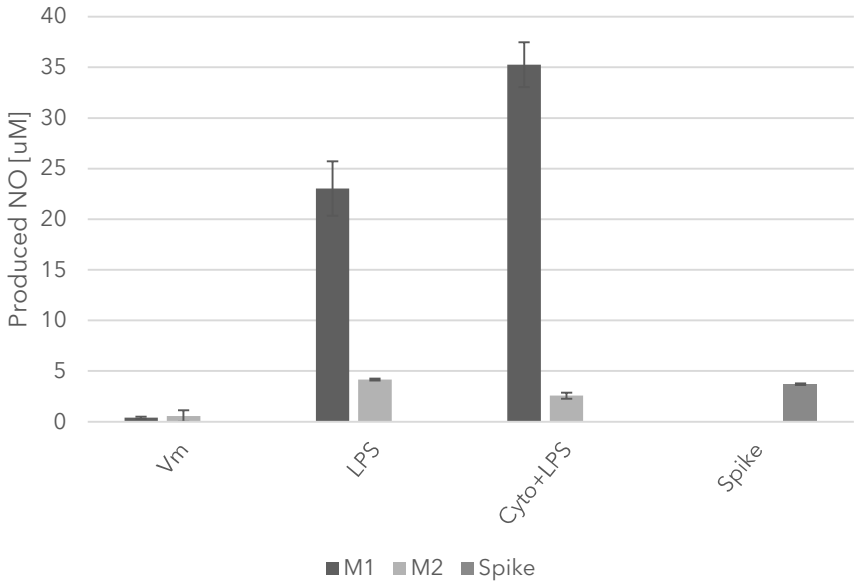


Figure 8: A representative NO assay of native, M1 or M2 cell treated with or without LPS, following 2 or 24 hour exposure to the polarizing cytokines.

As can be seen in figure 8, M1 or M2 cells without LPS stimulation ("Vm") show no significant NO production (<1 μM). This is in contradiction with experiment 1, where M1 cells without LPS stimulation show the strongest NO production out of all conditions (44,5 μM). Similarly, the spike samples, which were made in order to compare 2 hour LPS stimulation with 2 hour IFNγ stimulation as seen in experiment 1, does not show a similarly strong NO production (3,7 μM). The protocol where the polarizing cytokines were removed after 2 hours ("LPS") show a similar pattern as seen in experiment 1, as the M1 cells produce significantly more NO (23 μM) than the M2 cells (4 μM). The protocol where the cytokines were not removed ("Cyto+LPS") shows that same pattern, as M1 cells produce 35 μM NO, and the M2 cells produce 2,5 μM. Comparing the two protocols, the "Cyto+LPS" protocol shows both a small decrease in NO production in M2 cells, and a significant increase in NO production in M1 cells.

The same overall patterns as observed in experiment 1 are observed here. M1 cells with LPS still show strong NO production, especially when the cytokines were not removed after 2 hours, and M2 cells still show low NO production, which becomes even lower when the cytokines were not removed after 2 hours. As the NO production of M2 cells decreases slightly between protocols it can be inferred that the continuous presence of IL-4 and IL-13

has effect (for example by preventing the re-polarization of the M2 cells into a more pro-inflammatory form like the M1 cells), although it is likely that the cytokines have already reached most of their maximum effect after 2 hours of exposure. The continuous presence of IFNγ with the M1 cells shows a significantly stronger effect than 2 hours of exposure, as it has more time to sustain the effects of LPS on the NO production. After observing the unexpectedly high NO production of M1 cells with 2 hour IFNγ treatment in experiment 1, it was thought to be fruitful to examine the effects of 2 hours LPS treatment. Both the spikes as well as the 2 hour IFNγ treatment tested in experiments 2 and 3 show a significantly lower NO production than 2 hours IFNγ in experiment 1, which falls within expectations [https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7693038/]. Considering the anti-inflammatory effects of IL-10 were the central interest of this research, it was decided to follow the Cyto+LPS protocol for the following experiments, as the strongest inducible inflammatory response was induced with the M1 cells following the Cyto+LPS protocol.

The cell morphology between protocols was also examined (figure X and X+1). Vm cells incubated with fresh medium were either healthy, round cells or healthy and elongated. LPS cells (incubated with 2 hours cytokines) were clearly quite stressed, showing asymmetrical shapes, many were elongated and several cells had died during incubation. Cyto+LPS cells (incubated with 24 hours cytokines) were in general healthy, small round cells, much akin to Vm cells, although M2 cells show more stress and elongation.

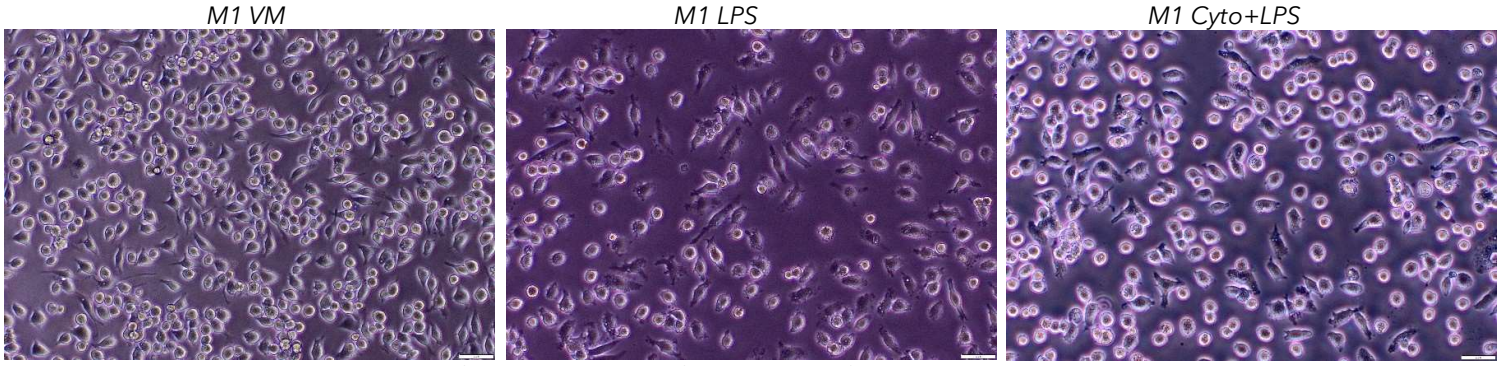


Figure 9: Photographs of cell morphology of M1 cells with fresh medium, LPS with 2 hours cytokine exposure and LPS with 24 hours cytokine exposure.

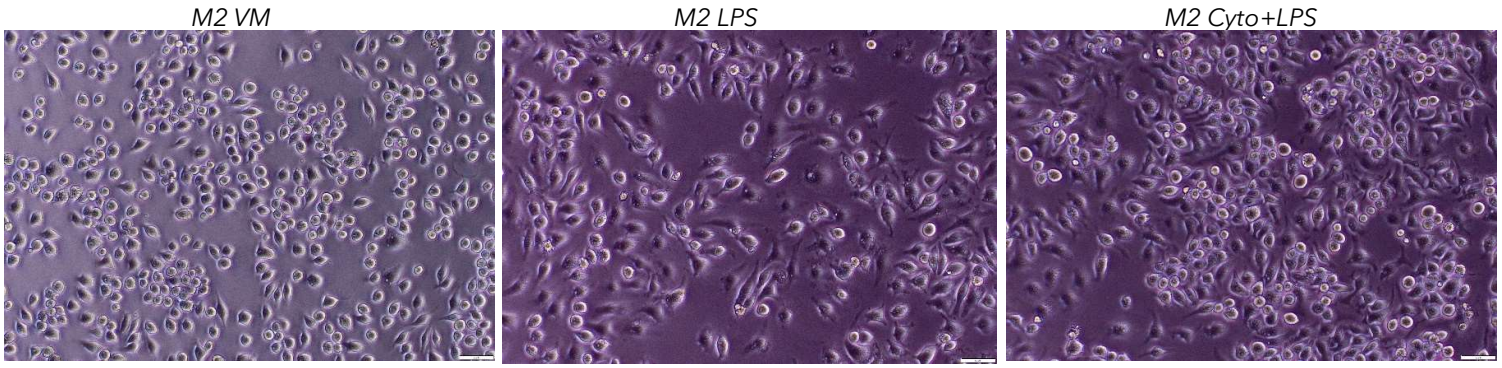


Figure 10: Photographs of cell morphology of M2 cells with fresh medium, LPS with 2 hours cytokine exposure and LPS with 24 hours cytokine exposure.

24-ST-04: Differences in IL-10 receptor α expression between RAW M1 and M2 cells (n=2)

In experiment 4, flow cytometry was performed in order to study the differences in IL-10Rα expression between M1 cells without LPS exposure, M1 cells with LPS exposure and M2 cells (figure 11).

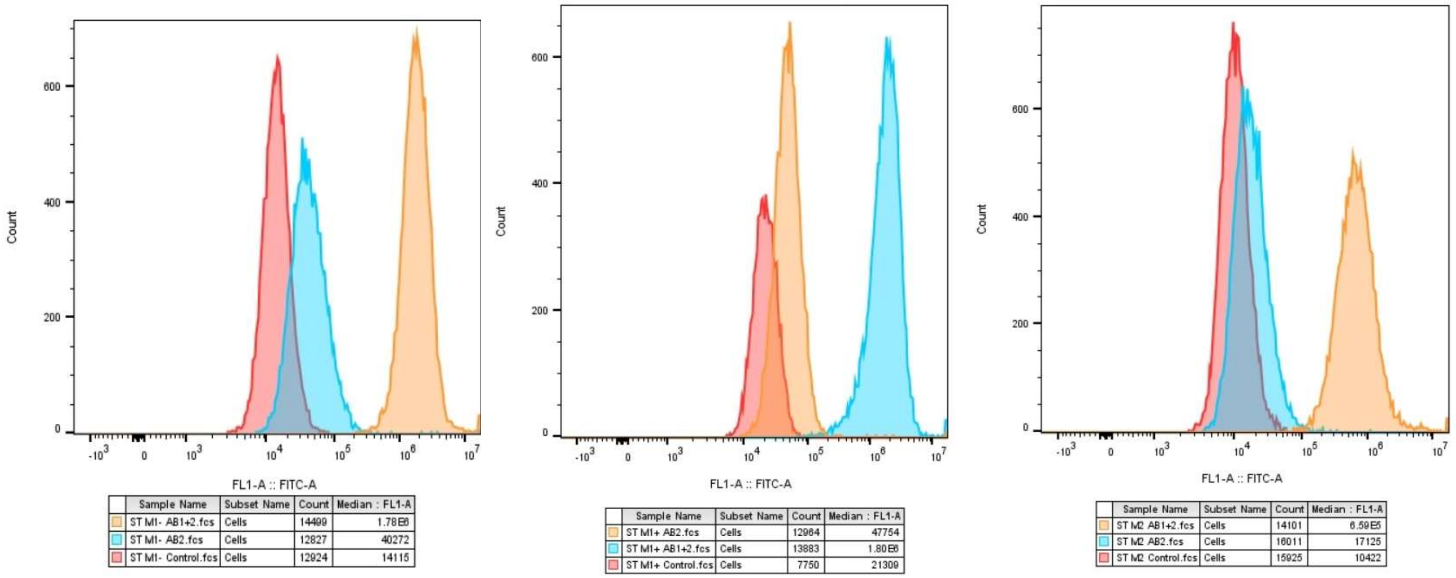


Figure 11: Histograms of IL-10Rα expression in M1 cells without LPS exposure (left), M1 cells with LPS exposure (middle) and M2 cells (right).

All histograms show clear distinction between peaks: the peaks corresponding to the control samples (without antibodies) show the lowest fluorescence, followed by the samples incubated with the fluorescently labelled antibody only, which shows some overlap with the control samples. This indicates that the antibodies "stick" a-selectively to the samples in low numbers. Finally, the samples incubated with both antibodies show by far the highest fluorescence and have little to no overlap with previous samples.

Fluorescence medians of the samples incubated with both antibodies ($1,78*10^6$ for M1 - LPS, $1,80*10^6$ for M1+LPS and $6,59*10^5$ for M2 cells) can be used to compare IL-10Rα expression between treatment conditions, where the higher fluorescence means a higher IL-10Rα expression. From this follows that M1 cells show a higher IL-10Rα expression than M2 cells, and that LPS treatment has little effect on the receptor expression in M1 cells. This indicates that IL-10 may act as a brake on the inflammatory process, mostly targeting M1 cells, possibly acting in the period where the inflammatory response transitions into a wound healing process.

Additionally, an NO assay was performed (figure 12). The medium of M1 cells without LPS treatment and M2 cells show comparable NO concentrations, being 1,27 µM and 1,35 µM respectively. The medium of M1 cells with LPS treatment showed a significantly higher NO concentration, that being 42,5 µM. These results are all in accordance with experiments 2 and 3.

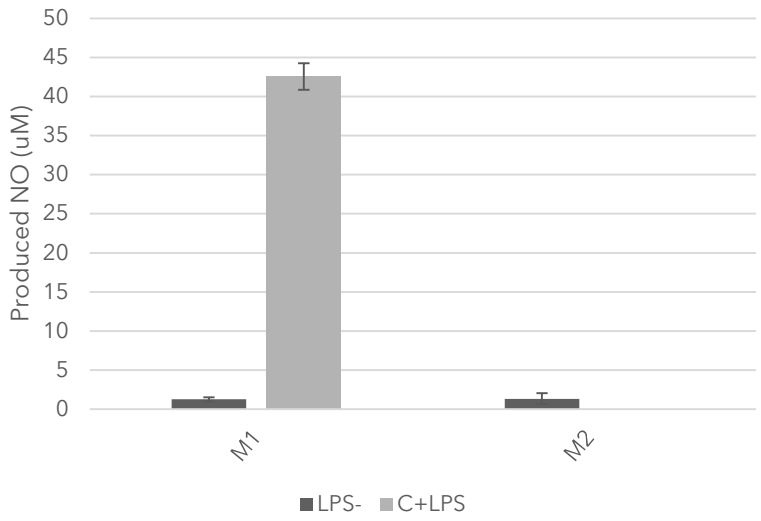
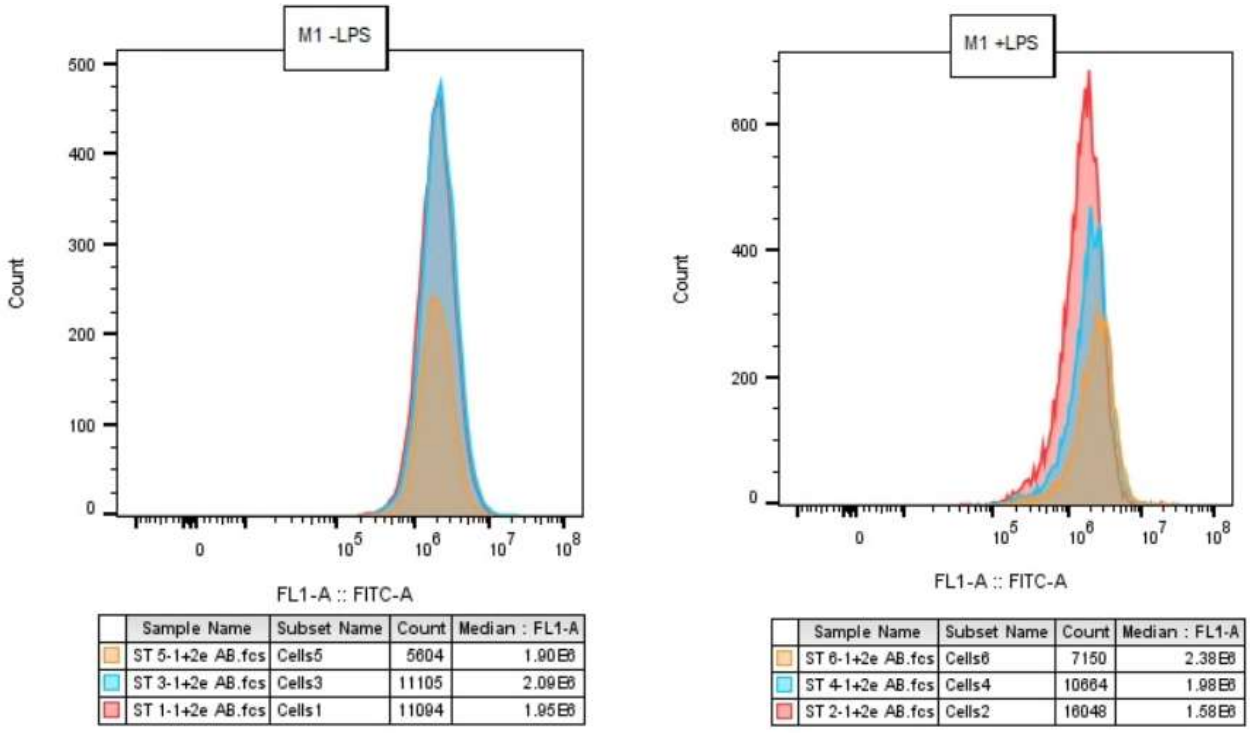


Figure 12: NO assay of the samples from experiment 24-ST-04.

24-ST-05: Effects of IL-10 treatments at different administration times on IL-10Rα expression and NO production (n=2)

After confirming the presence of the IL-10Rα on the M1 and M2 cells, flow cytometry was used to analyse the effects of IL-10 on the receptor expression (figure 13-18). Treatment conditions differed in presence of LPS and IL-10 at different timepoints. Additionally, samples of cells present in culture medium of the conditions M2 -LPS + IL-10 at T=0, and M2 -LPS + IL-10 at T=2 were taken, as many cells had released from the bottom of the well-plate by itself, which gave rise to the question whether those cells showed different characteristics as the cells which were still properly adhered.

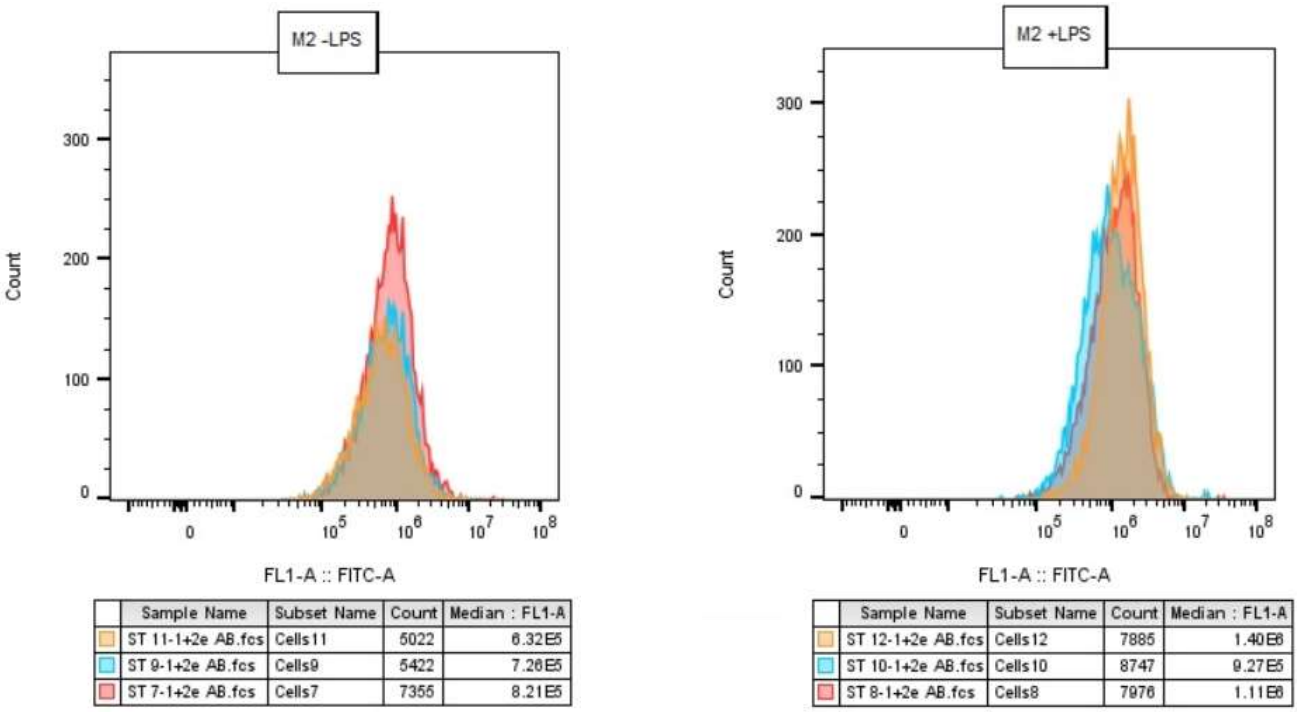


	Sample Name	Subset Name	Count	Median : FL1-A
	ST 5-1+2e AB.fcs	Cells5	5604	1.90E6
	ST 3-1+2e AB.fcs	Cells3	11105	2.09E6
	ST 1-1+2e AB.fcs	Cells1	11094	1.95E6

	Sample Name	Subset Name	Count	Median : FL1-A
	ST 6-1+2e AB.fcs	Cells6	7150	2.38E6
	ST 4-1+2e AB.fcs	Cells4	10664	1.98E6
	ST 2-1+2e AB.fcs	Cells2	16048	1.58E6

Figure 13: Flow cytometry histograms of M1 cells -LPS and +LPS with varying IL-10 treatments.

As can be seen in figure 13, all peaks perfectly overlap in M1 cells -LPS. From this, it can be concluded that IL-10 treatment does not up- or down-regulate the IL-10Rα. In M1 cells +LPS, although the median does slightly increase from no IL-10 (condition 2) to IL-10 at T=0 (condition 4), to IL-10 at T=2 (condition 6), the peaks mostly overlap. Therefore, it cannot be definitively concluded that IL-10 significantly up-regulates the IL-10Rα in M1 cells +LPS.

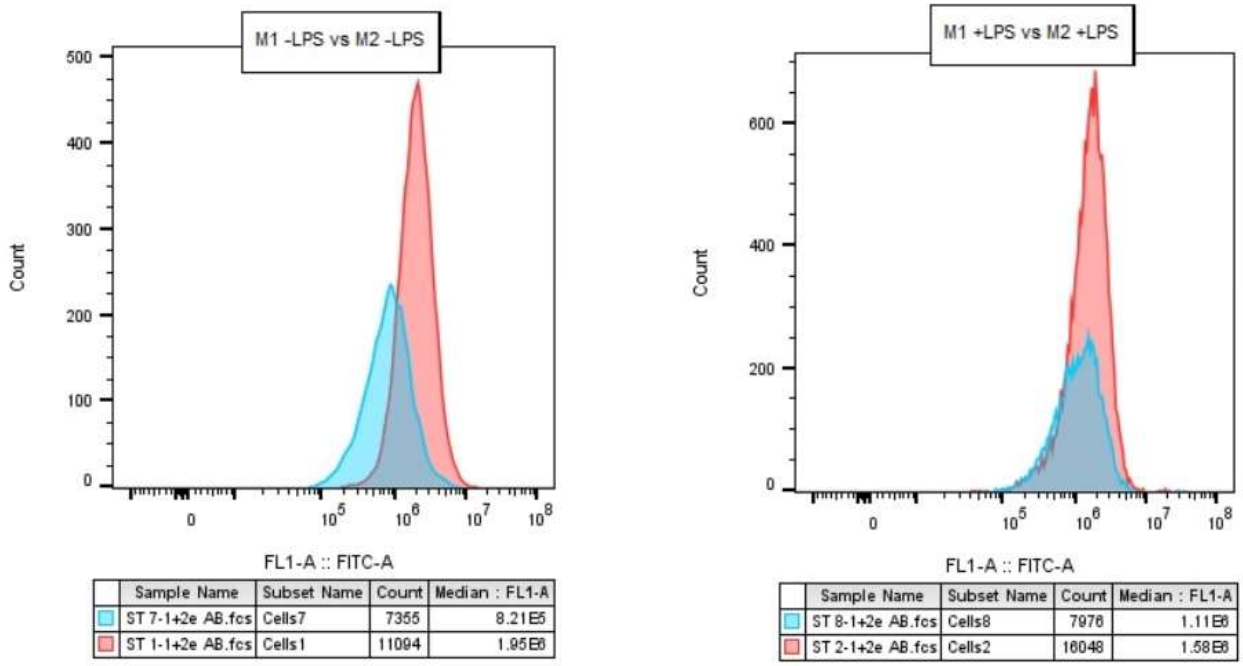


Sample Name	Subset Name	Count	Median : FL1-A
ST 11-1+2e AB.fcs	Cells11	5022	6.32E5
ST 9-1+2e AB.fcs	Cells9	5422	7.26E5
ST 7-1+2e AB.fcs	Cells7	7355	8.21E5

Sample Name	Subset Name	Count	Median : FL1-A
ST 12-1+2e AB.fcs	Cells12	7885	1.40E6
ST 10-1+2e AB.fcs	Cells10	8747	9.27E5
ST 8-1+2e AB.fcs	Cells8	7976	1.11E6

Figure 14: Flow cytometry histograms of M2 cells -LPS and +LPS with varying IL-10 treatments.

As can be seen in figure 14, both histograms show greatly overlapping peaks. This means that, even though the medians show some variation, it can be concluded that IL-10 does not strongly up- or down-regulate IL-10Rα expression in M2 cells.



Sample Name	Subset Name	Count	Median : FL1-A
ST 7-1+2e AB.fcs	Cells7	7355	8.21E5
ST 1-1+2e AB.fcs	Cells1	11094	1.95E6

Sample Name	Subset Name	Count	Median : FL1-A
ST 8-1+2e AB.fcs	Cells8	7976	1.11E6
ST 2-1+2e AB.fcs	Cells2	16048	1.58E6

Figure 15: Flow cytometry histograms of M1 cells -LPS vs M2 cells -LPS and M1 cells +LPS vs M2 cells +LPS.

As demonstrated in figure 15, there is a clear distinction between M1 cells -LPS and M2 cells -LPS, as was also observed in experiment 24-ST-04, where M1 cells (condition 1) express more IL-10Rα ($1{,}95*10^6$) than M2 cells (condition 7, $8{,}21*10^5$). Upon addition of LPS, this difference decreases slightly. M1 cells (condition 2) have a median of $1{,}58*10^6$, a small decrease compared to treatment without LPS, and M2 cells (condition 8) have a median of $1{,}11*10^6$, a small increase compared to treatment without LPS. From this, it can be concluded that M1 cells express more IL-10Rα than M2 cells, however that upon administration of LPS, the difference in expression decreases.

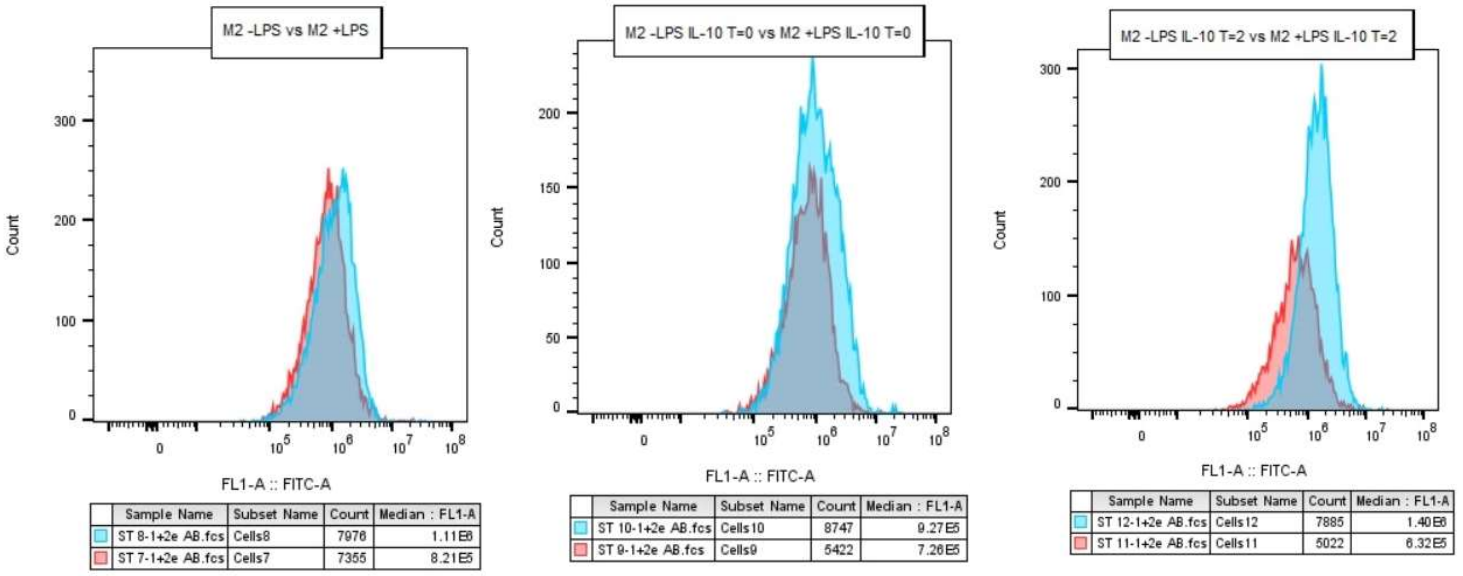


Figure 16: Flow cytometry histograms comparing M2 cells with varying IL-10 treatment.

As can be seen in figure 16, M2 cells without LPS treatment (conditions 7, 9 and 11) express slightly less IL-10Rα than M2 cells with LPS treatment (conditions 8, 10 and 12). The difference is small, and the peaks of IL-10 treatment at T=0h overlap almost completely, the trend is observable in each treatment group.

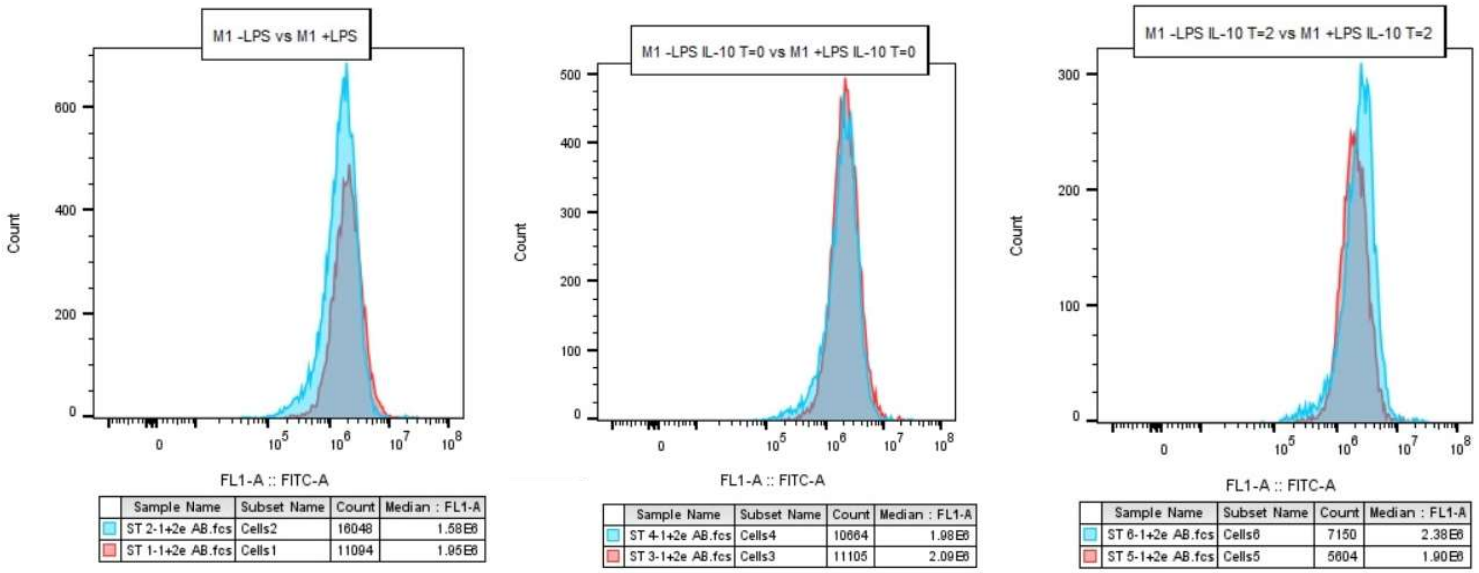
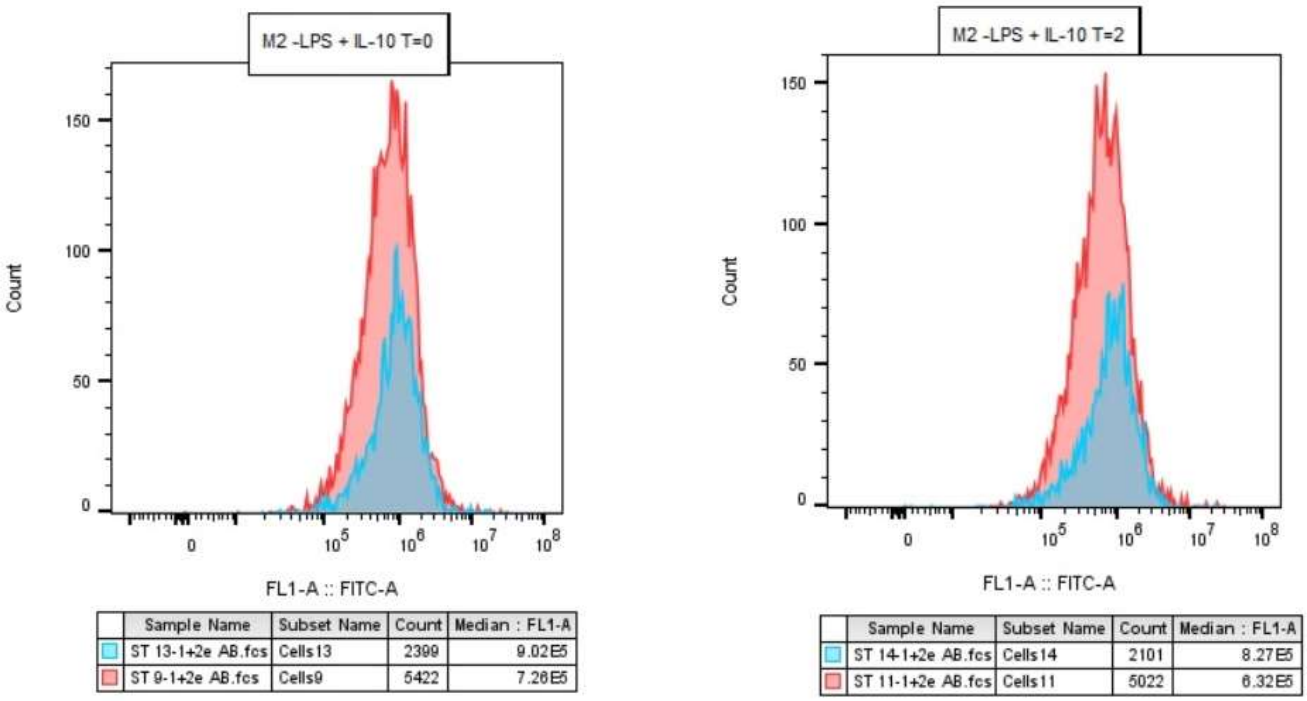


Figure 17: Flow cytometry histograms comparing M1 cells with varying IL-10 treatment

As demonstrated in figure 17, IL-10 does not show a clear significant effect in M1 cells. In M1 cells without IL-10 treatment, IL-10Rα expression decreases slightly (from $1,95*10^6$ in -LPS cells, to $1,58*10^6$ in +LPS cells), with IL-10 treatment at T=0h the median stay roughly equal ($2,09*10^6$ in -LPS cells, and $1,98*10^6$ in +LPS cells) and with IL-10 treatment at T=2h there is a small increase (from $1,90*10^6$ in -LPS to $2,38*10^6$ in +LPS). From this, it can be concluded that IL-10 does not affect IL-10Rα expression in M1 cells treated with LPS.



	Sample Name	Subset Name	Count	Median : FL1-A
	ST 13-1+2e AB.fcs	Cells13	2399	9.02E5
	ST 9-1+2e AB.fcs	Cells9	5422	7.26E5

	Sample Name	Subset Name	Count	Median : FL1-A
	ST 14-1+2e AB.fcs	Cells14	2101	8.27E5
	ST 11-1+2e AB.fcs	Cells11	5022	6.32E5

Figure 18: Flow cytometry comparing M2 cells in medium, as compared to adhered to the well-plate.

As demonstrated in figure 18, there is no apparent difference between cells in medium and cells still adhered to the well-plate. Although in both cases the medians slightly increase from well-plate to medium, in both cases the peaks completely overlap. This might also be caused by the strong decrease in cell number, as there are still less cells present in medium compared to adhered to the well-plate.

Additionally, an NO assay was performed using the sample medium. The same patterns as observed in previous experiments are observed here: M1 or M2 cells not treated with LPS show insignificant NO production. M2 cells treated with LPS produce NO concentrations of 7,9 µM (without IL-10 treatment), 5,4 µM (IL-10 at T=0h) and 5,6 µM (IL-10 treatment at T=2h). While IL-10 treatment results in a decrease in NO production, the reduction is small. M2 cells, as observed previously, produce much less NO than M1 cells: 48 µM (without IL-10 treatment), 54 µM (IL-10 at T=0h) and 56 µM (IL-10 treatment at T=2h). In contrast to the M2 cells or expectations however, IL-10 treatment seems to induce NO production in M1 cells.

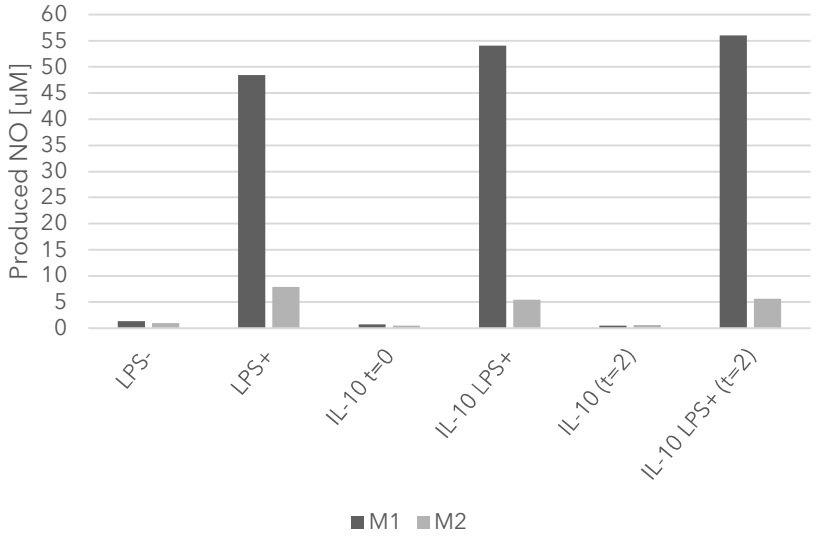


Figure 19: NO assay of experiment 24-ST-05.

24-ST-07: Effects of IL-10 pre-treatment on NO production of native RAW cells (n=2)

Considering that IL-10 had not shown a significant effect on the IL-10Rα expression, and that it had demonstrated a pro-inflammatory effect on the NO production, a new treatment protocol was drafted based on pre-treating cells with IL-10 before IFNγ stimulation [which would be tested via an NO assay (figure 20).

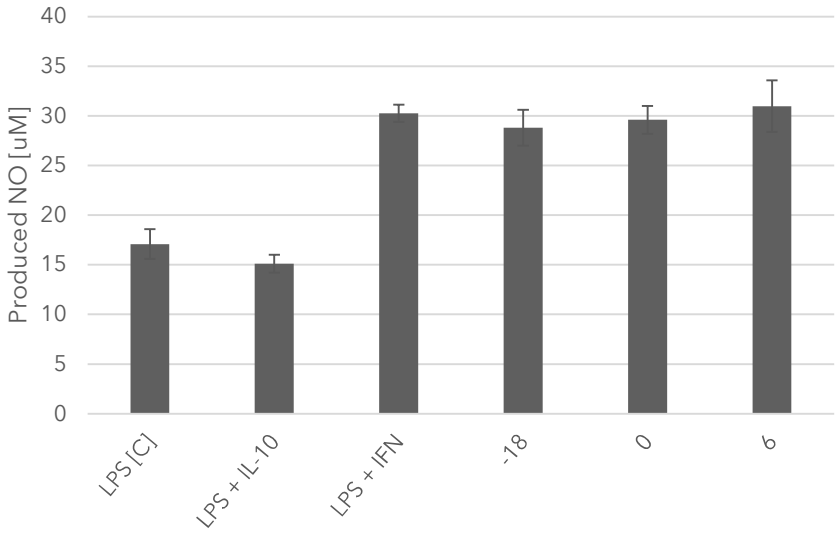


Figure 20: NO assay of experiment 24-ST-07.

As can be seen in figure 20, pre-, concurrent- or post-treatment of IL-10 to the RAW cells shows no significant reduction in NO production. The control sample (only LPS) and LPS+IL-10 at T=-18h show comparable NO concentrations, that being 17 µM and 15 µM respectively. LPS+IFNγ, IL-10 at T=-18h, T=0h and T=6h also show comparable NO concentrations, that being 30,2 µM, 28,8 µM, 29,6 µM and 31 µM respectively.

The control samples (treated only with LPS) and LPS + IL-10 at T=-18h cells show comparable NO concentrations, with a slight decrease with IL-10 treatment. LPS + IFNγ, as well as LPS + IFNγ with IL-10 treatment at T=-18h, 0h or 6h all show comparable NO concentrations. T=-18h shows a small decrease in NO production compared to the LPS + IFNγ, the decrease is nothing compared to the observed drop observed in This can mean that either the applied protocol proves ineffective in RAW 264.7 cells, or that a possible error was made somewhere during the stimulation period. Either way, the effect of IL-10 appears to be highly dependent on experimental setup, as both reductions and increases of NO concentration in macrophages-like cells have been reported.

In conclusion however, NO assay results have failed to show an anti-inflammatory effect of IL-10 on RAW 264.7 cells.

24-ST-08: Effects of IL-10 pre-treatment on TNF-α, TGF-β and IL-10Rα gene expression (n=2)

As previous experiments have failed to demonstrate a significant anti-inflammatory effect of IL-10 on RAW cells by means of NO assays, a final qPCR experiment was performed, in order to study the effects of IL-10 on gene expression. Genes examined were TNF-α, a pro-inflammatory cytokine, TGF-β, an anti-inflammatory cytokines, the IL-10Rα and finally β-actin, which served as household gene (figure 21).

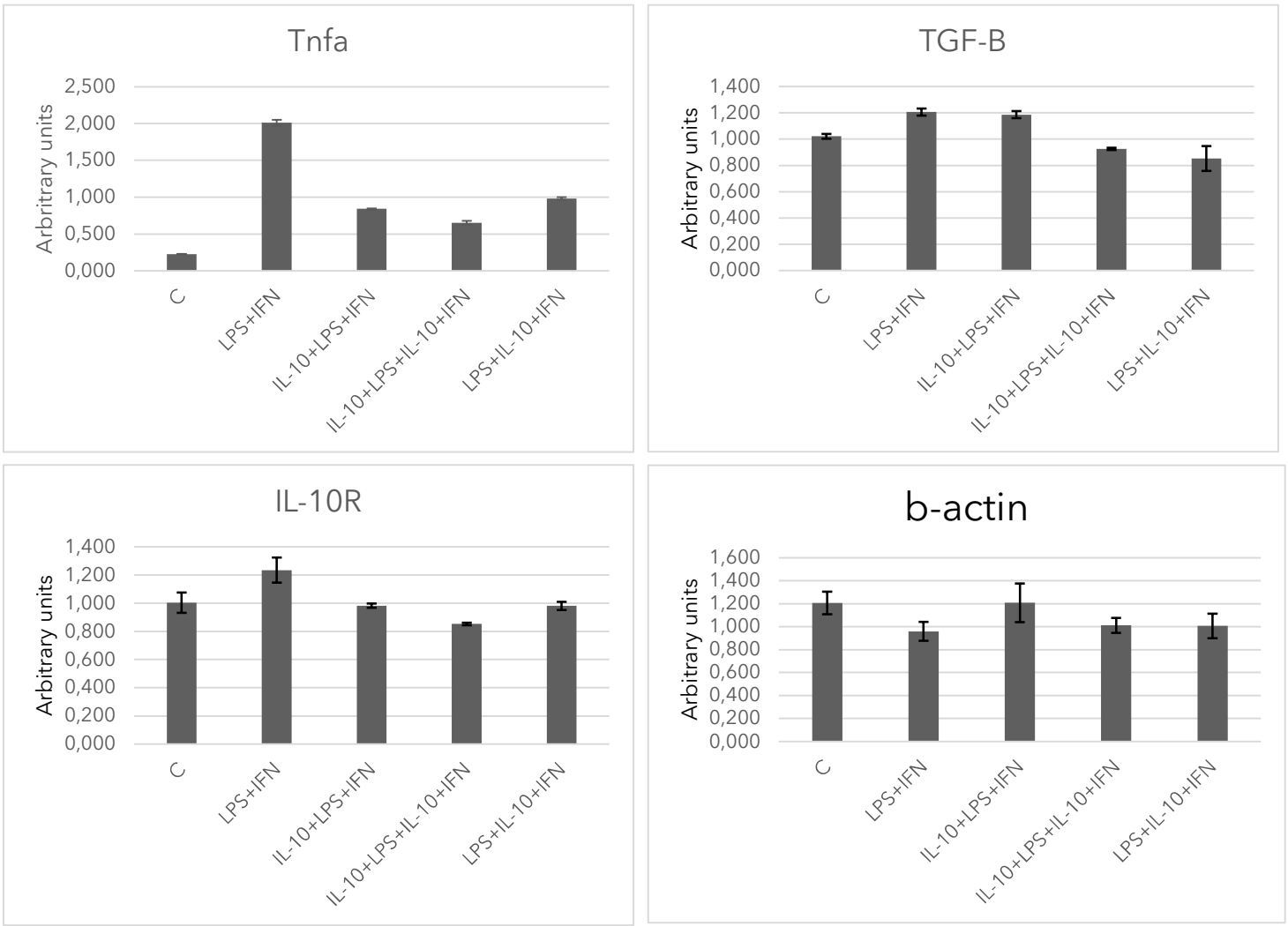


Figure 21: qPCR Graphs.

As can be seen in figure X, IL-10 treatment has no clear apparent effect on TGF-β or IL-10Rα gene expression. However, it shows a clear and significant decrease in TNF-α gene expression compared to a cell treated with LPS and IFNγ only. Considering that TNF-α is a pro-inflammatory cytokine, and upon administration of IL-10, the gene expression decreases, this is the first observed anti-inflammatory effect of IL-10. The graphs were not standardised for β-actin, as the β-actin values would, for the control and IL-10 + LPS + IFNγ condition, underestimate the actual values. Seeing as β-actin showed variable results and could not be used as proper household gene, its choice as such should be re-evaluated.

Conclusion

In conclusion, several analyses were performed in order to investigate the effects of IL-10 on M1, M2 and native RAW 264.7 macrophages. It was determined that treating RAW cells with 24 hours of the cytokines IFNγ for M1 cell polarization, and IL-4 + IL-13 for M2 cell polarization is preferred as it generates the strongest NO inflammatory response in M1 cells observed. Additionally, M1 and M2 cells treated with 24 hours cytokines were morphologically healthier than those treated with 2 hours cytokines. Using flow cytometry, it was proven that the protocol applied does indeed work, and that M1 cells, regardless of LPS treatment, express more IL-10Rα than M2 cells. IL-10 was not found to up- or down-regulate its own receptor in M1 or M2 cells. Disappointingly, IL-10 was also not found to have a anti-inflammatory effect on M1 or M2 cells on an NO level, regardless of pre-, concurrent- or post-treatment of IL-10. An anti-inflammatory effect was achieved on mRNA level, as IL-10 treatment down-regulated the TNF-α gene expression, leaving the TGF-β and IL-10Rα expression unaffected.

Future research should focus on examining and comparing the activity of the IL-10 derived peptides, P1-P4, with the effects of IL-10. This should be done using qPCR, focussing on the TNF-α gene expression.

This study evaluated the anti-inflammatory effects of IL-10, and its potential as an anti-fibrotic drug. Although IL-10 has demonstrated measurable anti-inflammatory effects on gene expression, no measurable effect on NO production was found. At this point in time, IL-10 may not be ready to be used in a clinical setting, although it is undeniable that IL-10 has potential to be used as important anti-fibrotic in the future.

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Appendices

Appendix A1: Raw data of experiment 24-ST-01

100	50	25	12,5	6,3	3,1	1,6	0,8	0
1,039	0,559	0,298	0,176	0,113	0,084	0,068	0,059	0,053
1,063	0,567	0,295	0,183	0,117	0,084	0,068	0,059	0,053
1,051	0,558	0,294	0,177	0,113	0,083	0,067	0,061	0,063

N -LPS	N +LPS	M2 -LPS	M2 -LPS	M1 -LPS	M1 +LPS
0,066	0,065	0,061	0,063	0,507	0,462
0,066	0,068	0,075	0,061	0,489	0,464

Figure A1-1: Raw absorbances of the NO assay of experiment 24-ST-01.

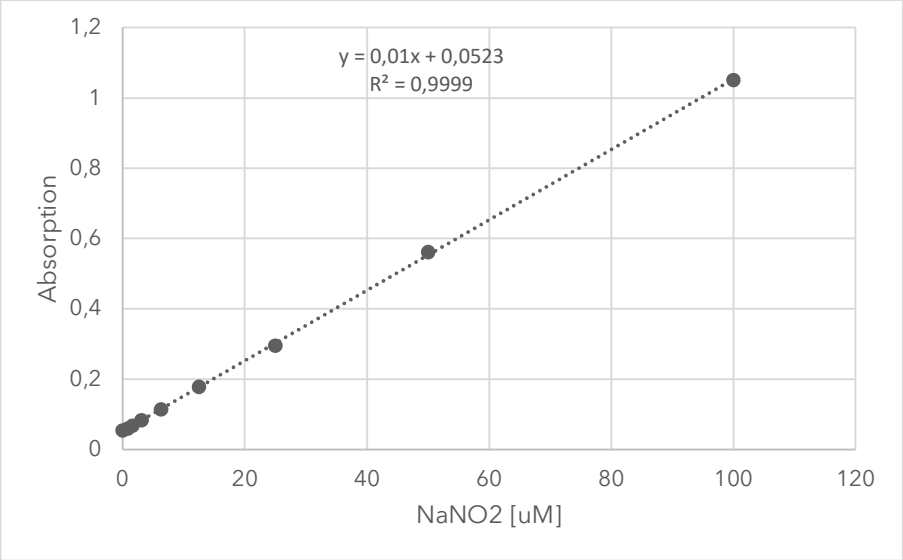


Figure A1-2: Calibration curve of the NO assay of experiment 24-ST-01.

Appendix A2: Raw data of experiment 24-ST-02

100	50	25	12,5	6,3	3,1	1,6	0,8	0
1,031	0,551	0,303	0,177	0,113	0,094	0,069	0,066	0,051
1,067	0,553	0,306	0,18	0,114	0,083	0,069	0,059	0,051
1,061	0,553	0,311	0,175	0,112	0,083	0,068	0,059	0,051

M1 Vm	M1 Vm	M1 LPS	M1 LPS	M1 C+LPS	M1 C+LPS	Spike1	Spike2
0,056	0,057	0,259	0,305	0,381	0,424	0,09	0,089
0,058	0,056	0,261	0,308	0,393	0,425	0,09	0,091

M2 Vm	M2 Vm	M2 LPS	M2 LPS	M2 C+LPS	M2 C+LPS
0,057	0,067	0,095	0,095	0,082	0,076
0,054	0,056	0,095	0,093	0,08	0,076

Figure A2-1: Raw absorbances of the NO assay of experiment 24-ST-02.

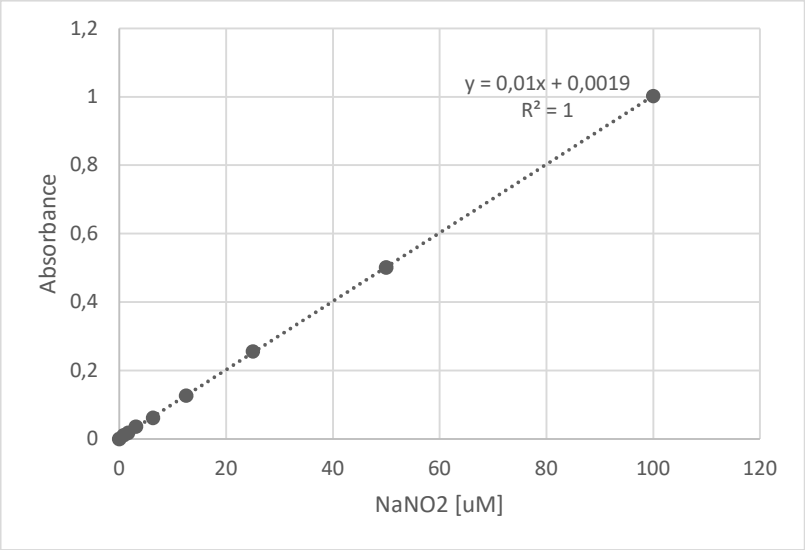


Figure A2-2: Calibration curve the NO assay of experiment 24-ST-03.

Appendix A3: Raw data of experiment 24-ST-03

100	50	25	12,5	6,3	3,1	1,6	0,8	0
0,997	0,545	0,301	0,171	0,111	0,083	0,067	0,06	0,052
1,035	0,545	0,293	0,17	0,113	0,081	0,067	0,059	0,052
1,018	0,528	0,286	0,173	0,109	0,079	0,066	0,06	0,053
M1 Vm	M1 Vm	M1 LPS	M1 LPS	M1 C+LPS	M1 C+LPS		Spike1	Spike2
0,057	0,055	0,479	0,472	0,559	0,543		0,16	0,167
0,058	0,057	0,484	0,465	0,547	0,534		0,161	0,162
M2 Vm	M2 Vm	M2 LPS	M2 LPS	M2 C+LPS	M2 C+LPS			
0,055	0,059	0,196	0,19	0,149	0,116			
0,057	0,059	0,193	0,187	0,142	0,116			

Figure A3-1: Raw absorbances of the NO assay of experiment 24-ST-03.

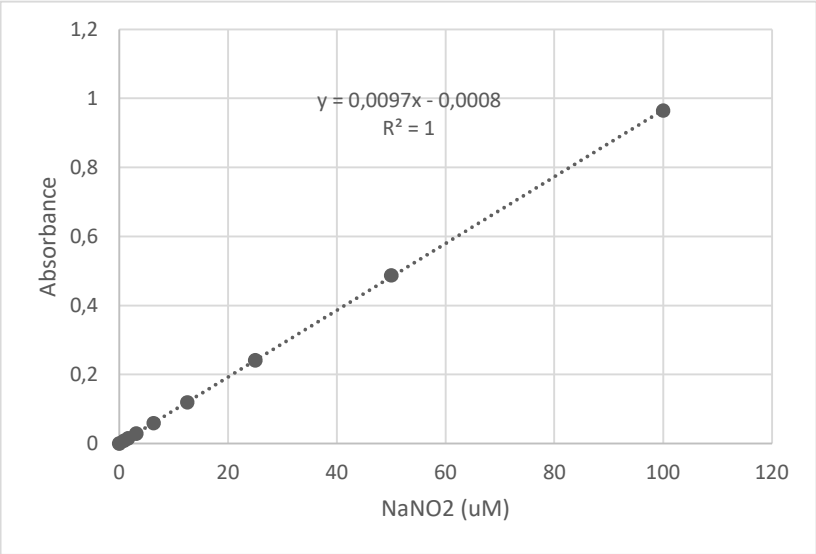


Figure A3-2: Calibration curve of the NO assay of experiment 24-ST-03, and 24-ST-04.

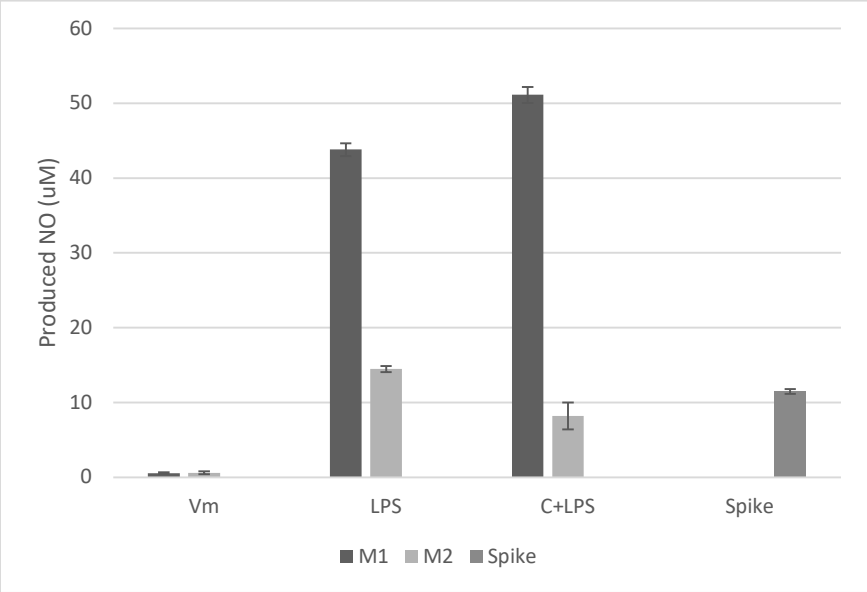


Figure A3-3: NO assay of 24-ST-03.

Appendix A4: Raw data of experiment 24-ST-04

M1 -LPS	M1 -LPS	M1 -LPS
0,065	0,06	0,067
0,065	0,062	0,064
M1 +LPS	M1 +LPS	M1 +LPS
0,493	0,458	0,464
0,443	0,459	0,461
M2	M2	M2
0,078	0,06	0,064
0,064	0,06	0,062

Figure A4-1: Raw absorbances of the NO assay of experiment 24-ST-04.

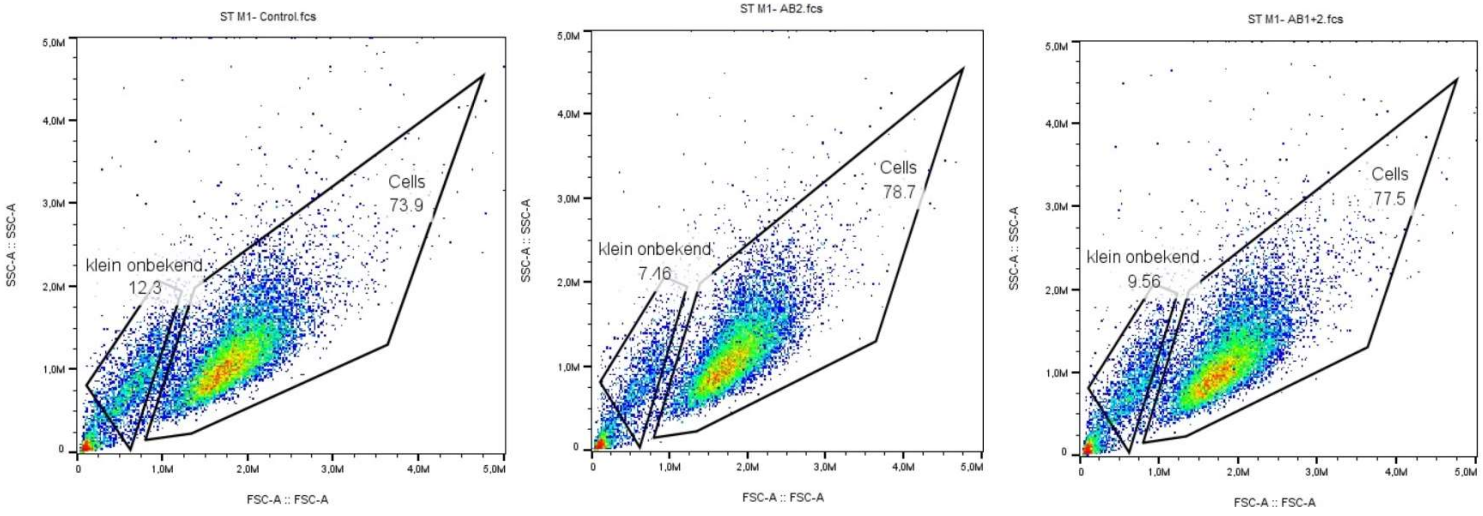


Figure A4-2: Gating strategy for the M1 -LPS flow cytometry samples of experiment 24-ST-04.

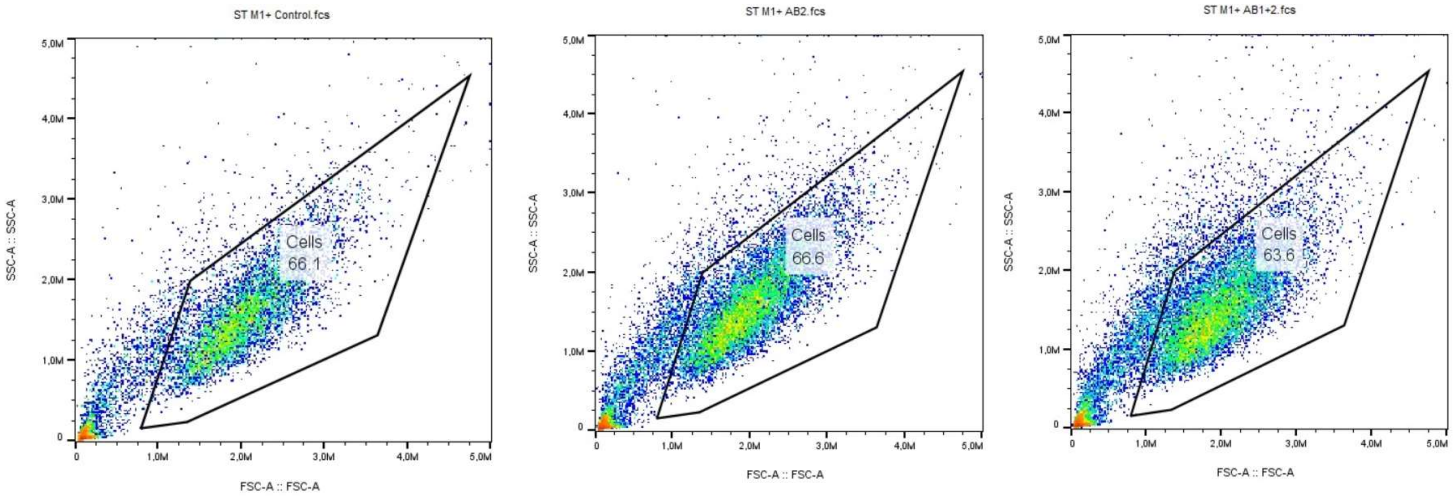


Figure A4-3: Gating strategy for the M1 +LPS flow cytometry samples of experiment 24-ST-04.

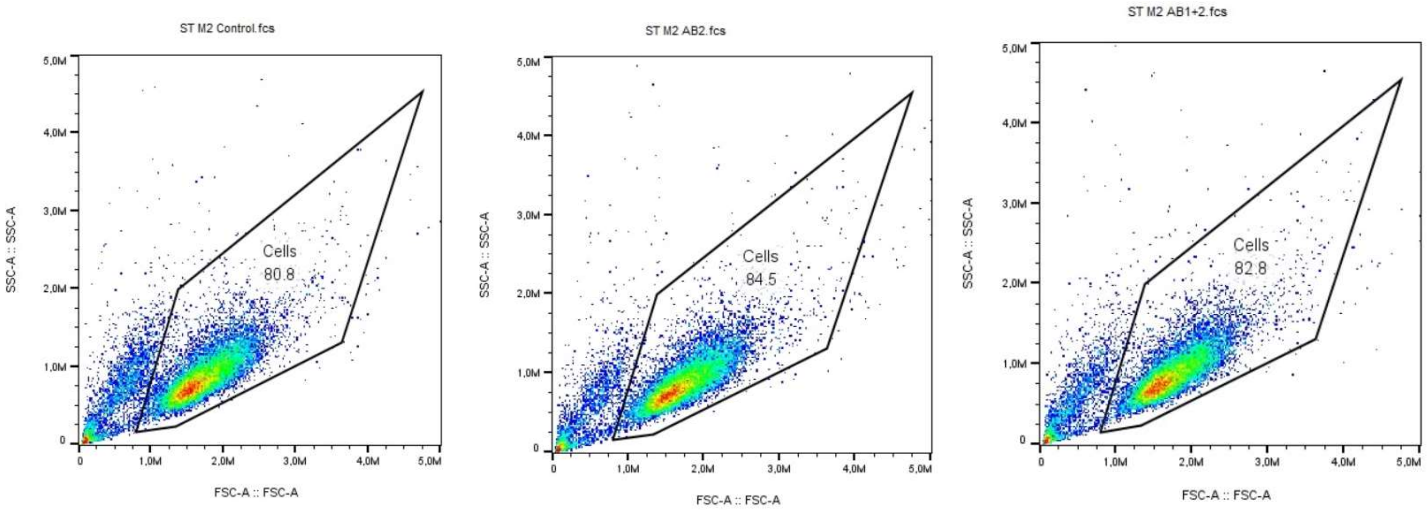


Figure A4-4: Gating strategy for the M2 flow cytometry samples of experiment 24-ST-04.

Appendix A5: Raw data of experiment 24-ST-05

100	50	25	12,5	6,3	3,1	1,6	0,8	0
1,031	0,537	0,3	0,184	0,116	0,085	0,069	0,064	0,054
1,043	0,56	0,301	0,181	0,118	0,087	0,07	0,062	0,054
1,044	0,55	0,308	0,178	0,116	0,085	0,07	0,063	0,054
1	2	3	4	5	6			
0,068	0,539	0,062	0,592	0,06	0,615			
0,07	0,527	0,063	0,585	0,06	0,6			
7	8	9	10	11	12			
0,065	0,134	0,06	0,11	0,064	0,109			
0,064	0,132	0,06	0,108	0,059	0,112			

Figure A5-1: Raw absorbances of the NO assay of experiment 24-ST-05.

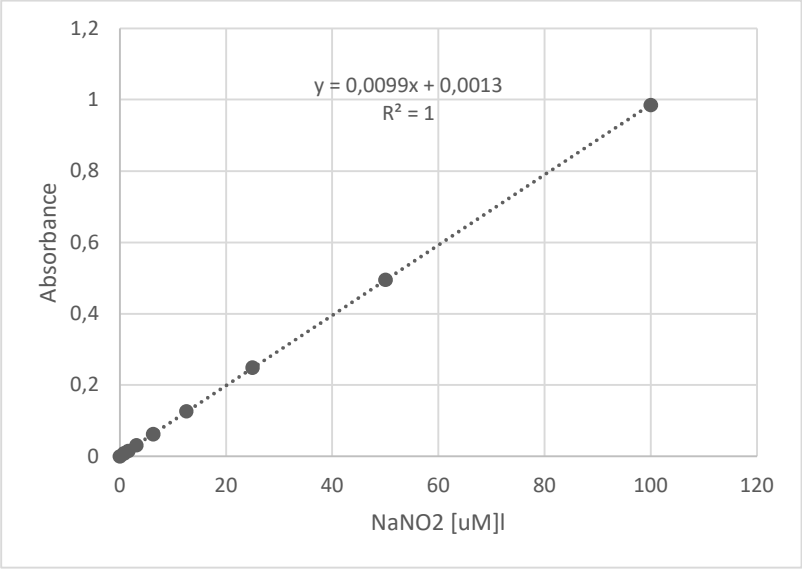


Figure A5-2: Calibration curve of the NO assay of experiment 24-ST-05.

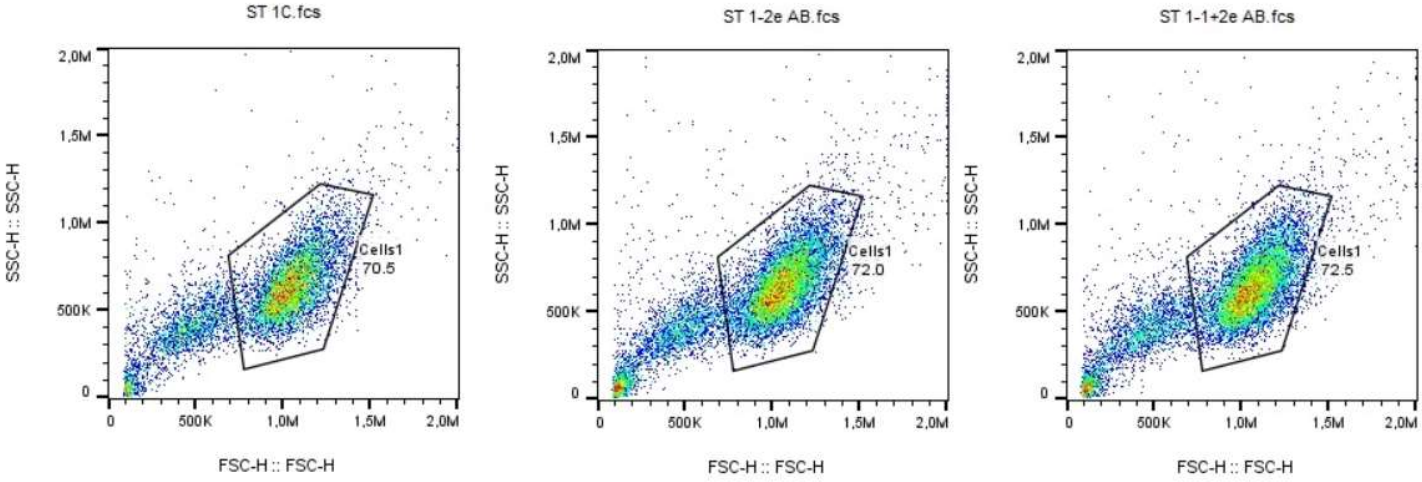
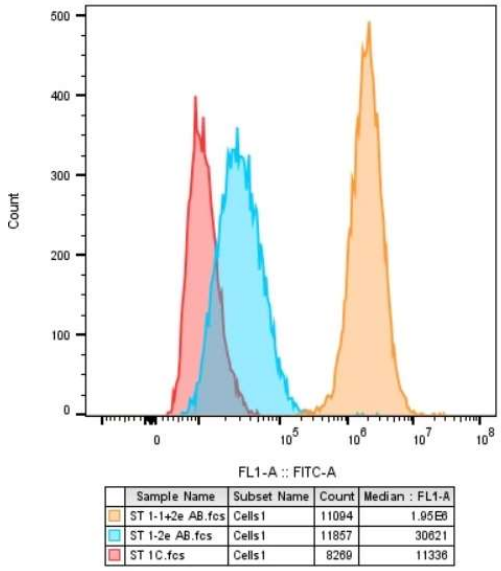


Figure A5-3: Gating strategy for treatment condition 1 of experiment 24-ST-05.



Sample Name	Subset Name	Count	Median : FL1-A
ST 1-1+2e AB.fcs	Cells1	11094	1.95E6
ST 1-2e AB.fcs	Cells1	11857	30621
ST 1C.fcs	Cells1	8269	11336

Figure A5-4: Control, secondary AB and primary + secondary AB histogram of treatment condition 1 of experiment 24-ST-05

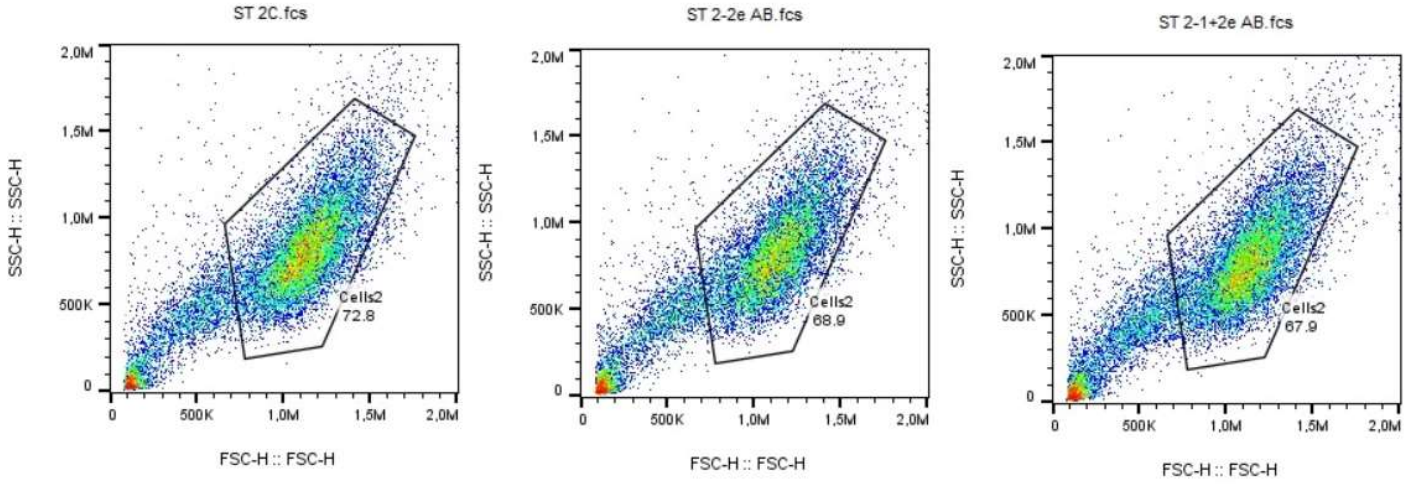


Figure A5-5: Gating strategy for treatment condition 2 of experiment 24-St-05.

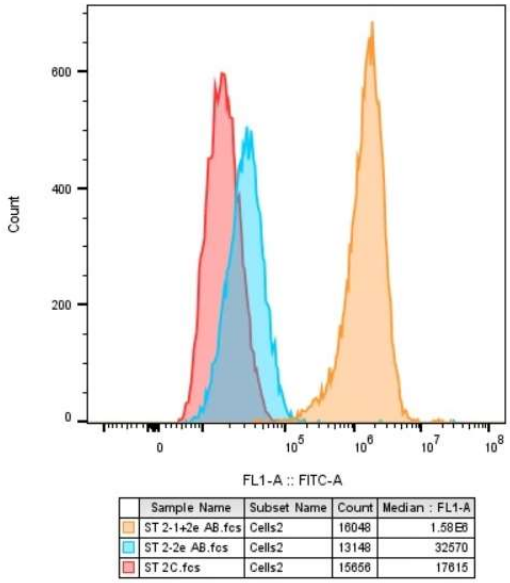


Figure A5-6: Control, secondary AB and primary + secondary AB histograms of treatment condition 2 of experiment 24-ST-05.

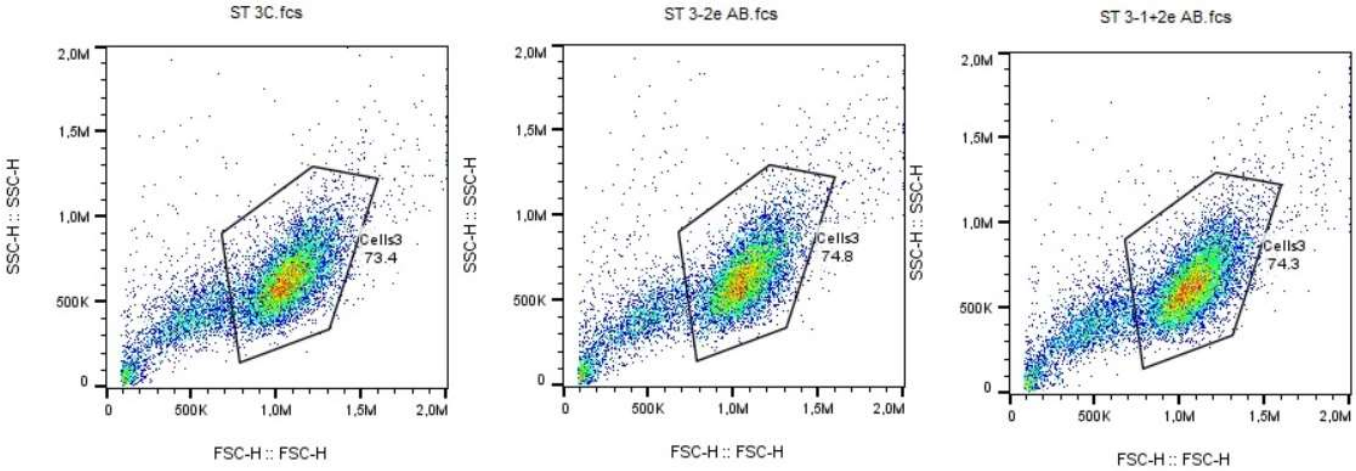
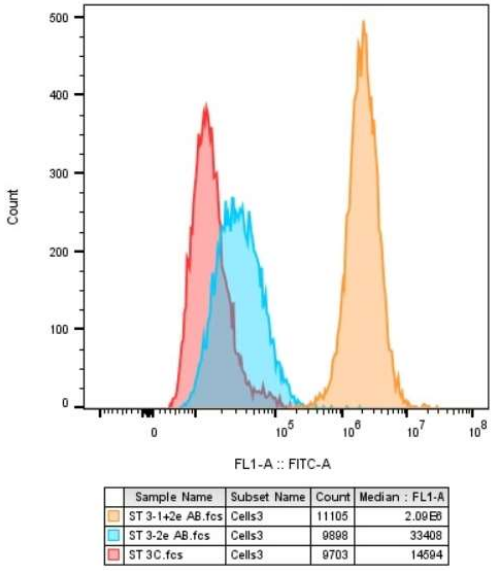


Figure A5-7: Gating strategy for treatment condition 3 of experiment 24-ST-05.



Sample Name	Subset Name	Count	Median : FL1-A
ST 3-1+2e AB.fcs	Cells3	11105	2.09E6
ST 3-2e AB.fcs	Cells3	9898	33408
ST 3C.fcs	Cells3	9703	14594

Figure A5-8: Control, secondary AB and primary + secondary AB histograms of treatment condition 3 of experiment 24-ST-05.

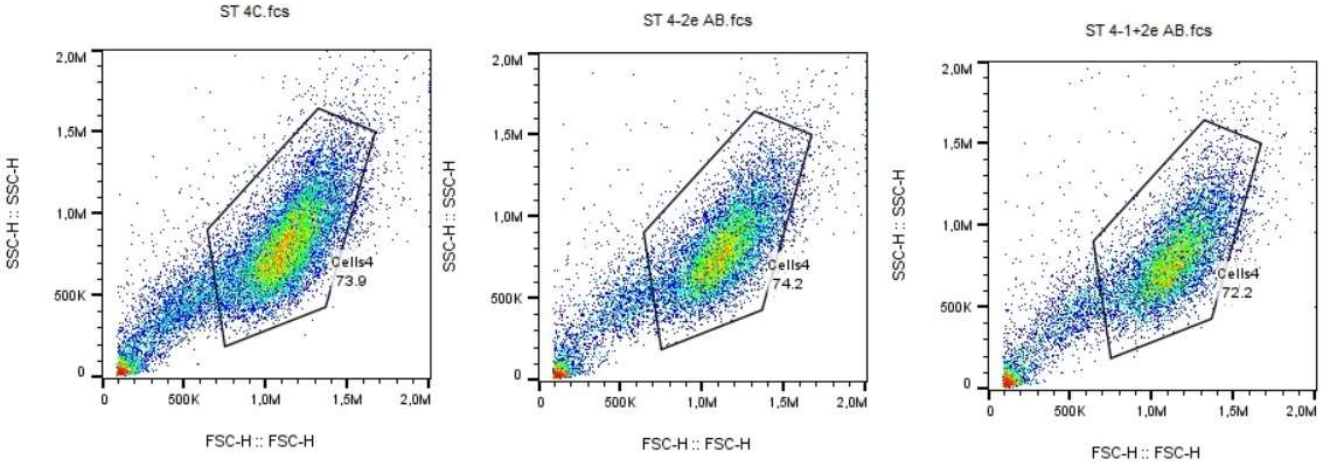
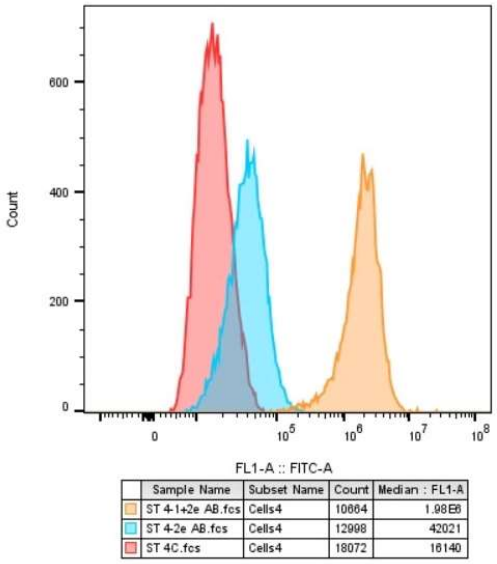


Figure A5-9: Gating strategy of treatment condition 4 of experiment 24-ST-05.



	Sample Name	Subset Name	Count	Median : FL1-A
	ST 4-1+2e AB.fcs	Cells4	10864	1.98E6
	ST 4-2e AB.fcs	Cells4	12998	42021
	ST 4C.fcs	Cells4	18072	16140

Figure A5-10: Control, secondary AB and primary + secondary AB histograms of treatment condition 4 of experiment 24-ST-05.

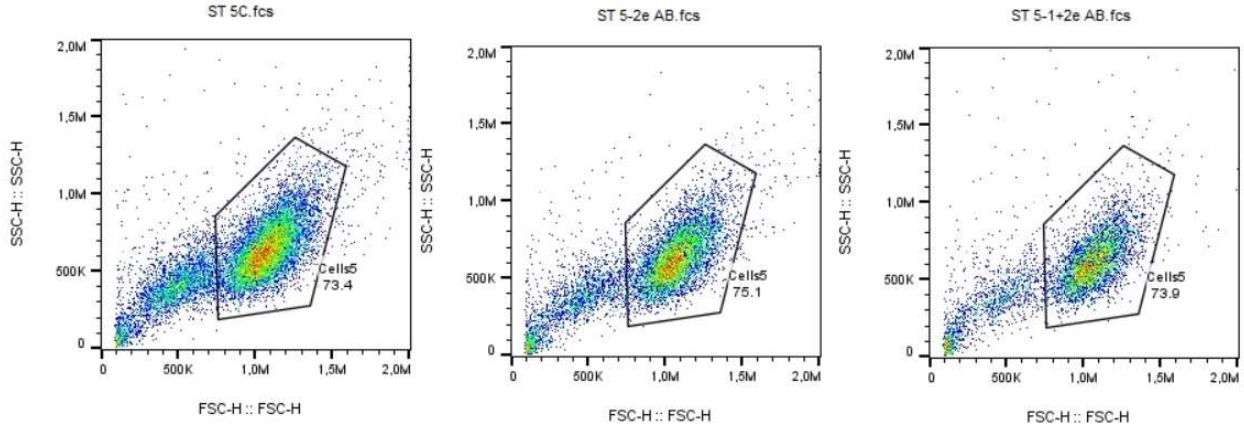
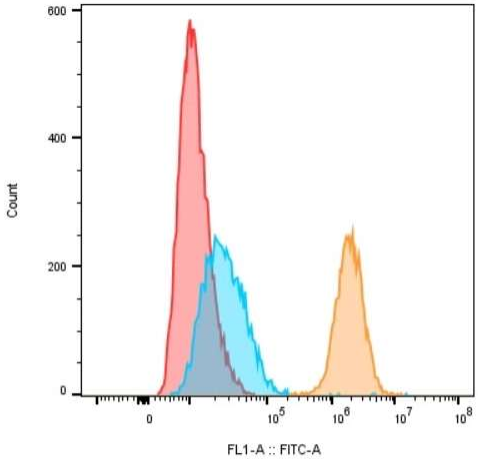


Figure A5-11: Gating strategy of treatment condition 5 of experiment 24-ST-05.



	Sample Name	Subset Name	Count	Median : FL1-A
	ST 5-1+2e AB.fcs	Cells5	5604	1.90E6
	ST 5-2e AB.fcs	Cells5	8412	26154
	ST 5C.fcs	Cells5	13095	11367

Figure A5-12: Control, secondary and primary + secondary AB histograms of treatment condition 5 of experiment 24-ST-05.

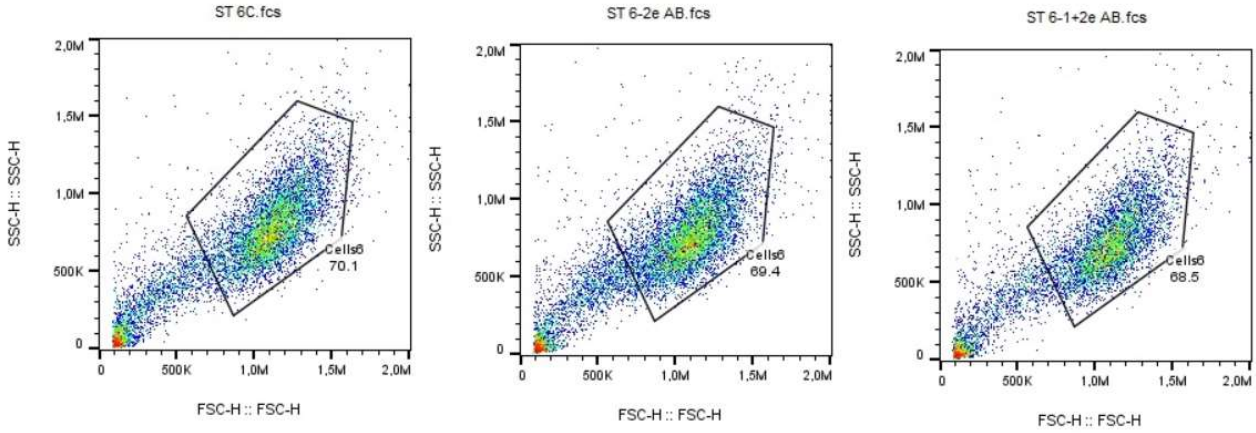
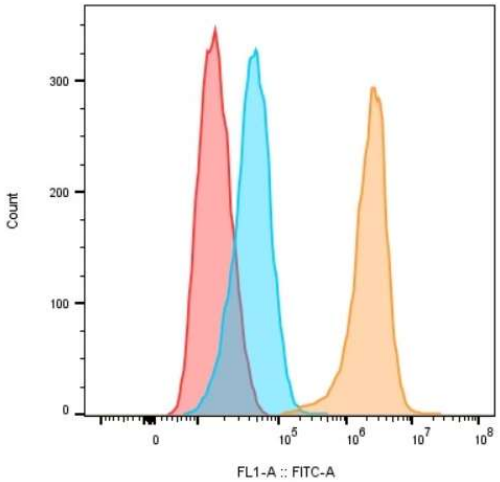


Figure A5-13: Gating strategy of treatment condition 6 of experiment 24-ST-05.



	Sample Name	Subset Name	Count	Median : FL1-A
	ST 6-1+2e AB.fcs	Cells6	7150	2.38E6
	ST 6-2e AB.fcs	Cells6	9118	47108
	ST 6C.fcs	Cells6	8977	16055

Figure A5-14: Control, secondary and primary + secondary AB histograms for treatment condition 6 of experiment 24-ST-05.

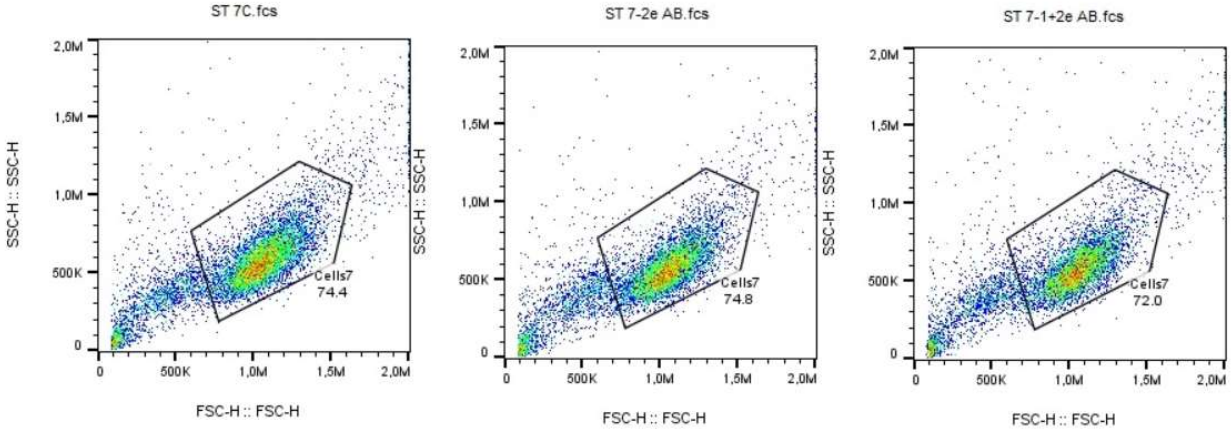
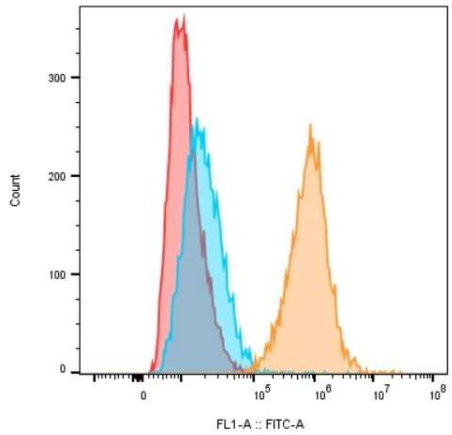


Figure A5-15: Gating strategy of treatment condition 7 of experiment 24-ST-05.



Sample Name	Subset Name	Count	Median : FL1-A
ST 7-1+2e AB.fcs	Cells7	7355	8.21E5
ST 7-2e AB.fcs	Cells7	8146	19230
ST 7C.fcs	Cells7	9214	11120

Figure A5-16: Control, secondary AB and primary + secondary AB histograms of treatment condition 7 of experiment 24-ST-07.

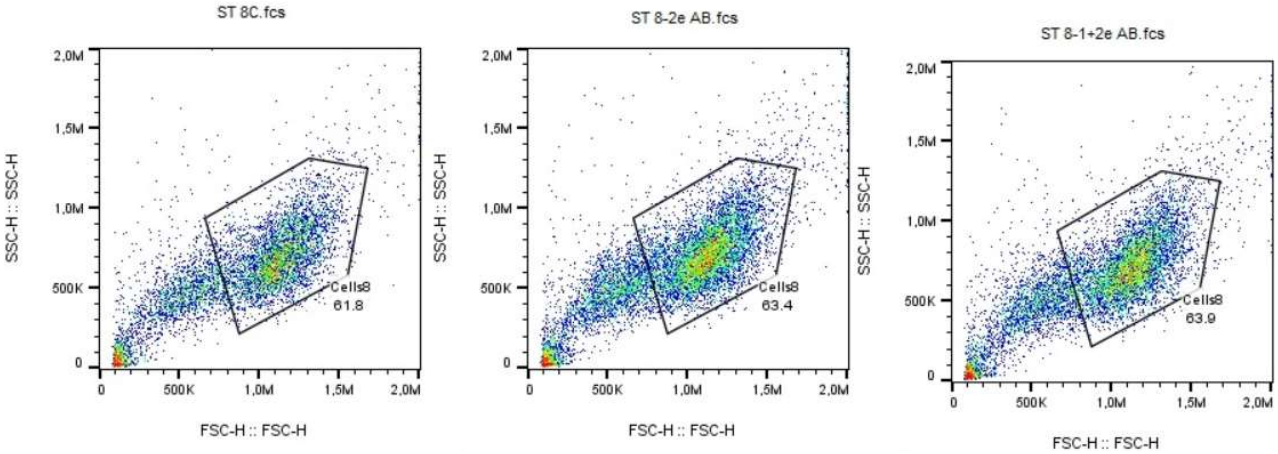
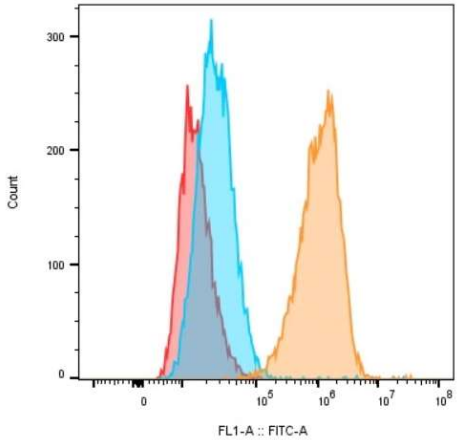


Figure A5-17: Gating strategy of treatment condition 8 of experiment 24-ST-05.



Sample Name	Subset Name	Count	Median : FL1-A
ST 8-1+2e AB.fcs	Cells8	7976	1.11E6
ST 8-2e AB.fcs	Cells8	8947	26808
ST 8C.fcs	Cells8	5916	14867

Figure A5-18: Control, secondary AB and primary + secondary AB histograms of treatment condition 8 of experiment 24-ST-05.

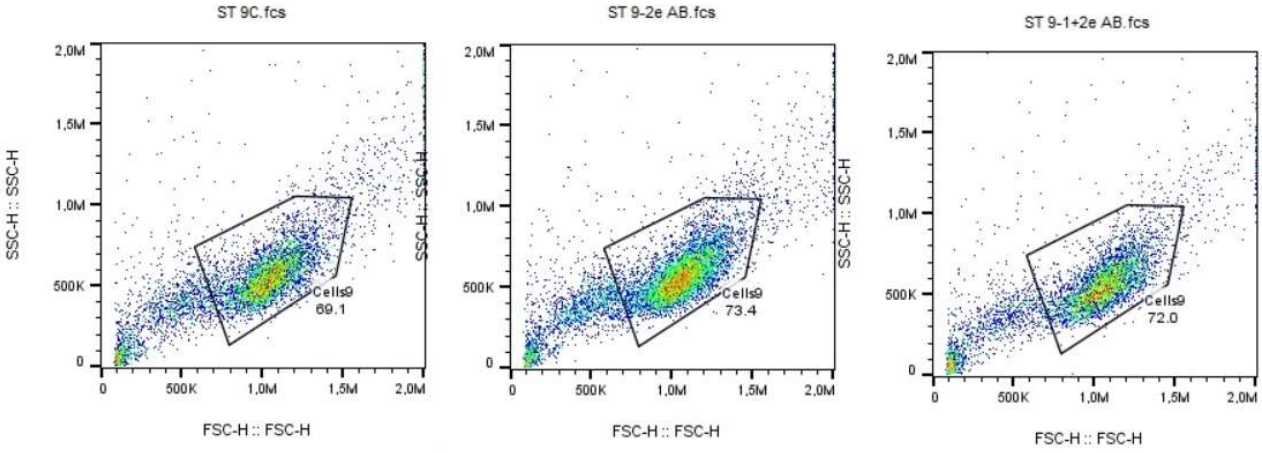
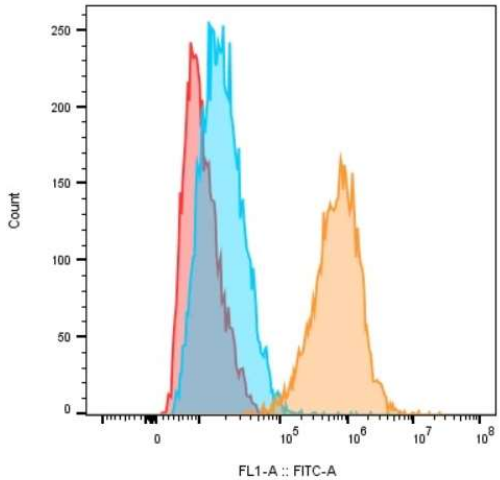


Figure A5-19: Gating strategy of treatment condition 9 of experiment 24-ST-05.



	Sample Name	Subset Name	Count	Median : FL1-A
	ST 9-1+2e AB.fcs	Cells9	5422	7.26E5
	ST 9-2e AB.fcs	Cells9	8371	18429
	ST 9C.fcs	Cells9	5946	10260

Figure A5-20: Control, secondary AB and primary + secondary AB histograms of treatment condition 9 of experiment 24-ST-05.

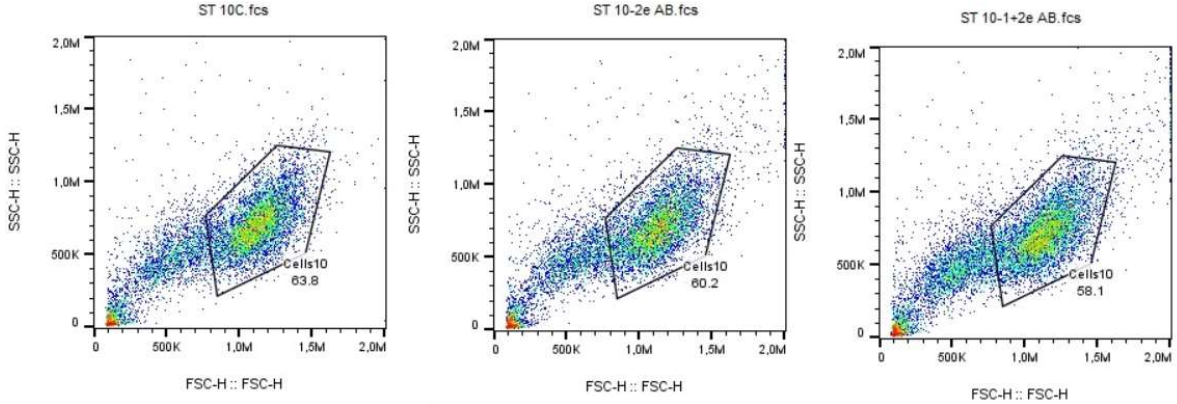
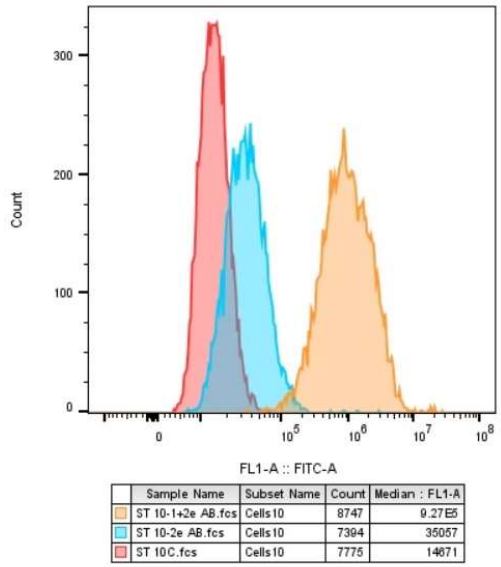


Figure A5-21: Gating strategy of treatment condition 10 of experiment 24-ST-05.



	Sample Name	Subset Name	Count	Median : FL1-A
	ST 10-1+2e AB.fcs	Cells10	8747	9.27E5
	ST 10-2e AB.fcs	Cells10	7394	35057
	ST 10C.fcs	Cells10	7775	14671

Figure A5-22: Control, secondary AB and primary + secondary AB histograms of treatment condition 10 of experiment 24-ST-05.

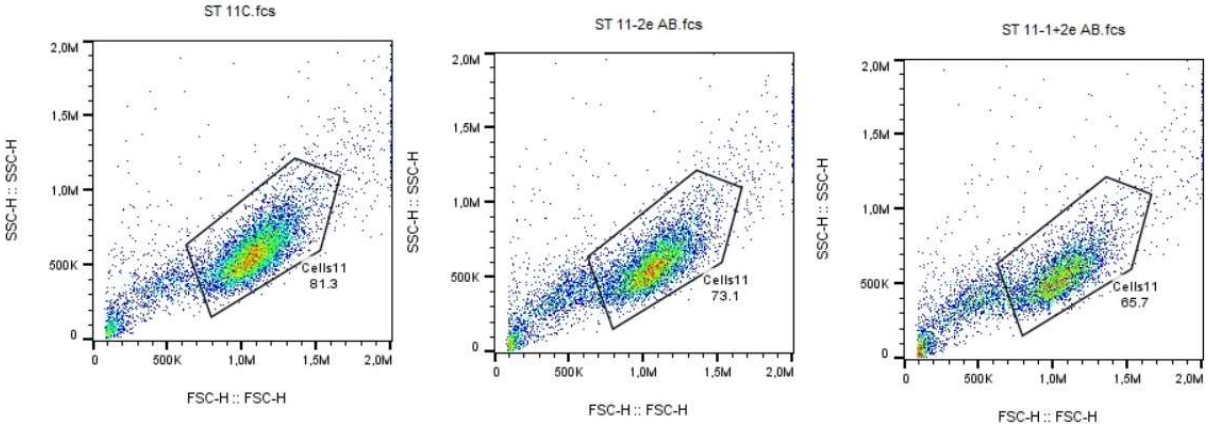
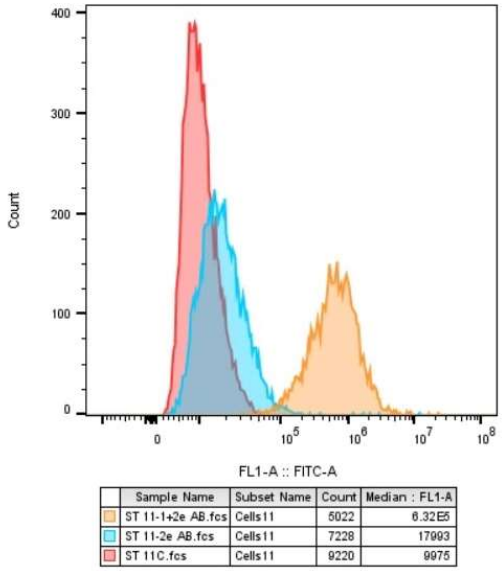


Figure A5-23: Gating strategy of treatment condition 11 of experiment 24-ST-05.



	Sample Name	Subset Name	Count	Median : FL1-A
	ST 11-1+2e AB.fcs	Cells11	5022	6.32E5
	ST 11-2e AB.fcs	Cells11	7228	17993
	ST 11C.fcs	Cells11	9220	9975

Figure A5-24: Control, secondary AB and primary + secondary AB histograms of treatment condition 11 of experiment 24-ST-05.

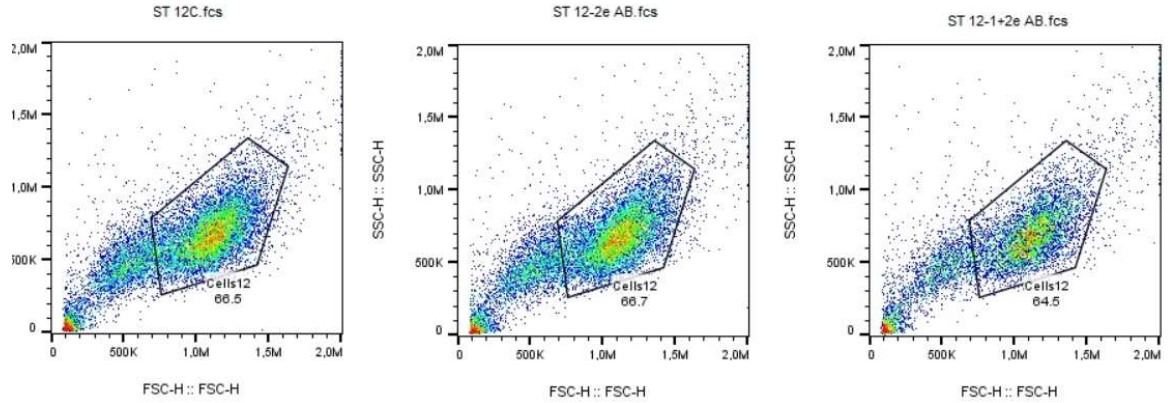
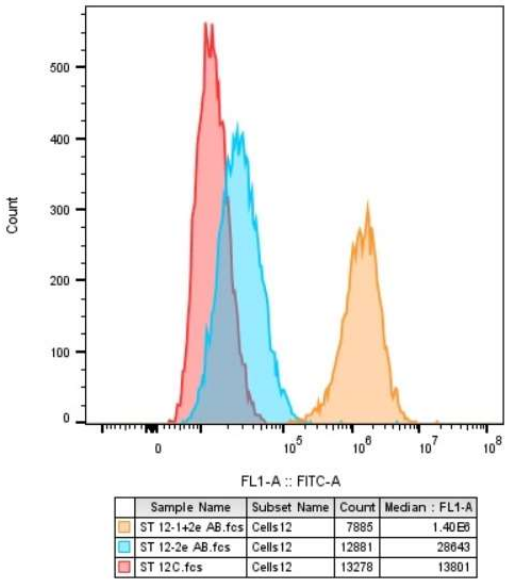


Figure A5-25: Gating strategy of treatment condition 12 of experiment 24-ST-05.



	Sample Name	Subset Name	Count	Median : FL1-A
	ST 12-1+2e AB.fcs	Cells12	7885	1.40E6
	ST 12-2e AB.fcs	Cells12	12881	28643
	ST 12C.fcs	Cells12	13278	13801

Figure A5-26: Control, secondary AB and primary + secondary AB histograms of treatment condition 12 of experiment 24-ST-05.

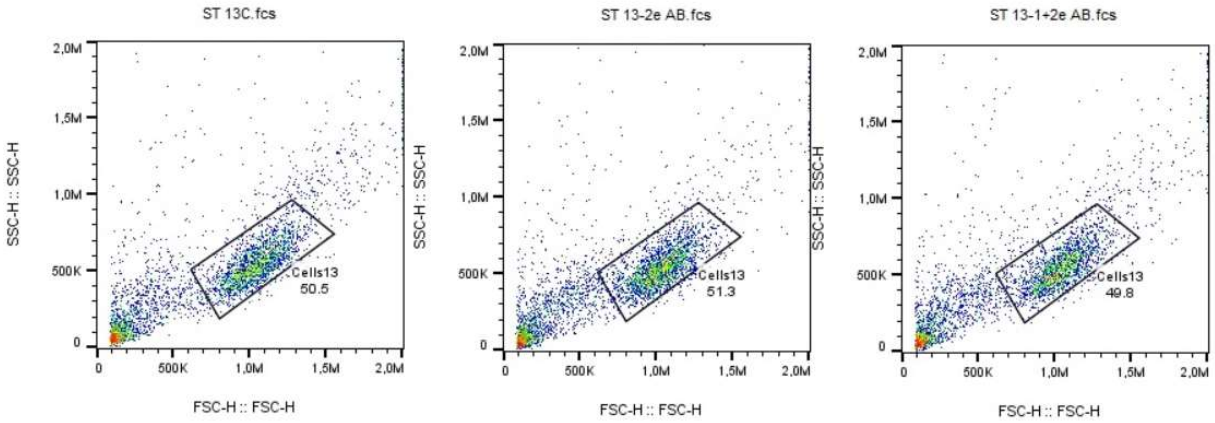
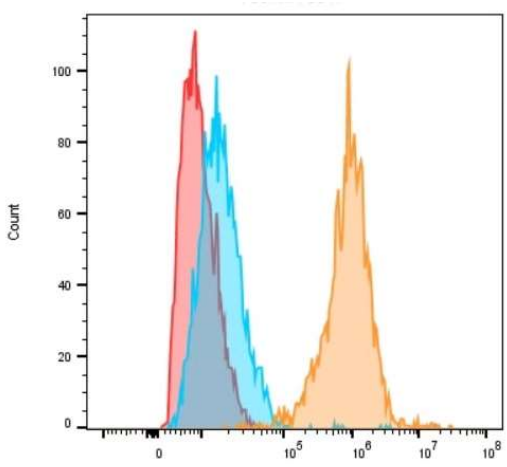


Figure A5-27: Gating strategy of treatment condition 13 of experiment 24-ST-05.



	Sample Name	Subset Name	Count	Median : FL1-A
	ST 13-1+2e AB.fcs	Cells13	2399	9.02E5
	ST 13-2e AB.fcs	Cells13	2655	16443
	ST 13C.fcs	Cells13	2612	8837

Figure A5-28: Control, secondary AB and primary + secondary AB histograms of treatment condition 13 of experiment 24-ST-05.

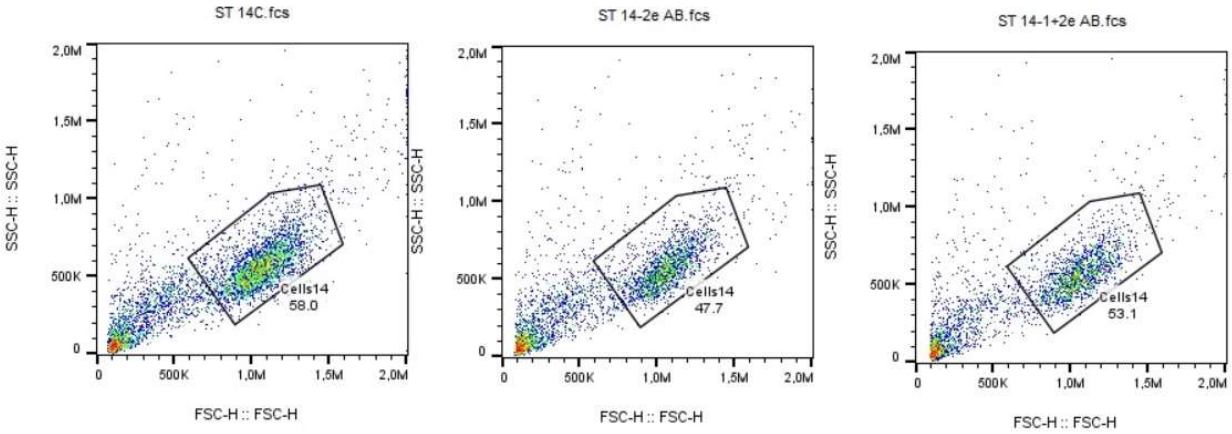
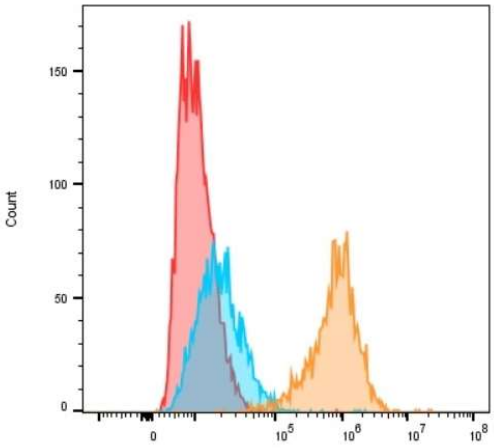


Figure A5-29: Gating strategy of treatment condition 14 of experiment 24-ST-05.



	Sample Name	Subset Name	Count	Median : FL1-A
	ST 14-1+2e AB.fcs	Cells14	2101	8.27E5
	ST 14-2e AB.fcs	Cells14	2295	19128
	ST 14C.fcs	Cells14	4040	10088

Figure A5-30: Control, secondary AB and primary + secondary AB histograms of treatment condition 14 of experiment 24-ST-05.

Appendix A6: Raw data of experiment 24-ST-07

100	50	25	12,5	6,3	3,1	1,6	0,8	0
1,719	0,919	0,501	0,281	0,167	0,113	0,084	0,07	0,055
1,645	0,902	0,501	0,28	0,167	0,112	0,084	0,069	0,055
1,62	0,891	0,5	0,283	0,167	0,111	0,084	0,07	0,054
C	C	-18	-18	0	0	6	6	
0,364	0,326	0,57	0,52	0,57	0,532	0,58	0,568	
0,367	0,321	0,542	0,503	0,561	0,523	0,614	0,513	
-IFNg -18	-IFNg -18	-IL-10	-IL-10					
0,332	0,301	0,544	0,576					
0,316	0,301	0,55	0,559					

Figure A6-1: Raw absorbances of the NO assay of experiment 24-ST-07.

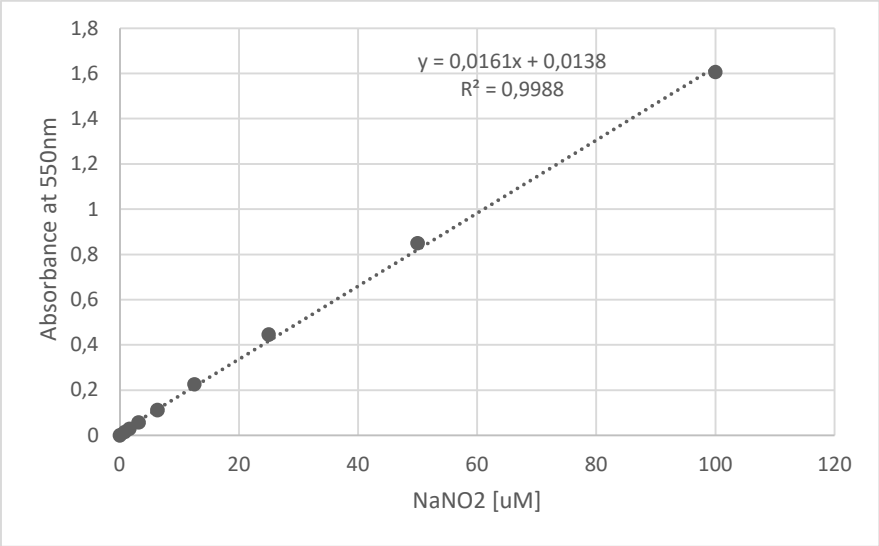


Figure A6-2: Calibration curve of the NO assay of experiment 24-ST-07.

Appendix A7: Raw data of experiment 24-ST-08

Well Positi	Sample Na	Target Name	CT	Quantity	Tm1	Tm2
N6	1	Stefan b actin	15,597	1,319	86,23	
O6	1	Stefan b actin	15,778	1,166	86,23	
P6	1	Stefan b actin	15,819	1,134	86,361	
N7	2	Stefan b actin	15,957	1,032	86,361	
O7	2	Stefan b actin	16,038	0,976	86,361	
P7	2	Stefan b actin	16,208	0,869	86,361	
N8	3	Stefan b actin	15,626	1,293	86,361	
O8	3	Stefan b actin	15,599	1,317	86,23	
P8	3	Stefan b actin	15,984	1,013	86,361	
N9	4	Stefan b actin	15,883	1,085	86,361	
O9	4	Stefan b actin	16,065	0,958	86,361	
P9	4	Stefan b actin	16,015	0,991	86,23	
N10	5	Stefan b actin	15,957	1,032	86,23	
O10	5	Stefan b actin	16,174	0,89	86,361	
P10	5	Stefan b actin	15,865	1,098	86,23	
O20		Stefan b actin	13,767	4,6	86,098	
O21		Stefan b actin	15,979	1,017	85,967	
O22		Stefan b actin	17,352	0,398	85,967	
O23		Stefan b actin	18,329	0,204	85,835	
O24		Stefan b actin	18,917	0,137	85,835	
P20		Stefan b actin	13,899	4,203	85,967	
P21		Stefan b actin	16,164	0,896	85,967	
P22		Stefan b actin	17,181	0,447	85,967	
P23		Stefan b actin	18,357	0,201	85,967	
P24		Stefan b actin	19,44	0,096	85,835	
N11	NC	Stefan b actin	36,66	0	88,466	
O11	NC	Stefan b actin	35,553	0	88,466	
P11	NC	Stefan b actin	33,327	0	89,124	
N5	STD0,25	Stefan b actin	17,972	0,25	86,361	
O5	STD0,25	Stefan b actin	17,976	0,25	86,361	
P5	STD0,25	Stefan b actin	18,22	0,25	86,361	
N4	STD0,5	Stefan b actin	17,04	0,5	86,23	
O4	STD0,5	Stefan b actin	17,209	0,5	86,23	
P4	STD0,5	Stefan b actin	17,023	0,5	86,23	
N3	STD1	Stefan b actin	15,999	1	86,23	
O3	STD1	Stefan b actin	16,056	1	86,23	
P3	STD1	Stefan b actin	15,57	1	86,098	
N2	STD2	Stefan b actin	14,979	2	86,23	
O2	STD2	Stefan b actin	14,935	2	86,23	
P2	STD2	Stefan b actin	14,897	2	86,23	
N1	STD4	Stefan b actin	13,983	4	86,23	
O1	STD4	Stefan b actin	13,971	4	86,23	
P1	STD4	Stefan b actin	14,211	4	86,23	

Figure A7-1: Raw CTs, quantities and melting points of b-actin qPCR of experiment 24-ST-08.

Well Position	Sample Name	Target Name	CT	Quantity	Tm1	Tm2
E12	NC	TGF-b	Undetermined		61,37	
F12	NC	TGF-b	Undetermined		61,37	
G12	NC	TGF-b	Undetermined		61,238	
E10	Sample 10	TGF-b	23,884	0,878	88,546	
F10	Sample 10	TGF-b	23,801	0,934	88,546	
G10	Sample 10	TGF-b	24,099	0,748	88,546	
E6	Sample 6	TGF-b	23,707	1,001	88,414	
F6	Sample 6	TGF-b	23,665	1,032	88,414	
G6	Sample 6	TGF-b	23,664	1,033	88,546	
E7	Sample 7	TGF-b	23,435	1,225	88,414	
F7	Sample 7	TGF-b	23,49	1,176	88,546	
G7	Sample 7	TGF-b	23,44	1,22	88,546	
E8	Sample 8	TGF-b	23,452	1,209	88,414	
F8	Sample 8	TGF-b	23,513	1,156	88,546	
G8	Sample 8	TGF-b	23,467	1,196	88,546	
E9	Sample 9	TGF-b	23,797	0,937	88,414	
F9	Sample 9	TGF-b	23,815	0,924	88,546	
G9	Sample 9	TGF-b	23,82	0,92	88,546	
E5	STD025	TGF-b	25,581	0,25	88,414	
F5	STD025	TGF-b	25,624	0,25	88,414	
G5	STD025	TGF-b	25,591	0,25	88,546	
E4	STD05	TGF-b	24,618	0,5	88,414	
F4	STD05	TGF-b	24,7	0,5	88,414	
G4	STD05	TGF-b	24,693	0,5	88,546	
E3	STD1	TGF-b	23,708	1	88,414	
F3	STD1	TGF-b	23,653	1	88,414	
G3	STD1	TGF-b	23,66	1	88,414	
E2	STD2	TGF-b	22,831	2	88,414	
F2	STD2	TGF-b	22,679	2	88,414	
G2	STD2	TGF-b	22,519	2	88,414	
E1	STD4	TGF-b	22,453	4	88,282	
F1	STD4	TGF-b	21,626	4	88,414	
G1	STD4	TGF-b	21,687	4	88,414	
A12	NC	TNF-a	Undetermined		61,238	92,504
B12	NC	TNF-a	Undetermined		61,37	86,172
C12	NC	TNF-a	Undetermined		61,106	79,048
A11	PC	TNF-a	17,502	23,018	86,172	
B11	PC	TNF-a	17,751	19,315	86,172	
C11	PC	TNF-a	17,382	25,041	86,172	
A6	Sample 1	TNF-a	24,114	0,219	87,623	
B6	Sample 1	TNF-a	24,074	0,226	87,623	
C6	Sample 1	TNF-a	24,057	0,228	87,623	

A7	Sample 2	TNF-a	20,98	1,991	87,623	
B7	Sample 2	TNF-a	20,983	1,987	87,623	
C7	Sample 2	TNF-a	20,933	2,058	87,623	
A8	Sample 3	TNF-a	22,191	0,849	87,623	
B8	Sample 3	TNF-a	22,22	0,832	87,755	
C8	Sample 3	TNF-a	22,211	0,837	87,623	
A9	Sample 4	TNF-a	22,628	0,624	87,755	
B9	Sample 4	TNF-a	22,556	0,657	87,755	
C9	Sample 4	TNF-a	22,52	0,673	87,623	
A10	Sample 5	TNF-a	21,972	0,991	87,755	
B10	Sample 5	TNF-a	22,018	0,959	87,755	
C10	Sample 5	TNF-a	21,971	0,991	87,755	
A5	STD025	TNF-a	23,993	0,25	87,623	
B5	STD025	TNF-a	23,908	0,25	87,623	
C5	STD025	TNF-a	23,845	0,25	87,623	
A4	STD05	TNF-a	22,915	0,5	87,491	
B4	STD05	TNF-a	22,926	0,5	87,623	
C4	STD05	TNF-a	23,078	0,5	87,623	
A3	STD1	TNF-a	21,867	1	87,491	
B3	STD1	TNF-a	21,985	1	87,623	
C3	STD1	TNF-a	21,96	1	87,623	
A2	STD2	TNF-a	21,085	2	87,491	
B2	STD2	TNF-a	20,887	2	87,623	
C2	STD2	TNF-a	20,972	2	87,623	
A1	STD4	TNF-a	19,759	4	87,491	
B1	STD4	TNF-a	20,042	4	87,491	
C1	STD4	TNF-a	20,157	4	87,623	
I12	NC	IL-10Ra	Undetermined		61,37	89,338
J12	NC	IL-10Ra	Undetermined		61,37	88,678
K11	NC	IL-10Ra	Undetermined		61,238	80,499
I6	Sample 11	IL-10Ra	24,662	1,058	80,895	
J6	Sample 11	IL-10Ra	24,703	1,029	80,895	
K6	Sample 11	IL-10Ra	24,865	0,921	80,895	
I7	Sample 12	IL-10Ra	24,523	1,163	80,895	
J7	Sample 12	IL-10Ra	24,47	1,207	80,895	
K7	Sample 12	IL-10Ra	24,322	1,335	80,895	
I8	Sample 13	IL-10Ra	24,786	0,972	80,895	
J8	Sample 13	IL-10Ra	24,745	1	80,895	
K8	Sample 13	IL-10Ra	24,78	0,976	80,895	
I9	Sample 14	IL-10Ra	24,964	0,861	80,895	
J9	Sample 14	IL-10Ra	24,995	0,843	80,895	
K9	Sample 14	IL-10Ra	24,975	0,855	80,895	
I10	Sample 15	IL-10Ra	24,756	0,992	80,895	

J10	Sample 15	IL-10Ra	24,825	0,946	80,895	
K10	Sample 15	IL-10Ra	24,744	1,001	80,895	
I5	STD025	IL-10Ra	26,68	0,25	80,895	
J5	STD025	IL-10Ra	26,613	0,25	80,895	
K5	STD025	IL-10Ra	26,735	0,25	80,895	
I4	STD05	IL-10Ra	25,78	0,5	80,895	
J4	STD05	IL-10Ra	25,837	0,5	80,895	
K4	STD05	IL-10Ra	25,781	0,5	80,895	
I3	STD1	IL-10Ra	24,784	1	80,895	
J3	STD1	IL-10Ra	24,864	1	80,895	
K3	STD1	IL-10Ra	24,873	1	80,763	
I2	STD2	IL-10Ra	23,754	2	80,895	
J2	STD2	IL-10Ra	23,764	2	80,895	
K2	STD2	IL-10Ra	23,916	2	80,763	
I1	STD4	IL-10Ra	22,666	4	80,895	
J1	STD4	IL-10Ra	22,636	4	80,895	
K1	STD4	IL-10Ra	22,493	4	80,763	

Figure A7-2: Raw CTs, quantities and melting points of TNF-α, TGF-β and IL-10Rα qPCR of experiment 24-ST-08.

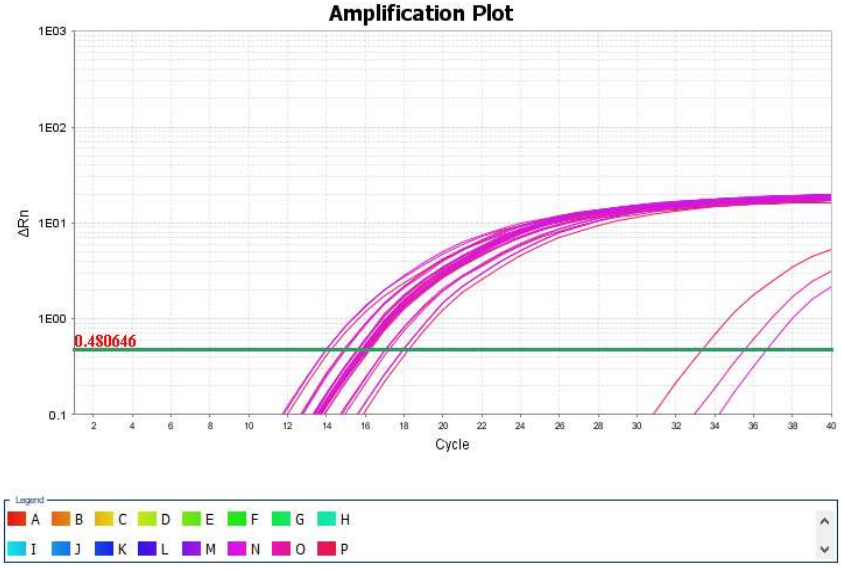


Figure A7-3: Amplification plot of β-actin qPCR samples, including standard curve and negative controls.

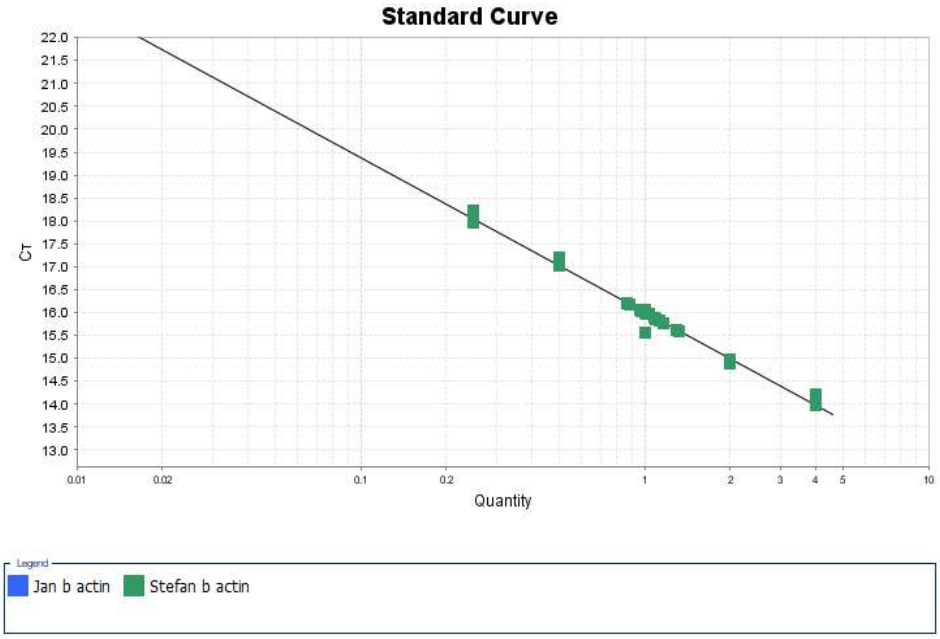


Figure A7-4: Standard curve of β-actin qPCR samples, including samples of interest.

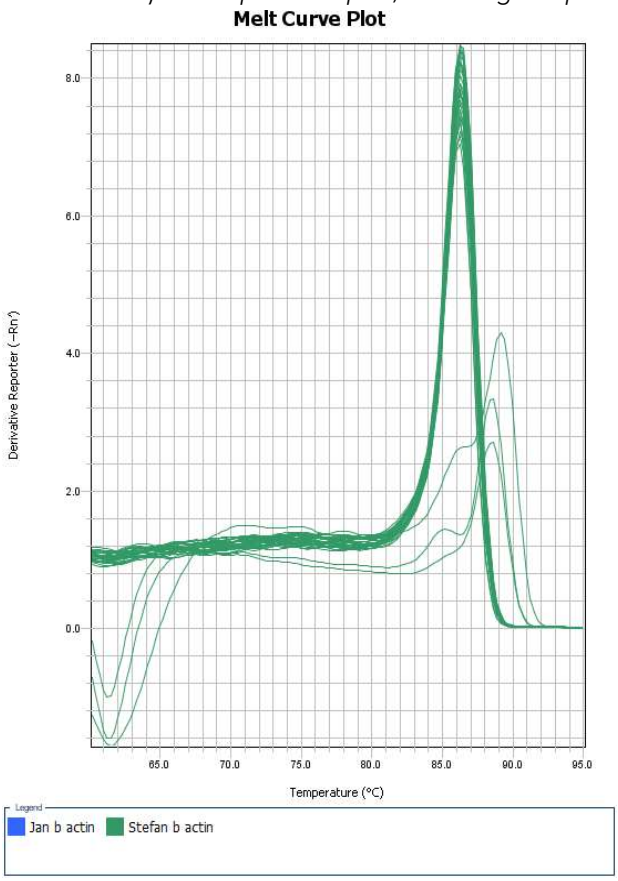


Figure A7-5: Melting curve of β-actin qPCR samples, including standard curve and negative controls.

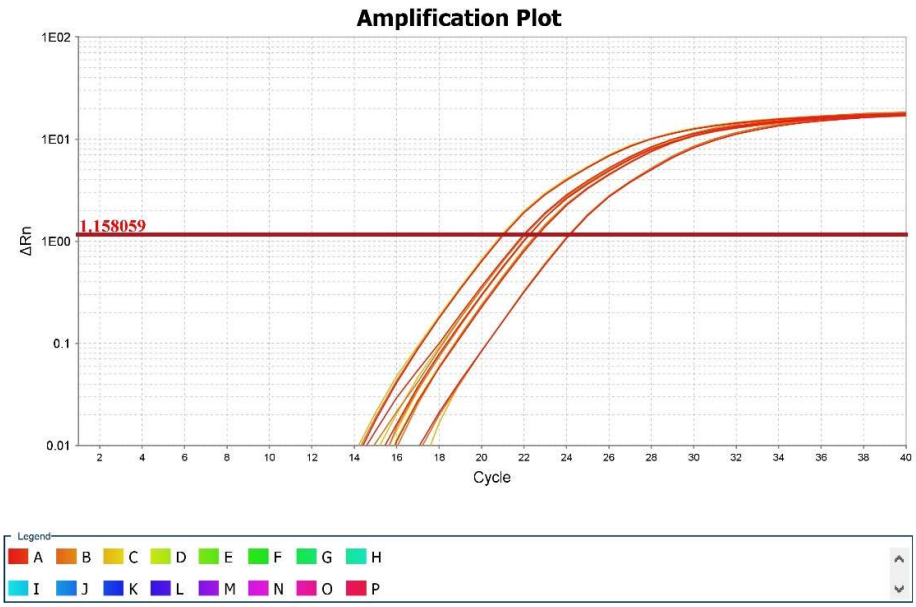


Figure A7-6: Amplification plot of TNF-α qPCR samples.

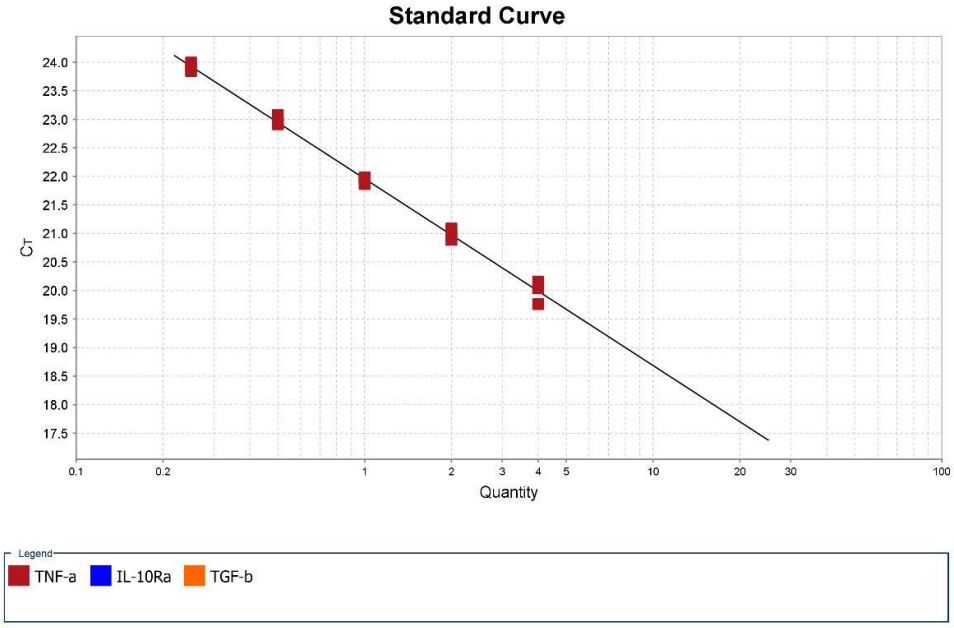


Figure A7-7: Standard curve of TNF-α qPCR samples.

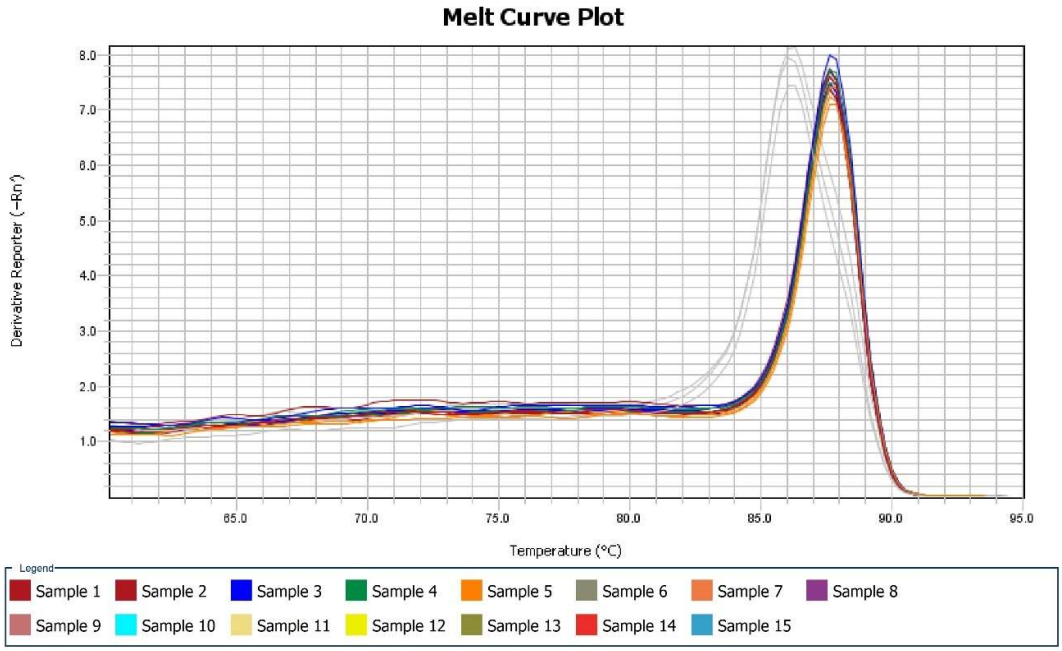


Figure A7-8: Melting curve of TNF-α qPCR samples.

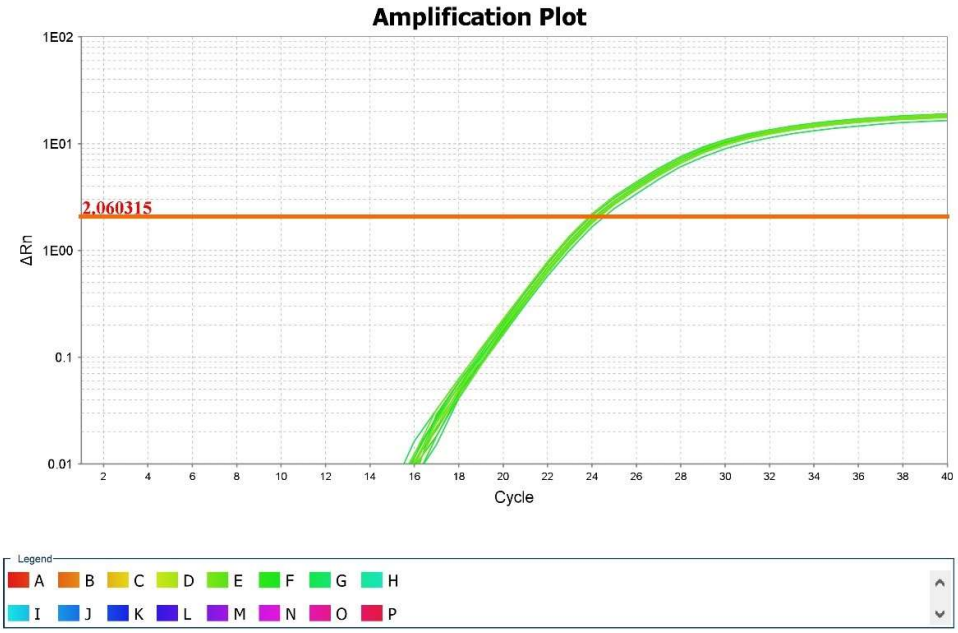


Figure A7-9: Amplification plot of TGF-β qPCR samples.

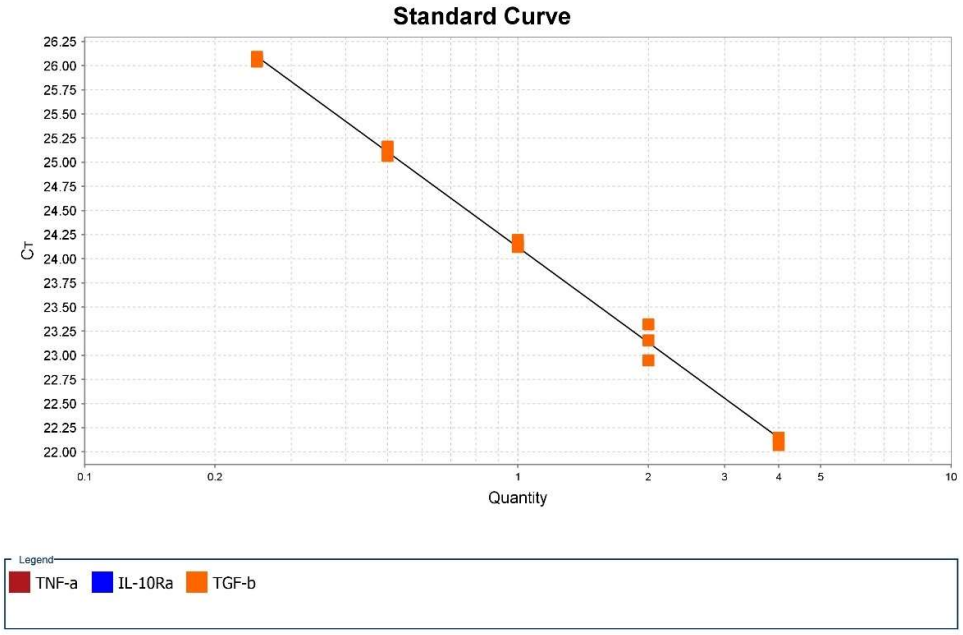


Figure A7-10: Standard curve of TGF-β qPCR samples.

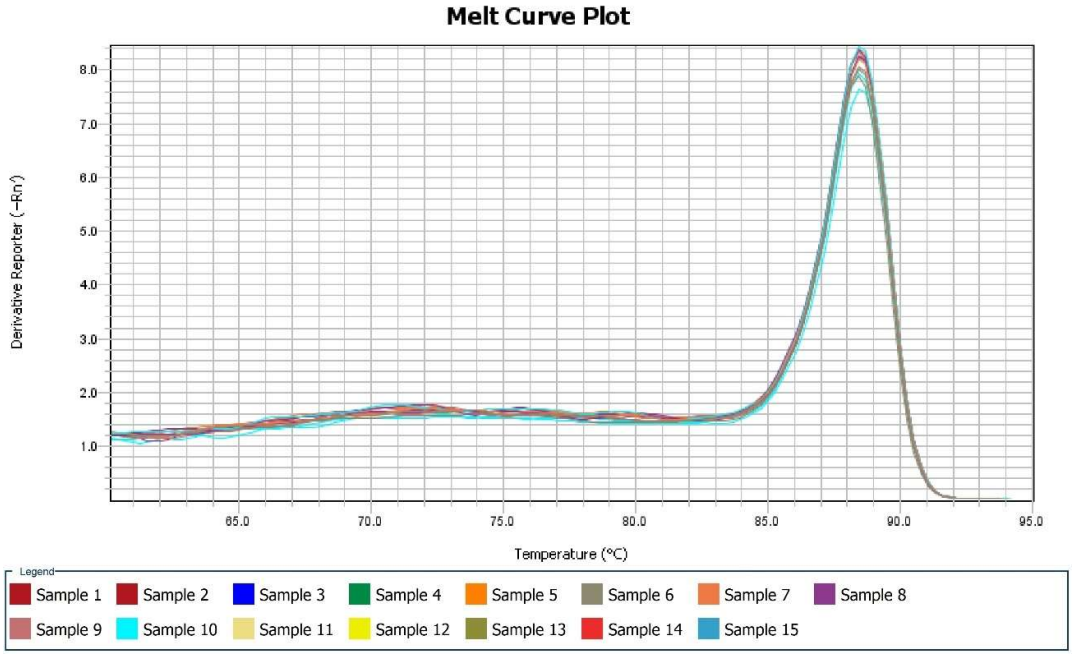


Figure A7-11: Melting curve of TGF-β qPCR samples.

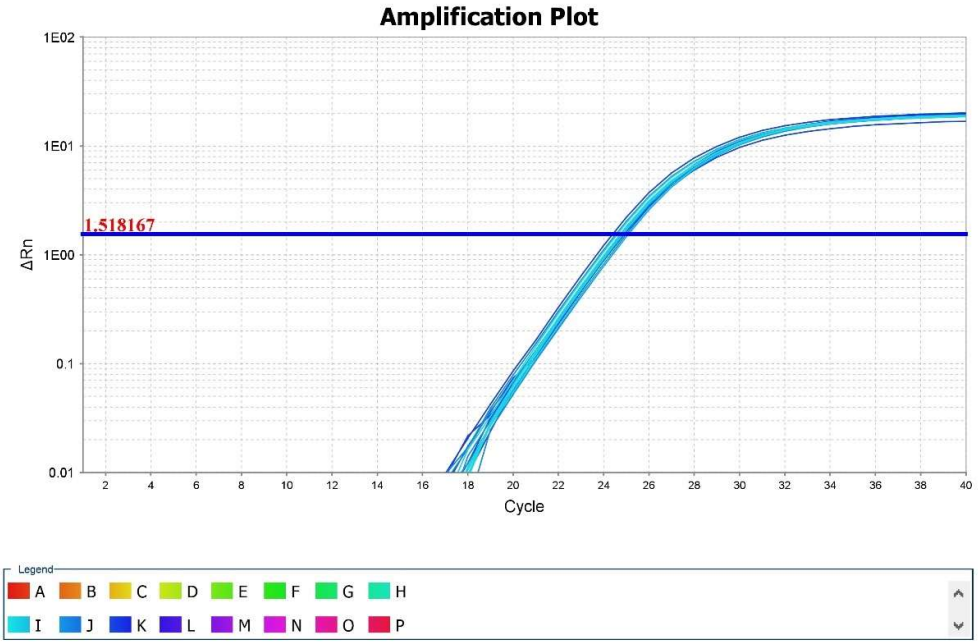


Figure A7-12: Amplification plot of IL-10Rα qPCR samples.

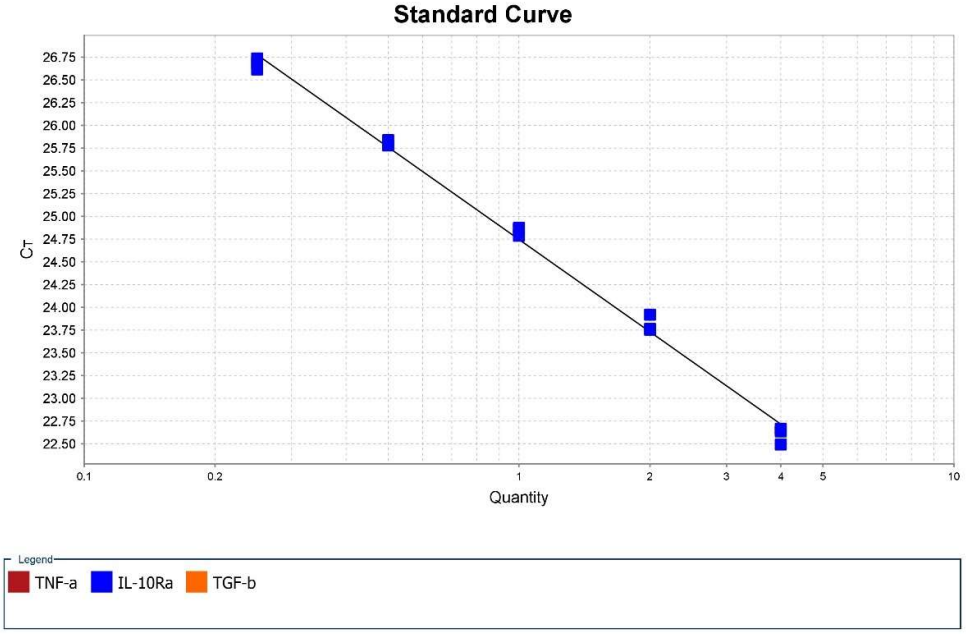


Figure A7-13: Standard curve of IL-10Rα qPCR samples.

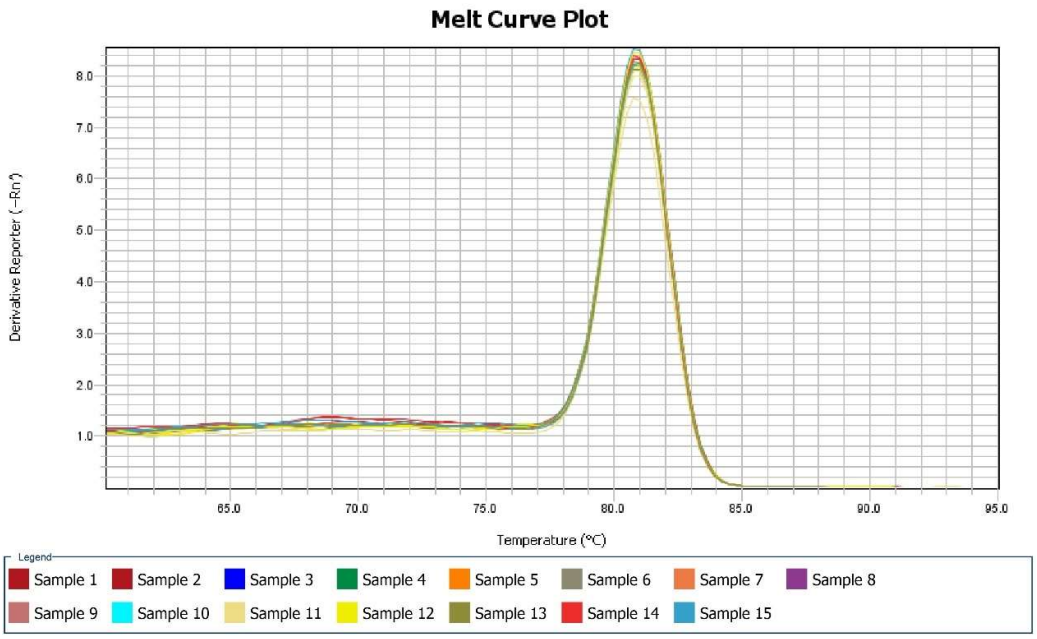


Figure A7-14: Melting curve of IL-10Rα qPCR samples.

Appendix A8: NO assay protocol

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 10 ul 100 mM NaNO2 in 1 ml medium ➔1 mM NaNO2
3. Make the standard curve:

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

The reaction:

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

Griess reagens:
Griess A: 2gr Sulfanilamide en 5 ml fosforzuur in total volume of 100ml MQ
Griess B: 200mg N-(1-Naphthyl)ethylenediamine dihydrochloride 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 A9: Flow cytometry protocol

FACS Protocol IL-10 alpha receptor in RAW cells

EDTA /Lidocaine solution:

- 10 mM EDTA (#406)
- 4,4 mg/mL Lidocaine (#56A)
- 10% FBS, added just before use
- dPBS

Staining buffer (SB):

- dPBS
- 2% FBS
- 5 mM EDTA

Antibody	Name	Cat#	Lot	Firma	Location	Dilution	Notes
1e AB	IL10Ra (M20)	sc-985	H2907	Santa Cruz.	Box 1 D7	1:50 in SB	
2e AB	GARPO AF488	A11034	2211209	ThermoFisher Scientific	Box F	1:200 in SB	Keep dark, fluorescent

Control

- Negative: Unstained cells. Don't add antibodies, instead add staining buffer.
- Background control: Only secondary antibody.
- Positive control: RAW M1 or M2 cells.

Protocol

Day 1: Seeding in 6 wells plate

- Seed cells in a 6-well plate in a density of $4*10^4$ cells/cm2 (for next day).
- Incubate at 37C overnight.

Day 2: Stimulation/Polarization

- Polarize the cells depending on the research question and cells.
- Incubate at 37C for 24h.

Day 3: Staining (in biochem lab)

Cell detachment

- View cells under microscope, check whether they are "happy," and take pictures.
- Take medium off, wash cells with dPBS.

- Add 2 mL of EDTA/Lidocaine solution to each well. Incubate for 30 mins at 37C.
- View plate under microscope to check whether they are happy.
- Pipette the medium up and down firmly in each well, to detach the cells from the bottom of the plate. View under microscope to check whether cells are detached and happy.
- Take 1 mL out of well, put in 2 mL Eppendorf.
- Repeat firm pipetting and check under microscope whether all cells have detached. Transfer last mL to the Eppendorf.
- Centrifuge each Eppendorf for 4 mins, 300g at RT.
- Check pellet, and discard EDTA/Lidocaine solution. Tap cells loose and add 1,5 mL of staining buffer.

Cell staining (in human lab)

- Count cells, and seed 200 uL of $25*10^4$ cells/mL suspension into each well of a round bottom 96-well plate.
- Centrifuge the plate for 5 mins at 300g, RT.
- Discard medium by flipping the plate and gently tapping it on a paper towel in one motion. Wash pellet by adding 200 uL staining buffer to each well. Pipette it up and down gently to resuspend pellet.
- Centrifuge plate for 5 mins, 300g, RT.
- Discard supernatant, by turning plate upside down and gently tapping on paper towel in one motion.
- Resuspend the pellet in 25 uL 1:50 diluted primary antibody (diluted in staining buffer) in designated wells, and incubate for 30 mins at 4C.
- After incubation, centrifuge plate for 5 mins at 300g, RT.
- Discard supernatant and wash twice with 200 uL staining buffer.
- Resuspend in 25 uL 1:200 secondary antibody (diluted in staining buffer) in designated wells, and incubate for 30 mins at 4C (Note: keep it in the dark as much as possible -> Use aluminum foil).
- Centrifuge the plate for 5 mins at 300g, RT.
- Wash cells with 200 uL staining buffer, resuspend and centrifuge for 5 mins at 300g, RT.
- Discard supernatant, add 200 uL staining buffer to each well and resuspend.
- Transfer well contents into FACS tubes.
- Centrifuge FACS tubes for 5 mins at 300g, RT.
- Discard supernatant.

Immediate flow cytometry

- Resuspend pellet in 100 uL dPBS, and measure on the cytoflex.

Flow cytometry next day (work in hood for every formaline step)

- Resuspend the pellet in 250 uL 4% formaline to each tube.
- Incubate for 20 mins in the dark, at RT.
- After incubation, add 500 uL staining buffer to each tube, and centrifuge for 5 mins at 400g, RT.
- Discard the supernatant (in specific formaline waste container) and resuspend pellet in 100 uL PBS.
- Store samples overnight in the dark, at 4C.
- (Next day) Before cytoflex, resuspend the cells just before measuring by pipetting up and down.

Appendix A10: Isolation of RNA protocol

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	Confluency	RNA yield (ng/ul)
3T3 6 well (10 cm2)		80-90	113 - 179
RAW 12 well (4 cm2)		80-90	166 - 180

March 2022

Appendix A11: RNA conversion into cDNA protocol

RNA conversion to cDNA

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

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

RT mix:

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

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

Converting RNA tot cDNA:

10 min 20 °C
30 min 42 °C
10 min 20 °C
5 min 99 °C
5 min 20 °C

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

After the reaction is completed:

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

Costs: € 3/sample

Basic materials Promega:

M-MLV Rev Transcriptase
cat nr: M1705 (= 5* 10.000 units)
per 10.000 U → 50 reactions
price: € 175,=/10.000 units

RNasin
cat. nr.: N 2515 (= 10.000 U)
per 10.000 U → 500 reactions
price: € 230,=/10.000 units

Random Hexamers
cat nr.: C1181 (=20 ug)
per 20 µg → 40 reactions
price: € 27,=/ 20 ug

dNTP's dATP, dCTP, dGTP, dTTP set 100 uM/nucl.
cat nr: U1245
each nucl. 400 ul → 5.000 reactions
price: €240,=

Appendix A12: qPCR + standard curve protocol

qPCR

qPCR + STD Curve.xlsx

Reserveren PCR machi iagenda.com (http://iagenda.com/) inloggen: c.reker-smit@rug.nl ww: NDTqpcr3211!

Creating Standard Curve:

1. Pool the undiluted cDNA of the samples that are assigned for the STD CURVE
2. Create the Standard Curve according to the tabel below:

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 (d 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 qPCR 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		

	1		2		3		4		5		6		7		8		9		10		11		12		
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	Primer
A	STD 4	STD 4	STD 2	STD 2	STD 1	STD 1	STD 0.5	STD 0.5	STD 0.25	STD 0.25	NC	PC	S1	S1	S2	S2	S3	S3	S4	S4	S5	S5	S6	S6	
B	S7	S7	S8	S8	S9	S9	S10	S10	S11	S11	S12	S12	S13	S13	S14	S14	S15	S15	S16	S16	S17	S17	S18	S18	
C	S19	S19	S20	S20	S21	S21																			
D	STD 4	STD 4	STD 2	STD 2	STD 1	STD 1	STD 0.5	STD 0.5	STD 0.25	STD 0.25	NC	PC	S1	S1	S2	S2	S3	S3	S4	S4	S5	S5	S6	S6	
E	S7	S7	S8	S8	S9	S9	S10	S10	S11	S11	S12	S12	S13	S13	S14	S14	S15	S15	S16	S16	S17	S17	S18	S18	
F	S19	S19	S20	S20	S21	S21																			
G	STD 4	STD 4	STD 2	STD 2	STD 1	STD 1	STD 0.5	STD 0.5	STD 0.25	STD 0.25	NC	PC	S1	S1	S2	S2	S3	S3	S4	S4	S5	S5	S6	S6	
H	S7	S7	S8	S8	S9	S9	S10	S10	S11	S11	S12	S12	S13	S13	S14	S14	S15	S15	S16	S16	S17	S17	S18	S18	
I	S19	S19	S20	S20	S21	S21																			
J	STD 4	STD 4	STD 2	STD 2	STD 1	STD 1	STD 0.5	STD 0.5	STD 0.25	STD 0.25	NC	PC	S1	S1	S2	S2	S3	S3	S4	S4	S5	S5	S6	S6	
K	S7	S7	S8	S8	S9	S9	S10	S10	S11	S11	S12	S12	S13	S13	S14	S14	S15	S15	S16	S16	S17	S17	S18	S18	
L	S19	S19	S20	S20	S21	S21																			
M	s1	s1	s2	s2	s3	s3	s4	s4	s5	s5	s6	s6	s7	s7	s8	s8	s9	s9	s10	s10	s11	s11	s12	s12	
N	s1	s13	s2	s14	s3	s15	s4	s16	s5	s17	s6	s18	s7	s19	s8	s20	s9	s21	s10	nc	s11	S23	s12	NC	
O	s13	s13	S14	S14	S15	S15	S16	S16	S17	S17	S18	S18	S19	S19	S20	S20	S21	S21	S22	S22	S23	S23	NC	NC	
P																									
	1		2		3		4		5		6		7		8		9		10		11		12		