Potential anti-fibrotic effects of interleukin-10 on fibroblasts

Author: Manolito Musters

Student number: s4447743

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Supervisor: Prof. Dr. Klaas Poelstra

Department: Nanomedicine & Drug Targeting (NDT)

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Abstract

Liver fibrosis is associated with many health problems like cirrhosis and liver cancers. This is caused by excessive accumulation of extracellular matrix(ECM) in the liver area after inflammation has occurred. This could be caused by alcoholism, obesity, diabetes or viral infections like hepatitis B and C. due to different cellular pathways is ECM formed and one of the main components are collagen, and also hyaluronic acid. In this thesis will the role of the anti-inflammatory cytokine IL-10 on liver fibrosis be researched on NIH 3T3 cells. Three assays: the NO assay which measures NO production which is a good indicator of inflammation. A collagen DHPAA assay was performed to see if collagen production is influenced by IL-10 and at last a qPCR measurement was performed to see if hyaluronic acid producing genes are upregulated under the influence of IL-10.

Findings showed that Il-10 had no distinctive effect on the NO release in the cell, because on its own it being an anti-inflammatory cytokine had no response. But in combination with INF-γ which induces inflammation, it gave no distinctive decrease or increase in NO release. The collagen DHPAA assay did not give sufficient results due to the positive control not being active enough and further testing did not give usable results. qPCR measurement gave upregulation of HAS1-3 genes when influenced under INF-γ + IL-10 + LPS which could indicate that more hyaluronic acid is being produced. Further studies are necessary to support this finding.

Introduction

Liver fibrosis

Liver fibrosis is excessive accumulation of extracellular matrix (ECM) proteins like collagens that occurs in many liver diseases[1]. After a long state of liver fibrosis the end state is cirrhosis when most states of morbidity and mortality takes place. But not only cirrhosis is the cause of problems, liver related problems increase exponentially with the progression of liver fibrosis. [2] Such problems are the likes of: hypertension, hepatocellular carcinoma or liver failure as a whole. Currently there are treatments to treat the underline causes to liver fibrosis but a direct therapy has not yet been discovered[2]. Currently Cirrhosis is the 11th most common cause of death followed by liver cancers at 16th place[3] Also liver transplantation is the second most common solid organ that is transplanted nowadays with 24% of organs being transplanted in 2022[4]. All of this is a still in a increase with diseases as obesity and diabetes still on the rise as of today who also contribute to liver fibrosis. Current causes of Liver fibrosis are the aforementioned diabetes and obesity but it can also as a response in viral infections like Hepatitis B and C. Overconsumption of alcohol which causes alcoholic liver disease (ALD). All of this underlines the importance of a effective therapy against liver fibrosis as it becomes a bigger thread against our health.

Cellular pathway of liver fibrosis.

To understand how create a therapy against the problem of liver fibrosis, it is necessary to understand its cellular pathways. This is to know how to approach the problem and if there is more research necessary to come up with a solution for the problem. Liver fibrosis occurs after several steps were different cells perform certain actions before actual fibrosis takes place.

Liver fibrosis occurs after years of persistent organ damage or very repetitive damage leading to a continuous inflammatory response inside the liver. This continues for years or even decades and in this period fibrosis of the liver is slowly progressing.

After acute inflammation following tissue damage, different cyto- and chemokines are released into the blood. Pro-inflammatory cytokines that are secreted are the likes of transforming growth factor beta (TGF-β), Interferon-gamma (INF-γ) or platelet derived growth factor (PDGF). blood clotting inducing mediators and matrix metalloproteinases are released to catch immune cells to their site of action and activate the innate immune system through antigen presentation[5]. The threats are eliminated and after inflammation, Phagocytes clean up the debris in the target area and also release anti-inflammatory cytokines[6].This leads to macrophages appearing on the stage which on their turn can activate stellate cells (HSC) and turn them into myofibroblasts. HSC's can also be activated by cytokines from immune cells or by DAMPs or PAMPs[7].

After activation myofibroblasts produce ECM but also activate other HSC's and produce cytokines and chemokines that attract immune cells[5]. This can cause an indefinite inflammatory reaction which leads to replacement of functional tissue into fibrotic tissue which is on itself not functional. Normally this stops after the wound has been remodeled but in a state of liver fibrosis the myofibroblast stay in the area and keep producing ECM. This principle together with constant damage from outside factors keep the kidney in an inflamed state while continuous production of non-functional ECM leads to damage to the organ.

IL-10's potential anti fibrotic properties

IL-10 is an anti-inflammatory cytokine that has a big role in reducing the inflammatory response after infection or cell damage[8]. It provides a negative feedback loop for inflammatory cytokines so wound healing can take place after disabling the thread at the site of action. Prevention of inflammation is one of the ways to prevent fibrosis to be formed. Another way is that IL-10 producing gene expression in adult wounds leads to more regenerative healing on its own [9]. This is done for example by potentially increase the High molecular weight-Hyaluronic acid (HMW-HA) in the ECM[9]. HMW-HA is anti-fibrotic on its own and also harbors anti inflammatory properties itself due to promoting collagen type III production and suppressing platelet formation and growth factor release[10]. All of these factors make IL-10 a possible target for treatment against liver fibrosis. However there are risks with IL-10 due to its immunosuppressive activity when administered for a longer period. Especially when liver fibrosis is induced by an antigen as is in the case of Hepatitis B and C patients. This causes potential prevention of liver fibrosis but gives the virus in this case more chance to damage the liver.

Inflammatory response

An inflammatory response can have different causes and can be caused by certain compounds or environments in which the cells are present. One of these compounds is Lipopolysaccharide (LPS) and this is a compound produced by gram negative bacteria. LPS is a molecule that consists of a short lipid part and a long polysaccharide chain that is present in the vast majority of gram negative bacteria like *Escherichia coli* or *Salmonella enterica*[11]. Interaction with LPS leads very often to an immune response because LPS is regarded as an endotoxin. It induces an inflammatory reaction in fibroblasts via Toll like receptor-4 (TLR4)[12]. This induces reactions were among others NO is produced which is measurable. TLR4 is also related to collagen I production, thus LPS can be used as a compound to induce inflammation, which should lead to an increased collagen production. NO is measurable from cells with a simple assay[13].

Extracellular matrix

Collagen is a very important substance in the formation of extracellular matrix. It is a protein that consist of a triple helix formation where alpha subunits are entangled around each other to give

strength and stability to this structure. Together with glycosaminoglycans it forms the connective tissue produced in the formation of scar tissue. Fibroblasts produce collagen when activated by e.g. immune cells or cytokines. If there is a dysregulation of collagen production from fibroblasts could it lead to fibrosis[14].

One of the most important glycosaminoglycans is hyaluronic acid (HA). This is a polysaccharide which is due to its carboxyl groups very negatively charged and very hydrophilic[15]. This leads to a attraction of water into the extracellular matrix that makes it a lot more flexible and prevents the matrix of being stiff and rigid. Also the difference in size gives different properties to the function of HA. High MW-HA stimulates macrophages in more inflammatory activity than low MW-HA which lead to macrophages enhancing pro-resolving functions. Also high MW-HA which forms a coat around the cells are responsible for a higher fluidity in the cells[15]. This is also in the very rigid form of liver fibrosis where HA is present in lower concentrations than in other types of ECM[16]. HAS1, HAS2 and HAS3 are notable genes that are responsible for the production of HA. These genes are responsible for differences in sizes of the HA chains were it was found that HAS3 produced smaller HA molecules than HAS1 and HAS2[16].

Aim of the experiment

IL-10 is a anti-inflammatory cytokine which has possibly anti fibrotic effects. In this thesis the effects of IL-10 on fibroblast activity is being tested while measuring different parameters. Parameters are the NO production, gene expression of HAS(1-3) and collagen production of the fibroblast cells. The proposed research question is thus: What are the effects of IL-10 on liver fibrotic properties in 3T3 fibroblastic cells?

Materials and methods

3T3 cells for research

For the experiments in this research paper NIH/3T3 cells were used for the project

Reagents

murine IFN-γ (CAT:315-05-100UG), Human IL-10 (CAT: 210-10), murine IL-10 (CAT: 210-10-10UG) murine PDGF (Cat: 315-18-10UG), Human TGF-β (CAT:100-21C-10UG), lipopolysaccharides from Escherichia coli (Merck, L2880-100MG), Collagen type 1 rat tail (4,24mg/mL stock, Corning 354236) collagenase for *clostridium histolyticum* (CAS no: 9001-12-1), 3,4-dihydroxyphenlyacetic acid (DHPAA(CAS no: 102-329))

NO assay

For the NO assays different time tables and thus were cultured with different densities depending on the experiment. NO assays were performed via the Griess[17] method with the medium above the cultured cells to measure the excreted NO. In experiment 1 3T3 cells were cultured in a 12 wells plate with a density of $5*10^3$ cells/cm^{2}for 24h. After cultivation of the cells 2 cells remained unstimulated and the rest was stimulated with 40ng/ml PDGF in new medium. After 3h of stimulation 2 wells got new medium again with 10ng/ml LPS and in 2 wells just 0,1 µL of 100µL solution was added. After 21h The NO samples were collected and the day after the NO assay was performed.

The rest off the NO assay based experiments had a different time table. Here it was chosen to culture the cells for 24h in a 12 wells plate in a density of $2*10^4$ cells/cm^2 and after 3h stimulate the cells with the stimulus of choice. In experiment 2 these stimulants were PDGF and INF-γ in concentrations of 40ng/mL for PDGF and 40ng/mL for INF-γ . After 3h of stimulation, a concentration of 100ng/mL LPS was added to a control group, PDGF stimulated and to INF-γ stimulated cells, while another set was present without LPS stimulation. Incubation of 24h followed after this and samples were taken and after 72h incubation samples were taken and tested for NO production.
Experiment 4 had the same time table as experiment 3 but the stimulants and cell density differed. The cells were cultured for 24h at a density of $3*10^3$ cells/cm^2. There was a negative control, a INF-γ group (40ng/mL), a IL-10 group (30ng/mL) and a INF-γ + IL-10 (40ng/mL + 30ng/mL) group and. After 3 hours of stimulation, The INF-γ group, the IL-10 group and the INF-γ + IL-10 group received 100ng/mL LPS stimulation and the negative control did not. Every group except the INF-γ group also had a second group that was tested without LPS addition after 3h. After 72h incubation NO samples were collected and not after 24h as in experiment 2.
Experiment 6 followed the setup of experiment 4 but differed in cell density. The cells were cultured again in 10 of the 12 wells for 24h in a density of $3*10^3$ cells/cm^2 and in the remaining 2 wells the cells were cultured at $2*10^4$ cells/cm^2 just as in experiment 4. Cells were stimulated with respectively: nothing, IL-10(30ng/mL), INF-γ(40ng/mL), IL-10 + INF-γ(30ng/mL + 40ng/mL) and in the wells with lower concentration cells INF-γ(40ng/mL), and after 3h each group had LPS (100ng/mL) added in half of the wells. The NO assays were performed according to the NO Assay protocol (Appendix I).
Experiment 8 was performed along experiment 7 as a control if something unexpected happened in the qPCR measurement. In this experiment 10 wells of a 6 wells plate with cells that were cultured in a density of $2*10^4$ cells/cm^2. After 24h of incubation there were 2 control groups, stimulation with: TGF-β(10ng/mL), INF-γ(40ng/mL) and INF-γ + IL-10 (40ng/mL + 30ng/mL). After 3h one control group and the stimulated groups were again stimulated with LPS (100ng/mL).

Collagen assay

The collagen assays were performed at the same 3T3 cells as the NO assays and is used to determine the collagen deposition of the cells under different setups.

In experiment 3 the experimental setup for the collagen assay were cells that were cultivated for 24h in a 6 wells plate in different densities. two wells had a density of $3*10^4$ cells/cm^2, two wells had a density of $1*10^4$ cells/cm^2 and the last two had a density of $5*10^3$ cells/cm^2. After this incubation period, one well of each density received stimulation of TGF-β(10ng/mL). medium of the $3*10^4$ cells/cm^2 group was collected after 24h for analysis. Medium was collected after 48h for the $1*10^4$ cells/cm^2 group and after 72h for the $5*10^3$ cells/cm^2 group. Analysis was performed according to the collagen assay protocol (Appendix II). For experiment 5 exactly the same setup was used except for a change in the collagen assay protocol. In experiment 3 a borate buffer of 0,5M was used, but in experiment 5 there was chosen for a borate buffer of 0,125M because of a bigger range of collagen measurement. Also the incubation time after the collagenase was added was prolonged from 1h to 20h.

Protein assay

After performing the collagen assay, a protein assay was performed to put the collagen assay into perspective. This assay was performed according to the Bio Rad DC Protein Assay protocol (Appendix IV).

Quantitative polymerase chain reaction (qPCR) measurement.

In experiment 7, 3T3 cells are cultured for 16h in two 12 wells plates in a density of $4*10^4$ cells/cm^2. After incubation the cells were stimulated with different compounds. There was a negative control, stimulation with: LPS, stimulation with TGF-β + LPS, stimulation with INF-γ + LPS and stimulation with INF-γ + IL-10 + LPS. After 3h of stimulation with cytokines the LPS was added to the wells that receive it and this was incubated for 1 hour. with these compounds the samples were collected and RNA isolation. cDNA conversion and qPCR analysis was conducted according to the corresponding protocol (Appendix XI). In each of the samples HAS(1-3) was quantified in comparison with the β-actin housekeeping gene.

Results

NO Assays.

The results section contain the results of the experiment and the raw data of these experiments are listed in Appendix VI-X. All of the experiments are done in duplicate unless stated otherwise. All positive controls worked indicating that the setup worked.

Experiment 1

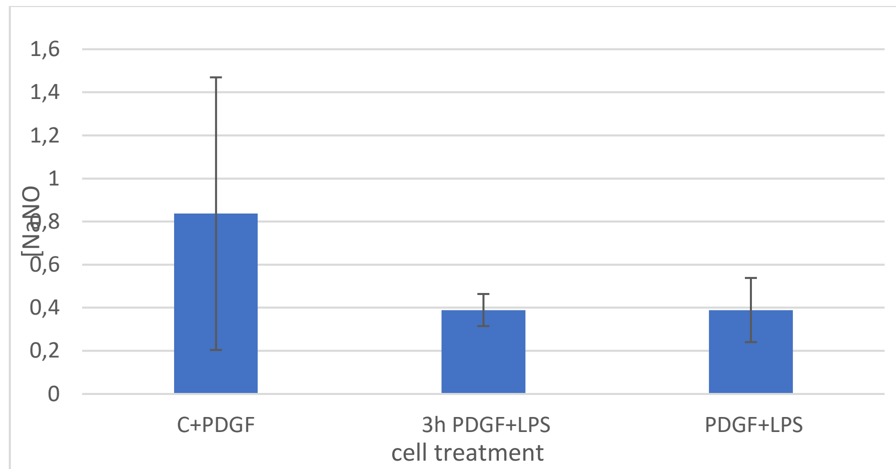


Figure 1: results of the NO Assay performed on 3T3 cells (µM). from left to right the [NO] is shown from only adding PDGF, 3h stimulation of PDGF and new medium with LPS and PDGF with added LPS.

In experiment 1 the effect of LPS was measured with co-stimulation of PDGF on 3T3 cells to possibly increase a response. As seen in figure 1, the control response gave a higher [$NaNO_2$] concentration than the other groups which were stimulated with LPS.

Experiment 2

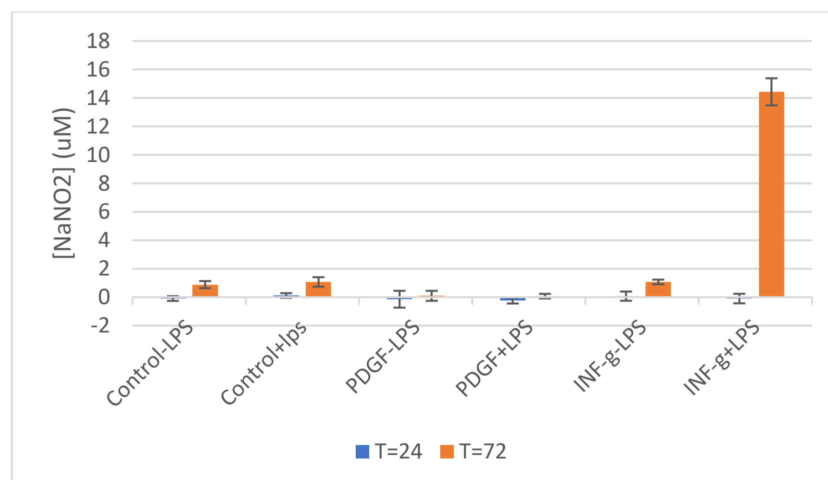


Figure 2: :Results of the NO Assay performed on 3T3 cells (µM). [NO] after stimulation with different compounds as depicted and after 3h with or without adding of LPS to the cells. Time of sample collection is 24h or 72h after LPS stimulation.

In experiment 2, the effect of various compounds was tested after different incubation times. Both the control groups saw an increased [NO] at T=72 compared to T=24h. In this effect there is not much difference visible when comparing the control group with LPS and without LPS.
In the PDGF group, there is almost no NO measurable in both the 24h and the 72h group. There is also no significant difference in LPS or no LPS addition with the PDGF group.
After adding INF-γ there is a big difference in [NO] between the 24h and 72h group, where after 72h the [NO] is much higher in comparison. Also when adding LPS to the INF-γ group, there was a big increase in [NO].

Experiment 4

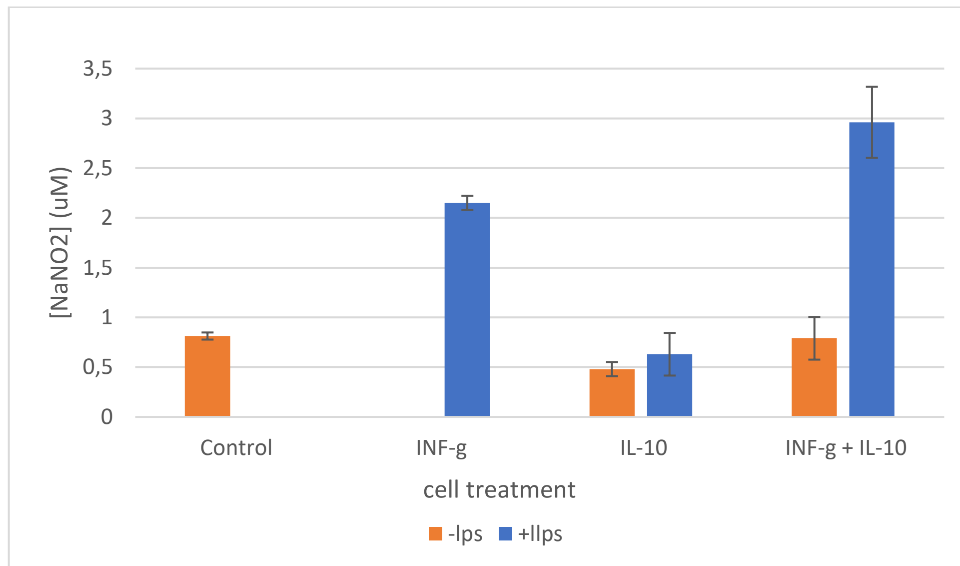


Figure 3: Results of the NO Assay performed on 3T3 cells (µM). [NO] after stimulation with with different compounds as depicted and after 3h with or without adding of LPS to the cells. The control is only present without LPS because with has not been tested. The INF-γ group is only tested with LPS because without LPS was not tested. All samples were taken after 72h of incubation stimulation with compounds.

In experiment 4 different setups were tested with or without the addition of LPS 3h after stimulation. The control group showed almost no release of NO. INF-γ did show a big response after LPS addition. Il-10 showed a slight increase in [NO] after LPS addition, but is far lower compared to the INF-γ with LPS. INF-γ + IL-10 showed a increase in [NO] after LPS addition in comparison without LPS. Without LPS the concentration is only a bit higher than IL-10 but the two are comparable. After LPS addition INF-γ gives a higher [NO] concentration when IL-10 is added than when it is not.

Experiment 6

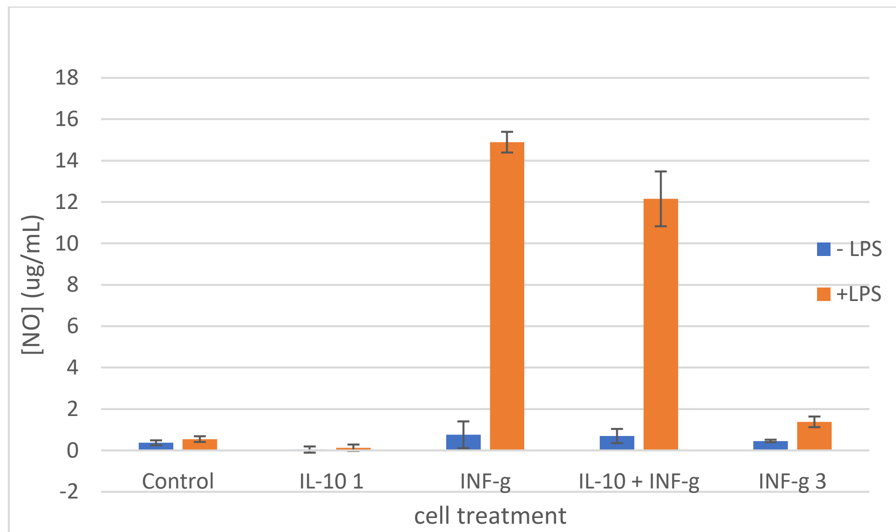


Figure 4: Results of the NO Assay performed on 3T3 cells (µM). [NO] after stimulation with different compounds as depicted and after 3h with or without adding of LPS to the cells. The last sample is the one with a lower cell count as said in the materials and methods. All the samples were tested with and without addition of LPS.

In experiment 6 again the [NO] was determined after addition of cytokines and with or without LPS. All the samples were incubated for 72h after stimulation. In the control group a light elevation is seen after LPS addition, but is very low in comparison INF-γ and IL-10 + INF-γ. IL-10 does not show any NO release or any difference after LPS addition. INF-γ shows a great NO release after adding LPS to the mixture in contrast with the sample without LPS. This same result is when IL-10 is added to the INF-γ but in less extent. The [NO] is lower after LPS addition than when only INF-γ is added. The sample without LPS addition show no NO release just as with the INF-γ and all the other graphs. The sample with the lower cell density gives a lower [NO] after LPS than at the higher cell density. Also in this case without LPS addition there is almost no NO release of the 3T3 cells.

Experiment 8

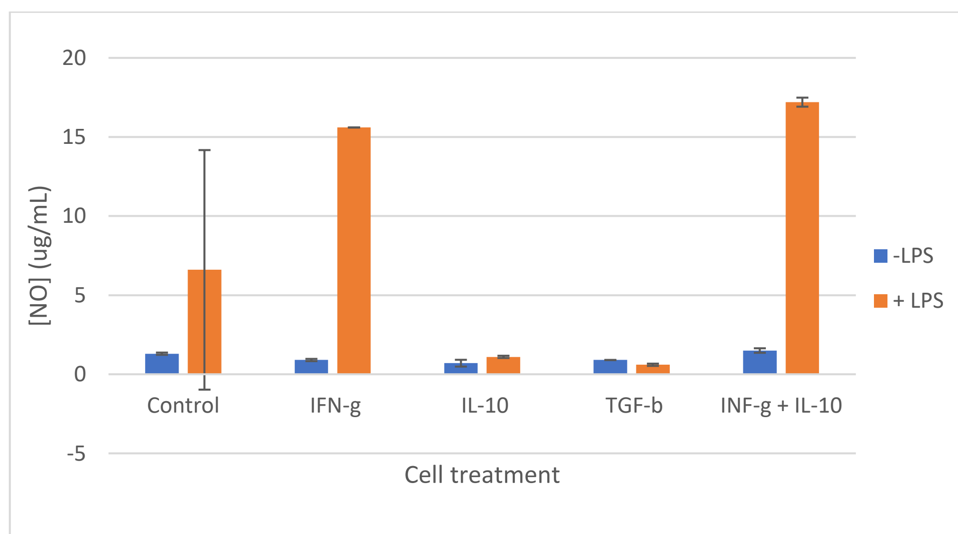


Figure 5: Results of the NO Assay performed on 3T3 cells (µM). [NO] after stimulation with different compounds as depicted and after 3h with or without adding of LPS to the cells.. All the samples were tested with and without addition of LPS.

In experiment 8 again the [NO] was determined after addition of cytokines and with or without LPS. All the samples were incubated for 72h after stimulation. In the control group a big elevation is seen after LPS addition, but is lower in comparison INF-γ and IL-10 + INF-γ. Also the standard deviation is very high and in comparison to other experiments in the same setting this is a high value.INF-γ shows a great NO release after adding LPS to the mixture in contrast with the sample without LPS. IL-10 does show a slight increase in NO release or any difference after LPS addition. There is no increase in NO release after addition of LPS to the TGF-β group. There is even a slight decrease in [NO]. When INF-γ and IL-10 are both added a big increase is shown after the addition of LPS. This has in this experiment the higher value. When LPS is not added is also releases more NO than the rest of the test groups.

Collagen Assay results

The results of the collagen assay are depicted below in graph form. All the raw data and the Collagen Assay protocol can be found at Appendix VIII. As stated in the materials and methods there are two differences between experiment 3 and 5, whereas experiment 3 uses a borate buffer of 0,5M and experiment 5 a borate buffer of 0,125M. In experiment 3 were the samples after adding collagenase incubated for 1 hour, and in experiment 5 the incubation period was 20 hours.

Experiment 3

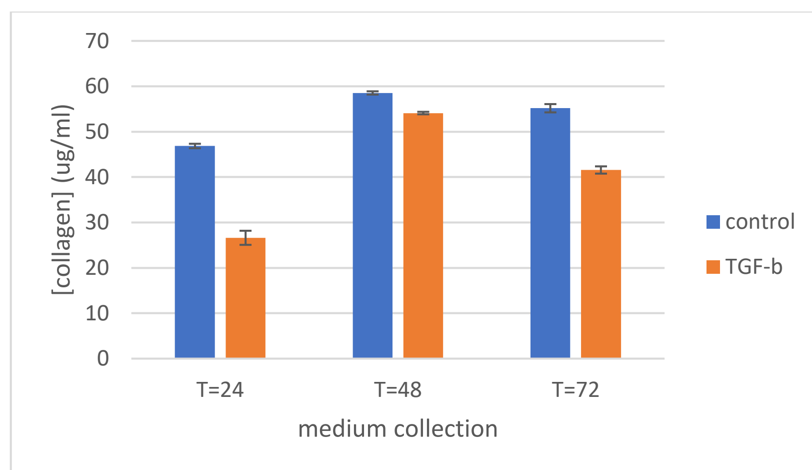


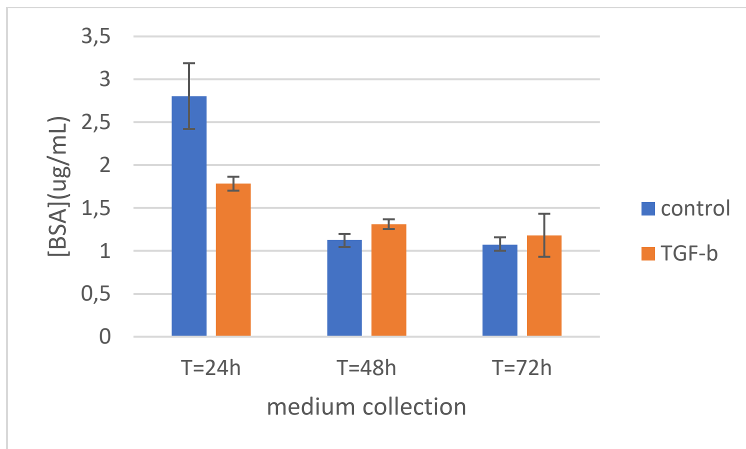
Figure 5: collagen assay results in (μg/mL) after stimulation with TGF-β on 3T3 cells. Samples were tested on different timepoints after stimulation with TGF-β. All samples had a control group and a stimulated group of TGF-β 24 hours after incubation. Timepoints are taken after stimulation with TGF-β

In the results of figure 5 is visible that there was a collagen production in all the test groups. In all the groups is also visible that less [collagen] is measured after administration of TGF-β than in the control group. There is in general more collagen found in the cells after 48h collection and 72h collection than in the 24h collection. There is a slight decrease in [collagen] between 48h and 72h.

Figure 6: protein assay performed on the cells used for the collagen assay. Bovine serum albumin is used as a reference for the standard curve.

In the results of figure 6 is visible what the protein concentration was in the cells that underwent experiment 3. This was used as a reference for if any unsuspected results showed up. In the results is visible that the control group of T=24h had a larger protein concentration than the rest of the

samples. Also the TGF-β at T=24h group had a slight increase compared with the other collection times.



Experiment 5

Experiment 5 was a collagen assay performed with a borate solution of 0,125M instead of 0,5M. Also the incubation after adding the bacterial collagenase was prolonged form 1h to 20h to increase fragmentation and thus a higher signal. Unfortunately was this experiment unsuccessful because the control at 24h after stimulation had such a big standard deviation, and both the TGF-β samples at T= 24h and 48h did not give a signal at all. Only T=72h gave a normal signal but the other samples gave such unusual signals that this experiment can be excluded from the total project. The reason for tis could be that the DHPAA could have been exposed to light and did not participate in the reaction in the expected manner.

qPCR results

Experiment 7

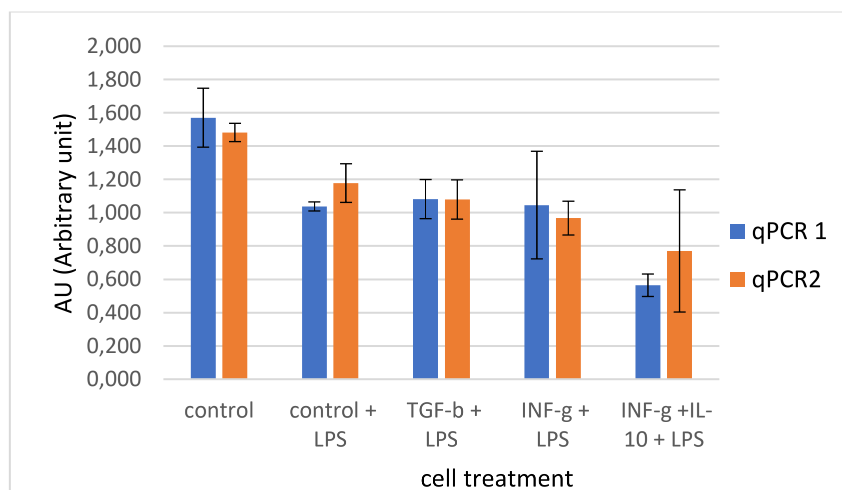


Figure 7: qPCR results of β-actin expression in 3T3 cells. B-actin expression show in different conditions. This graph is used as a reference for the HAS1-3 gene expressions. This experiment was conducted twice.

In figure 7 is seen how the housekeeping gene β-actin is expressed under different treatments. This was done twice. Visible is the difference in expression between the control group without LPS and the rest of the result. Both times the control had a higher β-actine expression than the rest of the

conditions. The other way around can be said for the group that was treated with INF-γ + IL-10 + LPS. This group showed twice a lower expression of the β-actin gene, however the second time it was with a large standard deviation. Every other group fluctuated around the 1,0.

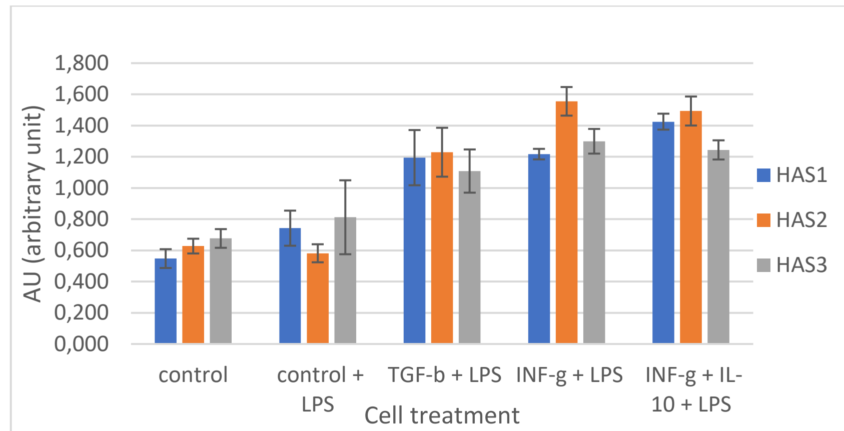


Figure 8: qPCR results of expression of 3T3 cells. HAS 1-3 mRNA expression relative to β-actin housekeeping gene. With different treatments. Cells were stimulated for 3 hours with cytokines and afterwards potentially stimulated with LPS for 1 hour.

In the results is visible that there is a general trend in gene expression. All the HAS genes have roughly the same expressions with some fluctuations among different situations. The control group has in general the lowest expression pf HAS genes, except for HAS2 when LPS was to a control group. The TGF-β group had also an increase in every gene in comparison to the two control groups. The INF-γ group had an increase in comparison to both control groups and the TGF-β group, with HAS2 having the highest expression. When IL-10 was added to INF-γ and LPS the result was almost the same as with the INF-γ group. HAS1 expression was higher in the IL-10 group than in the INF-γ only group, but further on the results were very similar.

Discussion

Experiment 1.

First was stated that we needed to induce an inflammatory reaction in the 3T3 cells before we could test the influence of IL-10. For this the NO assay was chosen because it is a quick and easy method to determine Inflammation by NO production levels. In experiment 1 we saw that the control group had a higher [NO] than the other two groups that were stimulated with LPS. Both the 3T3 cell that have had 3 hour of PDGF stimulation + LPS and constant PDGF and LPS stimulation had about half of the amount of NO production as the control + PDGF group. Normally you would expect NO increase after an inflammatory agent was added but in this case it was the other way around. This could be perhaps explained by a potential lower cell count after the addition of LPS because the [NO] was twice as high in the control group, but in comparison to the positive control was it also not very high (Appendix 6). To check if this was the case we could have performed a viability assay, such as a MTT assay. In this assay the water-soluble yellow dye MTT is converted by mitochondrial reductase into the insoluble formazan. Formazan can be solubilized and determined with an optical density of 570nm[18]. After this experiment it was found that 3T3 cells can get inflamed after exposure of a longer time[19].

Experiment 2

After Experiment 1 it was determined that the 3T3 cells were stimulated with LPS as an inflammation inducer and INF-γ as a pro inflammatory cytokine to enlarge the chances to see an inflammatory reaction. To enlarge our chances it was also chosen to increase the cell density because a lower amount of cells could have been the reason for the contradicting results of experiment 1. The experiment required the cells to incubate 24 hours after cytokine stimulation or 72h to see if there was a difference if cells were exposed for a longer time. This indeed had an effect on the release of NO out of the cell. Where just like in experiment 1 the cells that were incubated for 24h showed almost no NO production, showed cells that were incubated for 72h an increased NO release. The control group with and without LPS both released more NO than in experiment 1. The cells which were stimulated with PDGF showed no NO production even after 72h. This is in contrary with what was expected because PDGF increases cell proliferation and thus more cells were potentially there to produce NO, but the cells still did not produce NO.

The biggest NO production was seen at the stimulation of INF-γ + LPS addition after 72h. After 24h there was no NO production measurable but after 72h there was a lot to measure. This was in line with the expectation because INF-γ is pro-inflammatory and it was also expected that longer incubation should lead to higher NO production.

Experiment 3

After this successful finding it was chosen to perform a collagen assay on the 3T3 cells to see if these big inflammatory responses had an influence on the production of collagen by 3T3 cells. For this experiment was chosen to had two groups: a control group and a group stimulated with TGF-β where it is known that TGF-β is a promotor of the HAS1 gene expression[20]. This assay was primarily meant to see if this is a viable assay and TGB-β was used as a positive control. All the cells were incubated after stimulation with TGF-β by changing the medium with respectively 24h, 48h and 72h.

The calibration curve was correct and usable after testing (Appendix VIII). However the results gave a different picture than we expected. The 24h incubated cells indeed gave less collagen deposition then the 48h and 72h which was to be expected because cells had less time to produce collagen. There was no difference in collagen production between 48h and 72h in the control groups but slightly less even in the 72h group which was stimulated with TGF-β compared to the 48h TGF-β

group. The second thing that was evident after testing is that TGF-β stimulation gave less collagen production in the cells although it is stated differently in literature[20]. Something that has to be stated is that when before performing the assay a calibration curve was made and tested. When performing experiment 3, the collagenase was not added to the collagen calibration curve, and thus could the 3,4-DHPAA not react with the collagen fragments. So the calibration curve for this test did not work, therefore it was decided to use the previously made calibration curve to quantify the samples. This was actually made from the same line as the failed curve and thus it was not chosen to repeat the whole experiment. After this experiment it was decided to perform the experiment again.

The protein assay that was done to put the results into perspective gave mixed results. Where the later collection points showed corresponding results, showed T=24h a different story. At this time point was less collagen found in the cell. However the protein assay showed more protein that was present. This was an unexpected result which could not be clearly explained, thus was chosen to perform the experiment again.

Experiment 4

After the results of experiment 2 we wanted to first, find out if the effect of INF-γ is consistent and reproducible and second, what the effect is of IL-10 on the NO production. The experiment was performed at the normal cell density like it was mentioned in the materials and methods section.

In the results is once again visible that the INF-γ when stimulated lead to an increased [NO] in the cell medium. However the concentration was way lower than in the previous experiment and this can be traced back to the lower cell density. The control group once again showed some NO excretion but less than in experiment 2. The trend of the experiment is the same in comparison to experiment 2 but the absolute values are lower.

IL-10 gave similar responses as the control. This is as predicted because IL-10 has anti-inflammatory properties. Also with extra addition of LPS after 3h was there not much more NO release than without the NO. When added next to INF-γ gave it a bigger response than INF-γ without LPS in experiment 2 which is surprising because IL-10 is an anti-inflammatory cytokine. With LPS though, it is a completely different story. In this experiment it gave a stronger response than the sample which was stimulated with INF-γ alone. Which is again surprising due to the anti-inflammatory response.

Experiment 6.

In experiment 6 the aim was to compare the two different cell densities of the INF-γ samples from experiment 2 and 4. The results showed a low but comparable [NO] in both the control with and without added LPS. The same can be said about the samples where IL-10 was added after incubation.

INF-Y gave as expected again with the addition with LPS an increase in NO release witch was comparable with the one from experiment 2. The sample without LPS added showed some production but also had a high standard deviation thus cannot be said for certain that in this instance only INF-γ induced NO production. When adding IL-10 to INF-γ not much NO was produce but again just like in experiment 4 a big increase was visible after addition of LPS. But in this instance the [NO] was lower than in the samples with only INF-γ and LPS in contrary to experiment 4. At this point it cannot be concluded what the role of IL-10 is in the production of NO because the amounts of production in comparison to INF-γ are mixed. To compare with experiment 4 there was also a group with a lower cell density stimulated with INF-γ. This gave a similar response as in experiment 4 where both values were around 2,0 µg/mL NO which was as expected.

Experiment 8

Together with the qPCR measurement there was a 12 wells plate prepared to run alongside the qPCR experiment. This was done to control the qPCR results and if any surprises came up, it could be checked if the NO production was in line with the findings of the previous experiments.
This NO assay came up with “normal” results compared to the before gathered data from previous experiments. The only noticeable things from this experiments were that the control group with LPS addition gave a huge increase in NO production in comparison to previous experiments. However, the standard deviation was also very large thus this outcome is probably the result of a pipetting mistake. The other noticeable thing is that the INF-γ + IL-10 with LPS addition had this time a larger NO production that INF + LPS . The difference between the two is not quite big, so according to this experiment no certain conclusion can be drawn from the effect of IL-10 on the NO production. TGF-β was also tested for the first time but did not give rise to a increase in NO production which is a logical outcome due to its anti-inflammatory properties[21].

Experiment 8

The last experiment that was performed was to see what the effect of different cytokines was on the HAS(1-3) genes. This was done also because the collagen assay did not give sufficient results. Chosen was to compare the HAS(1-3) to a housekeeping gene β-actine for normalization. Also the β-actin housekeeping gene was tested twice because of fluctuation of expression in the different cell cultures. The control group had the most β-actin expression with and without LPS. All the other samples had the same amount of expression except for the IL-10 + INF-γ + LPS group in both tests. This could perhaps be because of an instability of β-actin as a housekeeping gene because the same result was found under every condition roughly, which makes it unlikely to have happened because of human error.

After this normalization experiment were the HAS 1-3 genes tested for expression. It was chosen not to compensate for fluctuations because of the wide range of the β-actin expression. After analysis it was concluded that the HAS genes all had similar gene expressions within their respective setup. The control showed the lowest expression as expected, because the 3T3 cells had no reason to form extra collagen. After adding LPS the HAS genes showed a slight increase in expression which also is logical as there is a inflammatory inducing agent present. But both these groups the control and LPS added groups had a lot less expression then the β-actin in comparison. TGF-β + LPS had a slight upregulation compared to the control and the β-actin but nu as high as expected. This is because TGF-β normally is involved with new fibrotic tissue formation and upregulates the HAS genes[22]. The samples that were stimulated with INF-γ and LPS had a also a slight increase in HAS expression. This was as suspected because in the NO assays INF-γ showed pro-inflammatory effects, but not much after short exposure. So an increase is visible especially when comparing it with the β-actine results. At last we saw a big increase in HAS expression after stimulating with INF-γ, IL-10 and LPS. A big increase in expression is visible for all 3 the HAS genes especially when having the β-actin in mind which had a much lower expression. This shows that a possible increase in Hyaluronic acid production could be induced with IL-10 in combination of a inflammatory environment. To fully validate this, this experiment must be conducted multiple times, but due to time limitations this was not possible. For the β-actin validation a MTT assay could be performed to establish the viability of the cells. The cells looked very alike under the microscope in terms of morphology and density so an assay could help to validate the results.

In a follow-up study it can be studied what type of hyaluronic acid is produced under certain conditions. This is because HMW-HA is associated with cell structure and remodeling of cells, and also are associated with anti-inflammatory properties[23].

Conclusion

The results of the NO assay showed that there is no definitive answer to the effect of IL-10 on the NO production of 3T3 cells. IL-10 on its own did not show any response on NO production but did in combination with INF-γ and LPS. INF-γ gave a strong inducing effect on NO production and when IL-10 was added to that mixture it gave both higher and lower results so no definitive conclusion can be drawn about the effect of IL-10 in a NO assay

The calibration line in the collagen DHPAA assay gave correct results that were promising to test the collagen production with. However after testing this gave a different result where the positive control did not respond as expected in the first experiment, and where the second assay did not give any sufficient results at all.

A qPCR run was performed to see if the hyaluronic acid producing genes (HAS1-3) were expressed more when the cells were stimulated with IL-10. This was done only with a combination of INF-γ and when added to INF-γ + LPS it showed a big increase in gene expression in all 3 HAS genes. This could mean that hyaluronic acid is produced more in the cells after stimulating to IL-10, but full disclosure cannot be given hence we tested it in combination with INF-γ and LPS.

For further research it would be wise to take al look at the collagen DHPAA assay and to test more with it to look where the problems that were arising in this thesis came from. Further on it would be necessary to perform more qPCR measurements to verify this result and to test more with IL-10 on its own to see its own effect. As earlier mentioned there is a difference in high and low molecular weight hyaluronic acid and perhaps the effect of those could be researched and as a follow—up it could be tested what kind of hyaluronic acid is formed after IL-10 stimulation.

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Appendix

Appendix I

3T3 Cell culture/harvesting experimental cells

All of these handlings must be performed in a laminar flow hood under sterile conditions.

- Sterilize the aspirator
- remove the medium inside a T25 flask
- Wash cells twice carefully with 5mL PBS and remove the PBS after each wash and sterilize the aspirator in between.
- Add 0,75 mL TEP solution and spread it across the bottom of the flask to loosen the cells from the bottom
- Watch under the microscope if cells have come loose from the bottom.
- Add 5mL 3T3 medium to dissolve the cells in medium and resuspend 3 times roughly to disconnect any clumps of cells.
- Pipette medium with cells from T25 into a 15 mL tube and resuspend to homogenize
- Pipette 8µL from the tube onto a Burker-Turk counting chamber
- Count the cells from three squares. Count only those within the second line, and only from two sides where the cells touch the line and from the two other sides do not count the cells that touch the second line.
- After counting three squares, take the average of these amounts and these are your cells*10^4/cm^2.
- Calculate how many cells you will need for the experiment with regards to the density of your well for an experiment or the density for your cell culture. and how much medium you will need to add.
- Take the calculated amount medium and pipette it into a tube.
- Add the calculated amount of cell suspension
- For a T25, pipette 5mL of new made cell suspension into the new T25
- For a 12 wells plate pipette 1 mL of the new made cell suspension into each well that you want to experiment in.
- For a 6 wells plate pipette 2 mL of the new made cell suspension into each well that you want to experiment in.

Appendix II
NO assay Protocol

All of these handlings must be performed in a laminar flow hood under sterile conditions.

- Watch how the cells look under the microscope and take pictures when abnormalities or differences are present
- Make solution with new medium and the desired concentration of stimulant.
- After cultivation sterilize the aspirator and remove the medium from the wells
- Add new medium on the cells and cultivate them on 37°C.
- Add 3h later if necessary the LPS directly onto the medium.
- Cultivate cells according to experiment respectively 24h, 48h or 72h.
- Pipette 500µL from every well into an Eppendorf tube. After incubation
- Store Eppendorf in -80°C until further use

Protocol NO assay

Materials:

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

Calibration curve of Sodium Nitrite ($NaNO_2$):

1. Prepare stock-solution: 100 mM $NaNO_2$-solution in MQ (0.69 g/100 ml)
   (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

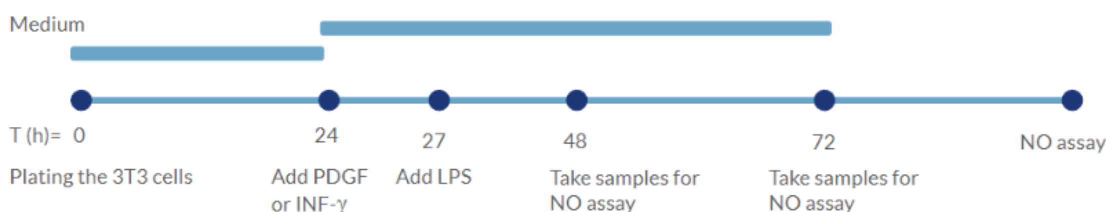

Figure 9: Timeline example for experiments conducted. All experiments were conducted in this matter unless stated otherwise in the materials and methods

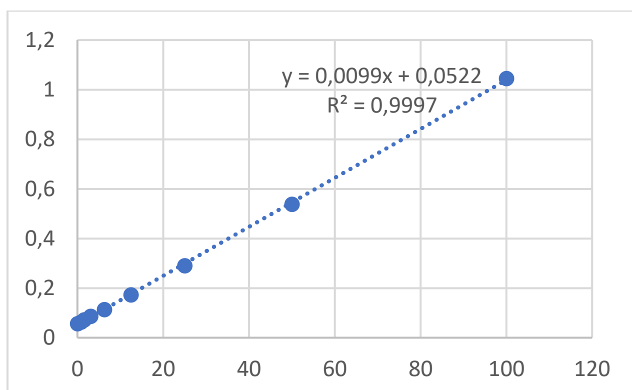

Figure 11: standard curve of NO assay used for [NO} determination

Appendix III

Collagen assay protocol

All of these handlings must be performed in a laminar flow hood under sterile conditions.

- Watch how the cells look under the microscope and take pictures when abnormalities or differences are present
- Make solution with new medium and the desired concentration of stimulant.
- After cultivation sterilize the aspirator and remove the medium from the wells
- Add new medium on the cells and cultivate them on 37°C.
- Cultivate cells according to experiment respectively 24h, 48h and 72h.
- Remove the medium with a sterilized aspirator.
- Wash the cells twice with 1mL PBS
- Add 400µL TEP to the well and shake the wells to remove the cells from the bottom.
- Add 1,5mL DMEM-FBS and resuspend the solution smoothly to get all the cells from the bottom
- Remove the medium and put it into a 2 mL Eppendorf tube and store it until further use in -80°C.

DHPAA Collagen Assay Protocol for 3T3 cells

- **Preparation of reagents:**
- Bacterial collagenase 0.1mg/ml (1.0µM): Dissolve the bacterial collagenase in 0,05M Tris buffer, pH 7.5 with 0,005M CaCl2 and store at -20°C until use. Further dilution of enzyme solution is done with the same Tris buffer. Do not vortex!
- Sodium Borate buffer (0,5M pH: 7.5)
  For 10ml: dissolve 0.310g boric acid in 10ml demi water, add 22.2mg of CaCl2 (5mM= 5.55mg/10ml). add 12.5mg NaOH, adjust pH with HCL to 7.5.
- Sodium borate (125mM pH 8.0)
  For 40ml: dissolve 0.310g boric acid in 40ml demi water and add 50mg NaOH, adjust pH to 8.0
- Sodium periodate (NaIO4) (1.25mM) in H20
  For 40ml: dissolve 10.7mg NaIO4 in 40ml demi water.
- 3,4-DHPAA (0.75mM) in H20: 250 µL per sample
- For 40ml: dissolve 5.05mg DHPAA in 40ml demi water.
- **Preparing calibration line:** Prepare the following calibration line. Store in -20°C until use.

Concentration collagen (µg/ml)	Collagen Stock (400ug/ml) (µL)	Water (µL)
0	-	2000
1	5	1995
3	15	1985
10	50	1950
30	150	1850

100	500	1500
300	1500	500

Preparing the cell samples

- In a 6-wells plate grow 3T3 cells till 80-90% confluence. For each treatment use 2 wells, which will be pooled after incubation (so 1 treatment is 2x2 wells with duplo). See table for cell density for seeding:

Incubation time	Number of cells seeded.
24H	$3 \cdot 10^4$
48H	$1 \cdot 10^4$
72H	$5 \cdot 10^3$

- Wash the cells 1x with PBS, add 400 uL trypsin to each well and detach the cells. To each well add 1.6mL media and homogenize the cells. Transfer each well to 2ml tubes. Centrifuge the cells for 5min at 300g to form a pellet and remove supernatant (this pellet can be stored at -80°C).
- When performing the assay: pull calibration line and samples from freezer and let it come to room temperature. Lyse the cell samples with 225 uL H2O and homogenize with the micro homogenizer. Once lysed pool the samples (so 2 samples become 1, with duplo so now 1x2 samples).

Enzymatic degradation:

- Use 200 µL of the supernatant/homogenized solution per sample.
  To this sample add: 20 µL of 0.1 mg/ml (1.0 mM) bacterial collagenase,
- 25 µL 500mM sodium borate buffer (**pH:7.5**) with 20mM CaCl2 and
- 5 µL H2O.
- Total volume = 250uL
- Mix solution, do not vortex!
- Incubate the samples for 1h at 37°C.

Fluorescence detection:

- After digestion spin down the samples for 5min at 300g. Collect 200 uL of the supernatant and transfer to a new tube.
- To the enzymatic solution (200 µL) add:
- 200 µL of 0.75mM 3,4-DHPAA
- 200 µL of 125mM sodium borate (**pH:8.0**)
- 200 µL of 1.25mM NaIO4.
- Vortex solution
- Reaction is immediately carried out for 15 min at 37°C.
- Fill black fluorescence 96-wells plate and place the pink adapter in the plate reader. The 96-wells plate goes on top of the adapter.

- Measure the fluorescence intensity: excitation: 375 nm, emission: 465 nm.

Appendix IV

Protein assay protocol

- Use the lysed cells from the previous described collagen assay

Prepare the standard curve with 20mg/mL stock BSA:

Standard (mg/mL)	BSA	Demi water (µl)
10	20µL of 20mg/mL	20
8	16µL of 20mg/mL	24
6	12µL of 20mg/mL	28
4	8µL of 20mg/mL	32
3	6µL of 20mg/mL	36
2	4µL of 20mg/mL	76
1	4µL of 20mg/mL	40
0.5	40µL of 1mg/mL	40
0.25	40µL of 0.5mg/mL	40
0.125	40µL of 0.25mg/mL	40
0.06	40µL of 0.125mg/mL	40
0.03	40µL of 0.06mg/mL	40

Protein assay:

- pipet in triplet 5µL of the standard concentrations in the wells of a 96 wells plate
- pipet in triplet 5µL of the samples in empty wells on the same plate
- pipet in triplet 5µL of H_2O (background measurement)
- prepare AS reagens: add 20 µL of assay reagent S per 1mL of reagent A
- add to each well 20µL AS
- add 200µL of reagent B to each well
- incubate for 15 minutes at room temperature
- calculate the protein concentration of the samples by interpolation in the standard curve

Appendix V

qPCR experiment protocol

- Watch how the cells look under the microscope and take pictures when abnormalities or differences are present
- Make solution with new medium and the desired concentration of stimulant.
- After cultivation sterilize the aspirator and remove the medium from the wells
- Add new medium on the cells and cultivate them on 37°C for 3h
- Add LPS directly into the medium and incubate for 1 additional hour

Continue after these steps with the RNA isolation of cultured cells.

RNA Isolation:

Prepare before starting (10 samples):
HB solution: Add 48 ul 1-Thioglycerol per 2,4mL ml of Homogenization Solution.

Harvest the samples:
do not wash with PBS

- Add 200 ul pre-chilled HB to each well, homogenize them with the pipet
- Poole the duplicates together due to low cell counts.
- 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 40 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
- Wait for 1h until the RNA is isolated and store it until further use in -80°C.

RNA conversion to cDNA

Precaution: tubes, tips en water must be RNase free.

- Measure the RNA yield using the nanodrop.
- If necessary, dilute sample with H_2O so a solution of 0,5µg in 5µL can be made for each sample

- **Prepare RT mix per sample (think about standard curve)**

  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_2O 1.65 ul (to get total vol. of 10 ul)

      Total volume: 10 ul

    Converting RNA tot cDNA:

    - Place tubes in PCR machine
    - Start the file: MLVCDNA
    - 10 min 20 °C
    - 30 min 42 °C
    - 10 min 20 °C
    - 5 min 99 °C
    - 5 min 20 °C
    - After the reaction is completed:
    - Spin the tubes (condensed water from the lids)
    - Store the samples at -20 until further use.

qPCR measurement protocol

Creating Standard Curve:

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

STD(ref)	V (µL)	H_2O (µL)
STD 4	100µL of pooled cDNA	150
STD 2	100µL of STD 4	100
STD 1	100µL of STD 2	100
STD 0.5	100µL of STD 1	100
STD 0.25	100µL of STD 0.5	100

Prepare the cDNA

- Dilute the cDNA samples after the conversion 10 times:
- Add 75 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

Prepare the Taq MasterMix (10 samples)

- 50µL Sybr Green Mix550
- 3µL primermix F+R (10µM)
- 27µL H_2O
- Total 80→ 8 ul/well
- cDNA 10* verdund→ 2 ul/well

Prepare the qPCR reaction

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

Appendix VI

Raw data experiment 1

Absorbance t = 24 hr	Control + PDGF	Control + PDGF	3h PDGF + LPS	3h PDGF + LPS	PDGF +LPS	PDGF +LPS
Absorbance 1	0,067	0,057	0,057	0,057	0,059	0,058
Absorbance 2	0,065	0,058	0,057	0,059	0,054	0,059
Average [NO]	0,863158	-	0,336842	-	0,494737	-
St Dev	0,632674	-	0,074432	-	0,148865	-

Appendix VII

Raw data experiment 2

Absorbance t = 24 hr	Control - LPS	Control + LPS	PDGF - LPS	PDGF + LPS	INF-γ- LPS	INF-γ +LPS
Absorbance 1	0,0605	0,0605	0,061	0,057	0,0635	0,059
Absorbance 2	0,0585	0,063	0,057	0,059	0,059	0,060
Average [NO]	-0,09524	0,119048	-0,14286	-0,2381	0,071429	-0,09524
St Dev	0,164957	0,122952	0,16265	0,560747	0,592215	0,109971

Absorbance t = 48 hr	Control - LPS	Control + LPS	PDGF - LPS	PDGF + LPS	INF-γ- LPS	INF-γ +LPS
Absorbance 1	0,072	0,069	0,0645	0,06	0,0715	0,207

Absorbance 2	0,0675	0,0745	0,858	0,0625	0,0585	0,217
Average [NO]	0,880952	1,071429	0,095238	0,071429	1,071429	14,42857
St Dev	0,164957	0,16265	0,592215	0,205738	0,324136	0,334465

Calibration curve									
	0	0,3	1,6	3,1	6,3	12,5	25	50	100
Absorbance 1	0,056	0,062	0,068	0,083	0,108	0,17	0,27	0,466	0,876
Absorbance 2	0,056	0,062	0,079	0,103	0,114	0,165	0,263	0,465	0,926
Average [NO]	0,056	0,062	0,0735	0,093	0,111	0,1675	0,2665	0,4655	0,901
St Dev	0	0	0,007778	0,014142	0,004243	0,003536	0,00495	0,000707	0,035355

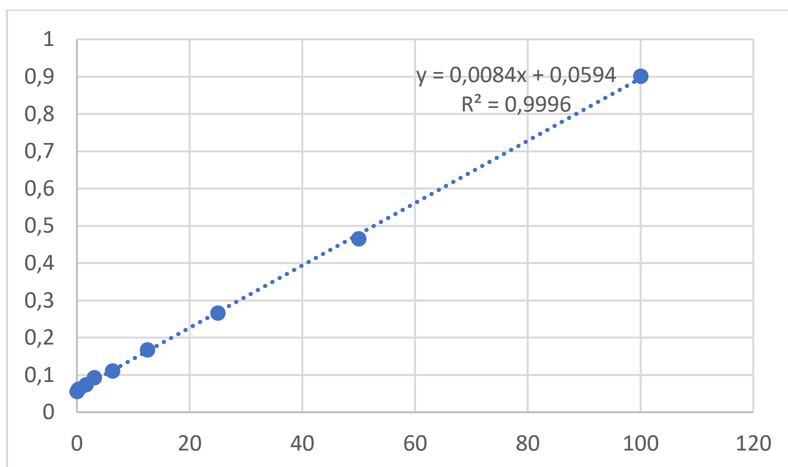

Figure 12: calibration curve of collagen assay.

Appendix VIII

Raw data experiment 3

Absorbance t = 24 hr						
	Control 24	TGF-β 24	Control 48h	TGF-β 48	Control 72	TGF-β 72

Absorbance 1	10794	8307	12231	11686	11817	10144
Absorbance 2	10709	8577	12294	11734	11976	10283
Average [NO]	47	28	59	54	56	42
St Dev	0,49	1,55	0,36	0,28	0,91	0,80

Absorbance t = 24 hr						
	Control 24	TGF-β 24	Control 48h	TGF-β 48	Control 72	TGF-β 72
Absorbance 1	0,424	0,337	0,234	0,261	0,225	0,273
Absorbance 2	0,488	0,322	0,248	0,72	0,242	0,224
Average [NO]	2,804	1,784	1,127	1,312	1,073	1,182
St Dev	0,383	0,081	0,071	0,057	0,086	0,251

Calibration curve							
	0	1	3	10	50	100	300
Absorbance 1	1712	1865	2481	2386	3387	6013	12168
Absorbance 2	1787	1900	1837	2352	3398	5733	12058
Absorbance 3	1840	1919	1797	2382	3479	7254	12840
Average absorbance	1779	1894	2038	2373	3421	6333	12355
Standard deviation	64,3	27,4	383,9	18,6	50,2	809,5	423,3

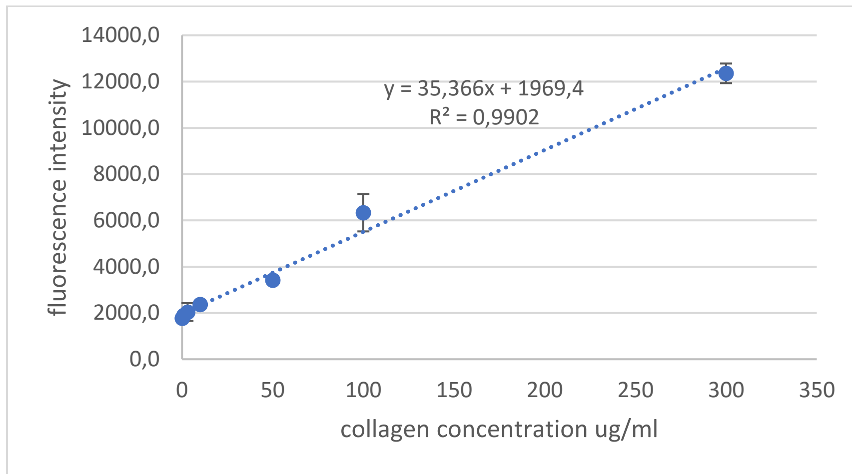

Figure 13: calibration curve of collagen assay.

Appendix IX

Raw data Experiment 4

Absorbance t = 24 hr	control	INF-γ + LPS	IL-10	IL-10 + LPS	INF-γ + IL-10	INF-γ + IL-10 + LPS
Absorbance 1	0,064	0,077	0,060	0,060	0,065	0,082
Absorbance 2	0,063	0,076	0,060	0,063	0,06	0,087
Average [NO]	0,813	2,152	0,485	0,636	0,788	2,960
St Dev	0,071	0,286	0,071	0,143	0,071	0

Appendix X

Raw data experiment 6

Absorbance t = 48 h without LPS	Control	IL-10	INF-γ	IL-10 + INF-IL-10 + INF-γ	INF-γ low density
Absorbance 1	0,063	0,060	0,064	0,069	0,064
Absorbance 2	0,062	0,060	0,067	0,061	0,63

Average [NO]	0,369	0,150	0,756	0,250	0,458
St Dev	0,119	0,150	0,342	0,648	0,060

Absorbance t = 48 h with LPS					
	Control	IL-10	INF-γ	IL-10 + INF-IL-10 + INF-γ	INF-γ low density
Absorbance 1	0,063	0,060	0,175	0,160	0,063
Absorbance 2	0,065	0,61	0,194	0,163	0,065
Average [NO]	0,548	0,131	14,8	12,1	1,38
St Dev	0,137	0,154	1,324	0,500	0,257

Appendix XI

Raw data experiment 7

β-Actin

Absorbance t = 72 h without LPS					
	Control	INF-γ	IL-10	TGF-β	IL-10 + INF-IL-10 + INF-γ
Quantity 1	16,360	17,031	16,863	16,734	17,847
Quantity 2	16,324	17,050	16,950	17,364	17,859
Quantity 3	16,647	16,996	17,095	-	17,970
Average [NO]	1,57	1,04	1,08	1,05	0,564
St Dev	0,177	0,027	0,118	0,323	0,067

First β-actin measurement	Control	INF-γ	IL-10	TGF-β	IL-10 + INF-IL-10 + INF-γ
Quantity 1	1,427	1,080	1,002	0,970	0,976
Quantity 2	1,484	1,199	1,169	1,031	0,603
Quantity 3	1,534	1,254	1,067	0,902	0,732
Average [NO]	1,48	1,18	1,08	0,97	0,77
St Dev	0,055	0,116	0,117	0,101	0,367

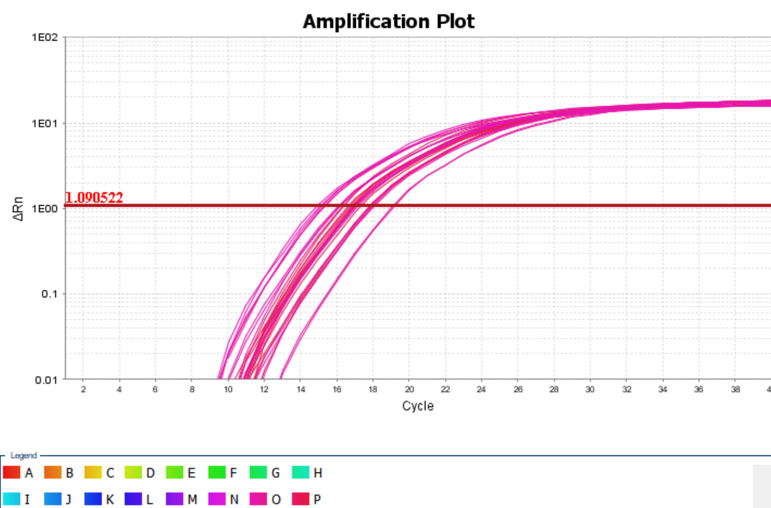

Figure 10: amplification plot of β-actin housekeeping gene measurement.

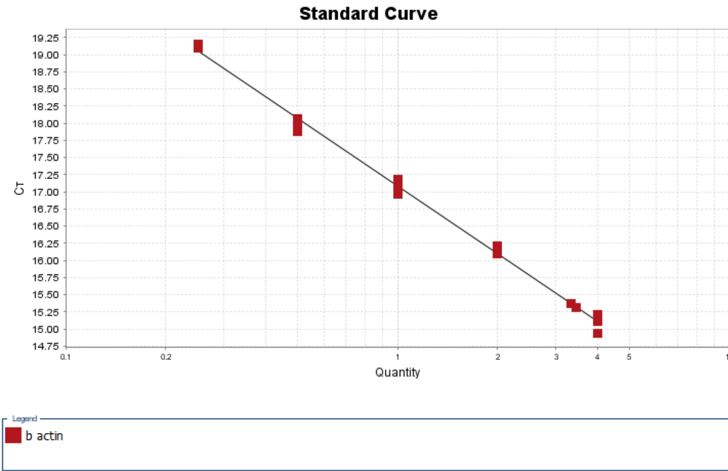

Figure 11: standard curve of made where quantity values are related with.

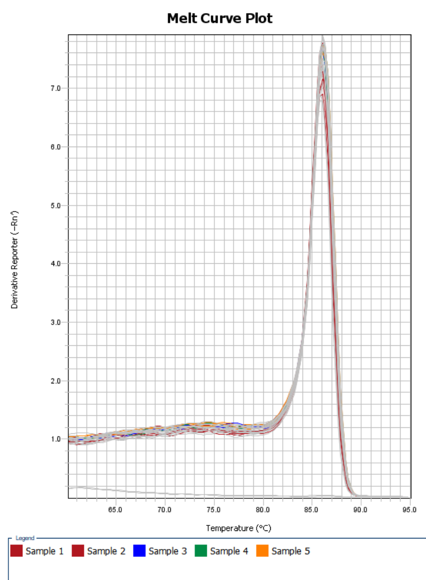

Figure 12: Melt curve of β-actine samples. Shows if there are any other substances within the mix with a different melting point

HAS1-3 genes

HAS1 gene expression	Control	INF-γ	IL-10	TGF-β	IL-10 + INF-IL-10 + INF-γ
Quantity 1	0,615	0,776	1,363	1,207	1,377
Quantity 2	0,527	0,834	1,011	1,189	1,418
Quantity 3	0,500	0,617	1,209	1,255	1,479
Average [NO]	0,547	0,742	1,194	1,217	1,425

St Dev	0,060	0,112	0,177	0,034	0,051

HAS2 gene expression					
	Control	INF-γ	IL-10	TGF-β	IL-10 + INF-IL-10 + INF-γ
Quantity 1	0,645	0,517	1,064	1,609	1,437
Quantity 2	0,664	0,629	1,248	1,449	1,441
Quantity 3	0,574	0,598	1,375	1,608	1,600
Average [NO]	0,628	0,581	1,229	1,555	1,493
St Dev	0,047	0,058	0,156	0,092	0,093

HAS3 gene expression					
	Control	INF-γ	IL-10	TGF-β	IL-10 + INF-IL-10 + INF-γ
Quantity 1	0,621	-	0,958	1,296	1,285
Quantity 2	0,740	0,645	1,232	1,380	1,274
Quantity 3	0,669	0,980	1,135	1,222	1,173
Average [NO]	0,676	0,812	1,108	1,229	1,244
St Dev	0,060	0,237	0,139	0,079	0,061

Appendix XII

Raw data experiment 8

Absorbance t = 72 h without LPS	Control	INF-γ	IL-10	TGF-β	IL-10 + INF-IL-10 + INF-γ
Absorbance 1	0,065	0,061	0,058	0,061	0,066
Absorbance 2	0,066	0,062	0,061	0,061	0,68
Average [NO]	1,34	0,94	0,74	0,89	1,49
St Dev	0,071	0,071	0,214	0	0,143

Absorbance t = 72 h with LPS					
	Control	INF-γ	IL-10	TGF-β	IL-10 + INF-IL-10 + INF-γ
Absorbance 1	0,171	0,207	0,064	0,059	0,22
Absorbance 2	0,065	0,207	0,063	0,058	0,22
Average [NO]	6,65	15,6	1,14	0,64	17,2
St Dev	7,57	0,00	0,071	0,071	0,286