UNIVERSITY OF ICELAND
FACULTY OF MEDICINE

Appendix A

Optimization of the THP-1 human monocyte cell culture

Before choosing the conditions for activation and stimulation of the THP-1 cells, several different concentrations of stimulus and time-points were tested. The results are presented in this appendix.

1 Cytokine secretion by THP-1 cells as activated monocytes

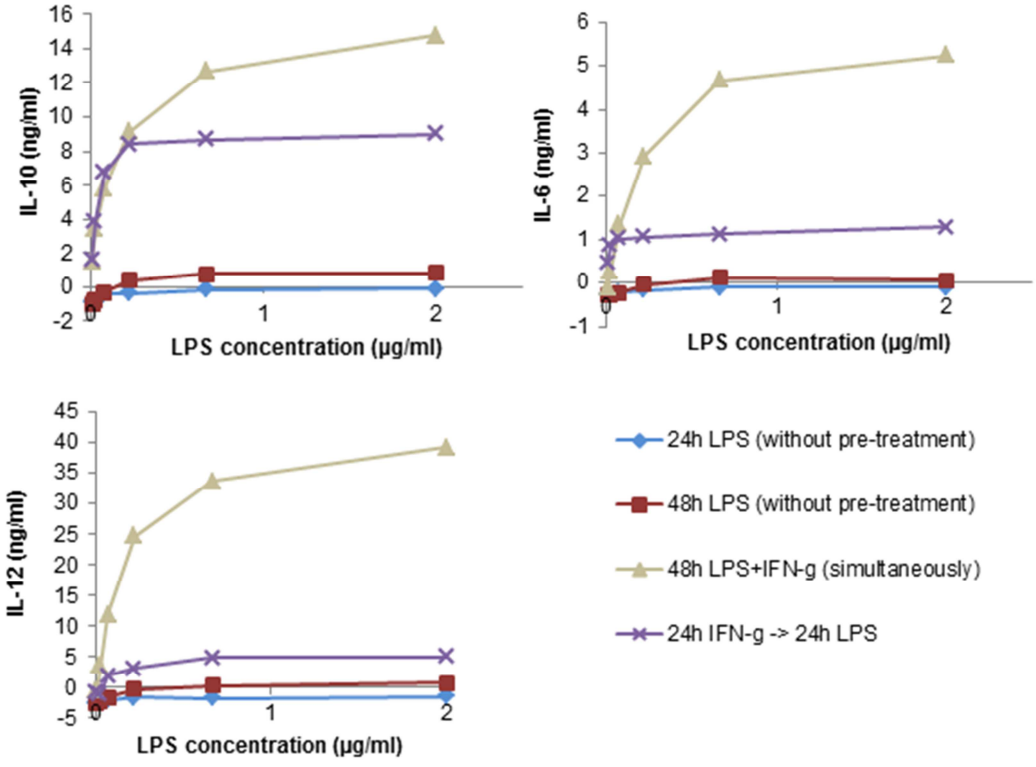

Figure A 1. Cytokine secretion by THP-1 cells stimulated with different concentrations of LPS with or without pre-treatment with IFN-γ.

THP-1 cells were seeded in 48 well culture plates at $5x10^5$ cells/ml, 1 ml/well and either pre-treated with 200 U/ml IFN-γ for 24 hours and stimulated with LPS for another 24 hours, stimulated with LPS without IFN-γ for 24 or 48 hours or lastly, stimulated with LPS and IFN-γ simultaneously for 48 hours. Cell culture supernatants were collected and stored until cytokine concentrations were measured using ELISA.

Without pre-treatment with IFN-γ there was negligible secretion of the cytokines IL-10, IL-6 and IL-12p40 (Figure A 1). Simultaneous treatment with IFN-γ and LPS for 48 hours led to the most production of the cytokines, although at lower concentrations of LPS, pre-treatment of the cells with IFN-γ for 24 hours prior to stimulation with LPS led to similar concentrations of IL-10 and IL-6 in the medium as simultaneous addition of IFN-γ and LPS.

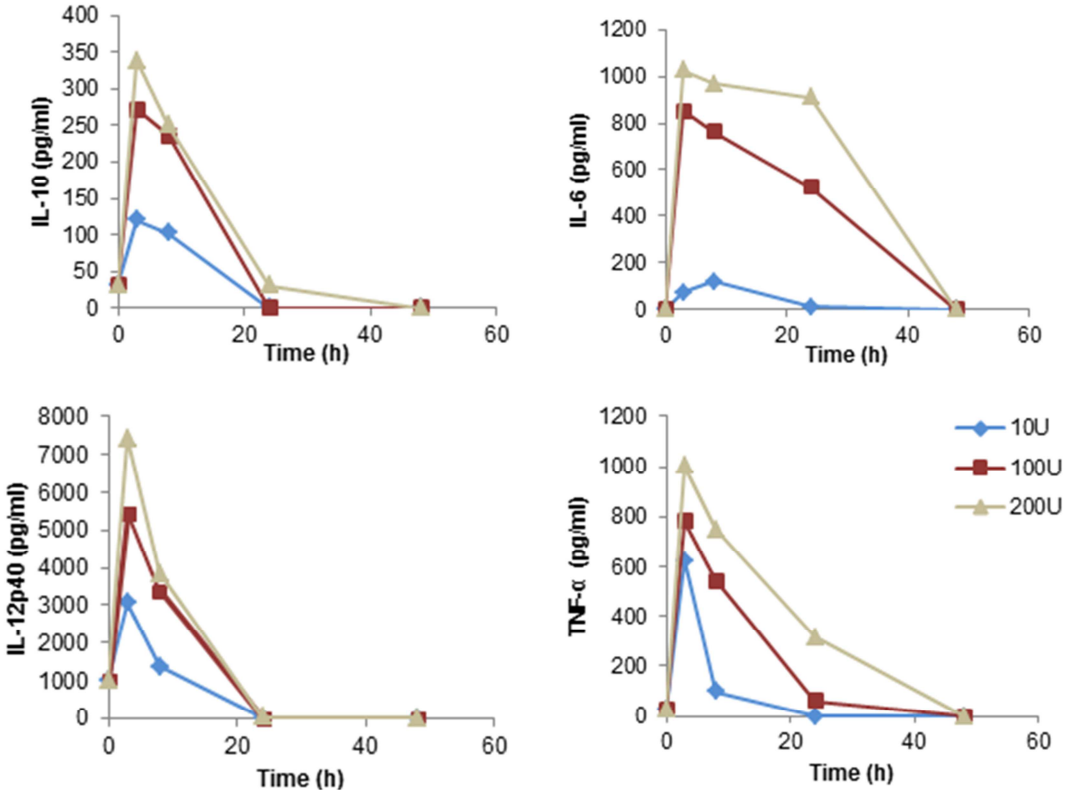

Figure A 2. Time course of cytokine secretion by THP-1 monocytes pre-treated with different concentrations of IFN-γ.

THP-1 cells were seeded in 48 well culture plates at $5x10^5$ cells/ml, 1 ml/well and treated with 10, 100 or 200 U/ml IFN-γ for the indicated times before stimulation with 0.5 µg/ml LPS for additional 48 hours. Cell culture supernatants were collected and stored until cytokine concentrations were measured using ELISA.

Secretion of all of the cytokines peaked at 3 hours of pre-treatment and fell rapidly after that (Figure A 2). Pretreatment of the cells with 100 or 200 U/ml of IFN-γ showed similar results, whereas pretreatment with 10 U/ml of IFN-γ resulted in less secretion of the cytokines.

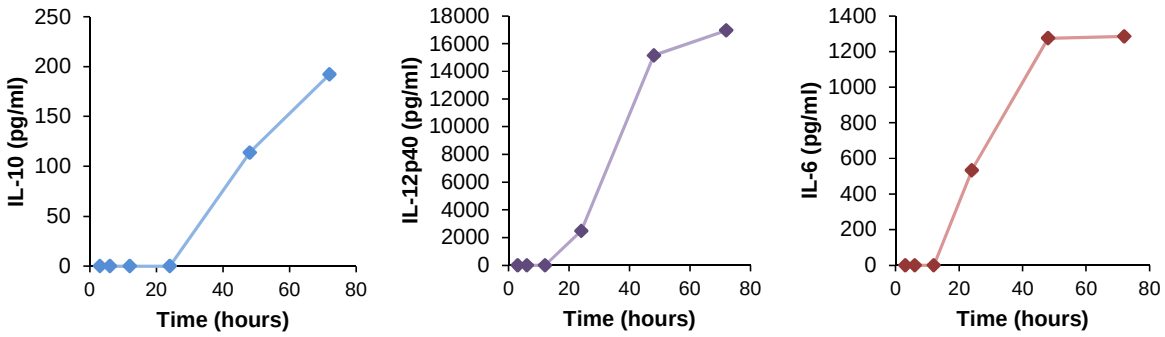

Figure A 3. Time course of cytokine secretion by THP-1 monocytes treated simultaneously with IFN-γ and LPS.

THP-1 cells were seeded in 48 well culture plates at $5x10^5$ cells/ml, 1 ml/well and treated simultaneously with 100 U/ml IFN-γ and 0.5 µg/ml LPS for the indicated time. The culture supernatants were collected and frozen until measured.

When treated simultaneously with IFN-γ and LPS the THP-1 monocytes secreted negligible amount of TNF-α (data not shown).

IL-6 concentrations reached maximum at 48 hours, IL-12p40 concentration increased slightly between 48 and 72 hours, but the concentration of IL-10, a typical "late" cytokine kept rising after 48 hours (Figure A 3). Given that the ELISA measures the total amount of cytokines secreted into the culture medium (cytokines do not get broken down at any significant rate at these time-points according to previous results from the lab), a plateau of cytokine levels suggests that cytokine secretion has stopped. When comparing the 48 hour simultaneous stimulation with the 3 hour pre-treatment followed by 48 hour stimulation (Figure A 2) it can be observed that the THP-1 cells secreted similar amounts of IL-10, but less IL-12p40 and IL-6 when pre-treated, however the grand difference was that the pre-treatment seemed to be necessary to drive the cells towards TNF-α secretion.

Therefore, 3 hour pre-treatment with 100 U/ml IFN-γ followed by 48 hours of LPS stimulation was chosen. Additionally, given the results in Figure A 1, the optimal concentration of 0.75 µg/ml of LPS was ultimately chosen for experiments with natural products.

2 PMA differentiated macrophage-like THP-1 cells

THP-1 cells were differentiated into a macrophage-like phenotype by treating them with 100 ng/ml PMA for 48 hours to differentiate them into macrophage-like adherent cells. The PMA and any non-adherent (undifferentiated) cells were washed off with a wash buffer (PBS with 1% bovine serum albumin (BSA)) and fresh medium added to the wells along with the compounds tested and the adherent cells were stimulated with different concentrations of LPS. When optimizing the culture for PMA treatment to differentiate the THP-1 monocytes into a macrophage-like phenotype a similar approach to what was described in the previous chapter was used.

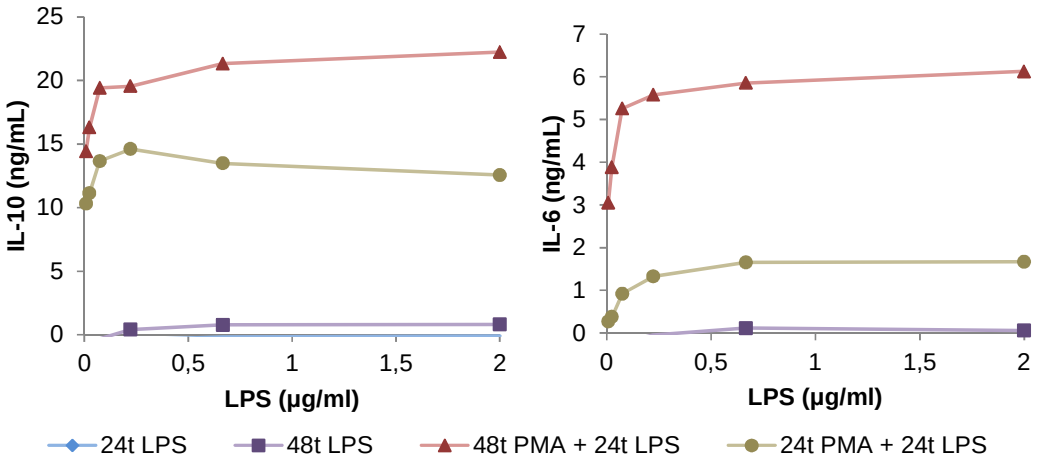

Figure A 4. Different combinations of PMA pre-treatment before stimulation with LPS.

The THP-1 cells were seeded as usual (see Figure A 1 legend) and then pre-treated with or without 100 ng/ml PMA for 24 or 48 hours. The cells were then washed with BSA in PBS and fresh medium added as the cells were stimulated with LPS in growing concentrations (max 2 µg/ml) for 24 or 48 hours. No IL-12p40 was measureable under these circumstances (data not shown). TNF-α was not measured in these samples. 24 hour LPS stimulation alone caused no measurable cytokine secretion in any LPS concentration tested.

When the THP-1 cells were treated with PMA the cells became adherent. After PMA pre-treatment the PMA-containing culture medium was removed and thus all undifferentiated cells were removed during this washing step. Trypan blue staining of the removed culture medium revealed that the cells not adherent at this step were very few and not viable (data not shown), indicating that the majority of the cells had differentiated and adhered.

As before, 24 and 48 hour stimulation with LPS alone was not enough to cause a measurable secretion of cytokines. Also, THP-1 cells differentiated with PMA secreted no measurable amount of IL-12p40 (data not shown). For both IL-10 and IL-6 secretion, 48 hour PMA pre-treatment caused more cytokine secretion than 24 hour pre-treatment (Figure A 4). As PMA alone caused no measurable cytokine secretion (data not shown) the cytokine secretion is the result of the following LPS stimulation.

These results suggest that 48 hours of pre-treatment would be better to reach optimal cytokine secretion.

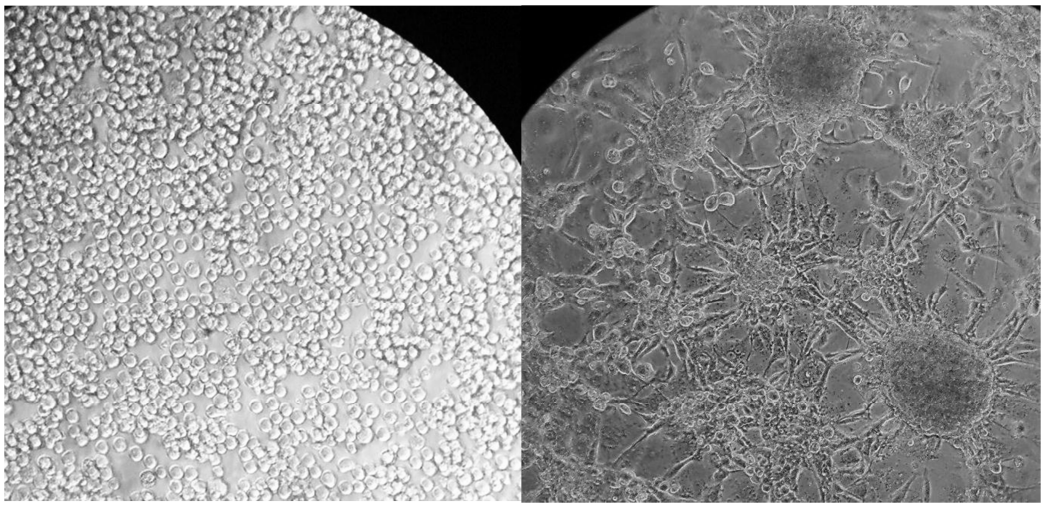

Figure A 5. THP-1 cells before (left) and after (right) PMA treatment for 48 hours and LPS stimulation for 24 hours.

During the same experiment as in Figure A 4, the cells were photographed before treatment and 72 h later after the PMA and LPS treatment (48 h PMA + 24 h LPS) using a standard digital camera, pointed into the microscope The method of photography is crude and impossible to scale correctly; these images are merely a way to emphasize the drastic phenotypic difference seen after the PMA and LPS treatment.

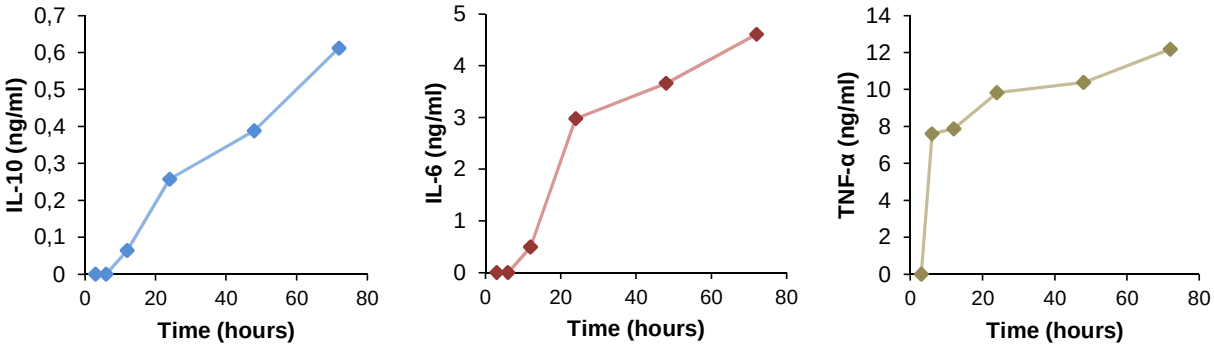

Figure A 6. Time course of cytokine secretion after 48 hour pre-treatment with PMA.

THP-1 cells were seeded as usual (Figure A 1 legend) and pre-treated with 100 ng/ml PMA for 48 hours. They were then washed, re-suspended in fresh medium and stimulated with 0.5 µg/ml LPS for the indicated amount of time (max 72 hours). The cell-culture media was then collected and cytokine concentrations measured using ELISA. As always the PMA treated cells secreted no IL-12p40

After the 48 hour PMA pre-treatment (Figure A 6) and subsequent LPS stimulation, there was some measurable secretion of the three cytokines measured, especially TNF-α. This secretion then steadily rose and had not reached maximum at 72 hours when the measurements were terminated.

Since all treatment conditions never induced IL-12p40 secretion, the use of PMA pre-treatment was abolished.

3 The effect of cell passaging on THP-1 monocyte proliferation and cytokine secretion

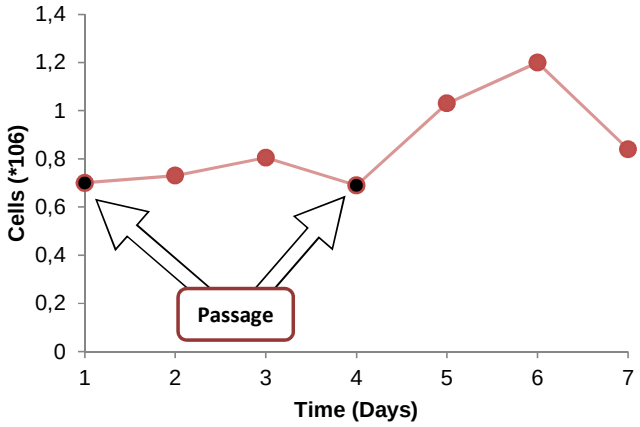

Figure A 7. Changes in proliferation of THP-1 cells over the course of 7 days.

Identical experiments were set up each day of the week. The cells were seeded as usual (Figure A 1, legend), pre-treated with 100 U/ml IFN-γ for 3 hours before stimulating them with 0.5 µg/ml LPS for 48 hours. Any adherent cells were then scraped of the culture surface using a cell scraper and the cells counted using trypan blue staining in a Countess cellcounter. The supernatants were collected and stored for cytokine measurements. Black dots represent days where the cells were passaged (the cells were passaged twice a week by counting and re-suspending the cells in an organized manner based on the time passing between passages).

Since the THP-1 cells are passaged twice a week, the time between splits is not even (leaving 3 and 4 days between splits, Figure A 7). After passaging the cells take a growth spurt, proliferating rather rapidly, but this appears to be more apparent in the 4 day passage period than the 3 day passage.

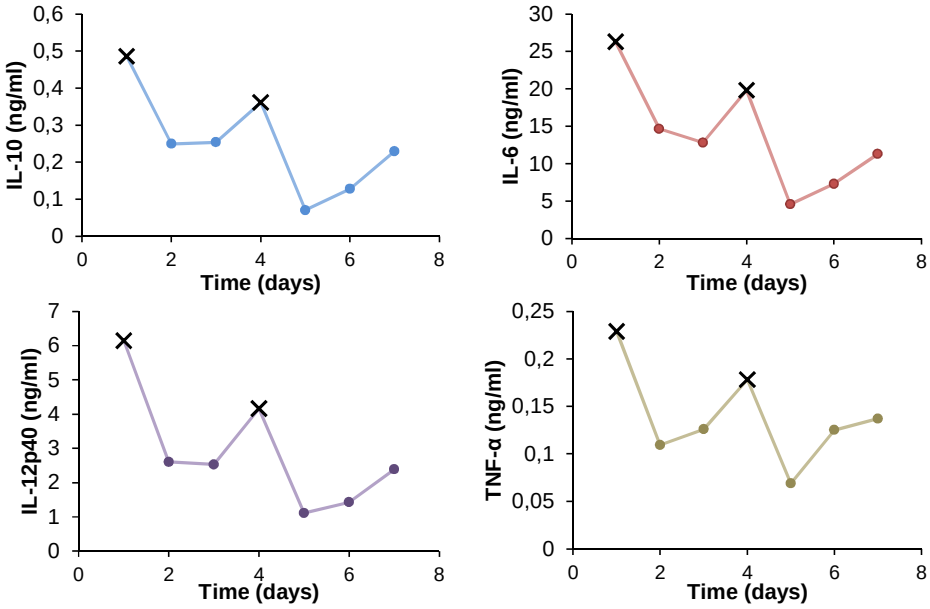

Figure A 8. THP-1 monocyte cytokine secretion in experiments started each day over the course of 7 days.

Cytokine levels in the supernatants from the culture described in the legend of Figure A 7 were measured using ELISA. Days marked with an "X" are days where the cells were passaged.

Figure A 8 demonstrates that when the THP-1 cells were actively proliferating, they secreted less cytokines. Again, this trend was less pronounced in the 3 day passage period than the 4 day period. This suggests that in order to achieve adequate reproducibility of experiments, the state of the cells in respect to passage times must be taken into account.

Once this information had been gathered it was decided that all experiments would begin the day before the cells were to be passaged.