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Maastricht University

Faculty of Medicine and Life Sciences
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Master of Biomedical Sciences

Master's thesis

Genetically engineered pro-regenerative macrophages as a treatment for spinal cord injury

Maika Dehaes

Thesis presented in fulfillment of the requirements for the degree of Master of Biomedical Sciences, specialization Molecular Mechanisms in Health and Disease

SUPERVISOR :
Prof. dr. Niels HELLINGS

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Genetically engineered pro-regenerative macrophages as a treatment for spinal cord injury*

Dehaes Maika[1], Veeningen Naomi[2], Lemmens Stefanie[2], Franssen Aimée[2], Hendrix Sven[2], Hellings Niels[2]

[1]Department of medicine and life sciences, Biomedical Research Institute, Universiteit Hasselt, Campus Diepenbeek,
Agoralaan Gebouw C - B-3590 Diepenbeek
[2]Immunology and infection research group, Biomedical Research Institute, Universiteit Hasselt, Campus Diepenbeek,
Agoralaan Gebouw C - B-3590 Diepenbeek

*Running title: *Super macrophages to treat spinal cord injury*

To whom correspondence should be addressed: Hellings Niels, Tel: +32 (11) 26 92 68; Email: niels.hellings@uhasselt.be

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ABSTRACT

Macrophages play a critical role as essential effectors of the innate immune system. In response to different stimuli, they are capable to adopt either a pro-inflammatory M1 or an anti-inflammatory M2 phenotype. After a spinal cord injury (SCI), the neuroprotective M2 macrophages are outbalanced by the neurotoxic M1 macrophages, which limit functional recovery. To increase the amount of M2 macrophages after SCI, this study aims to create super macrophages by overexpressing different M2 genes or migration-mediating receptors in naive macrophages to induce polarisation towards an M2 phenotype. Monocyte/macrophage-like RAW264.7 cells were either transfected with M2 markers FIZZ1 or YM1 or stimulated towards an M1 or M2 phenotype using LPS or IL-10, respectively. BMDMs were transduced with CCR2, a chemokine receptor. RT-qPCR was used to determine the gene expression of macrophage markers (FIZZ1, YM1, CD206, Arg-1, TGF-β, IL-6, CD38, CD86, TNF-α, iNOS). Protein expression was evaluated through western blot. Although overexpression of FIZZ1 or YM1 was achieved in RAW264.7 cells, no significantly higher expression of M2 markers (CD206, Arg-1, TGF-β, CD163) was found in transfected cells compared to untreated controls. M1 markers IL-6, CD38, and CD86 were significantly higher expressed in M1 stimulated cells compared to FIZZ1 or YM1 transfected cells, while expression of M1 markers TNF-α and iNOS did not differ between these groups. In addition, CCR2 overexpression was not achieved in BMDMs. To conclude, overexpression of FIZZ1 or YM1 in RAW264.7 cells cannot induce a phenotypical switch into M2 but, unexpectedly, might trigger pro-inflammatory characteristics in RAW264.7 cells.

INTRODUCTION

Annually, between 250 000 and 500 000 people worldwide suffer from a spinal cord injury (SCI) (1). The most common etiologies of a traumatic SCI are motor vehicle accidents and falls, followed by violence and sports (2). These injuries are characterised by a sudden traumatic blow to the spine which fractures, dislocates, or compresses at least one vertebra (1, 3). Symptoms related to such spinal injuries can vary widely. Typically, the location of the injury determines the affected body part(s) as well as the severity of the symptoms. In general, higher spinal injury levels are related to more severe symptoms (1). For example, a cervical SCI commonly results in tetraplegia or paralysis of all four limbs and the torso, while thoracic SCI causes paraplegia without affecting the arms, and a lumbar SCI results in sensory and motor loss in the hips and legs (1).

In traumatic SCI, the process where a mechanical force delivered to the spine results in structural damage of the vertebra(e), is known as the primary injury. Axons, neuronal cell

membranes, and blood vessels are damaged and neuronal transduction across the lesion is disrupted, affecting the spinal blood flow and causing a spinal shock (4). This spinal shock causes temporary disabling of the spinal cord below the lesion which can continue for several days (4). The structural and cellular damage of the initial primary injury generates an inflammatory environment and triggers the secondary injury cascade (3, 4). Secondary injury includes processes like vascular damage, ionic imbalance, free radical formation, inflammation, and cell death, leading to progressive tissue degeneration by initiating neuroinflammation and oxidative stress (3).

As the primary injury quickly initiates processes leading to the beginning of the secondary injury - which can continue more than six months -, the focus of therapeutic intervention is preventing or antagonizing secondary injury while promoting neuronal regeneration and axonal regrowth to ultimately save spinal tissue (4). Neuroinflammation is one of the main secondary injury pathways responsible for a pro-inflammatory setting that limits functional recovery after SCI. Therefore, this strong inflammatory response has been a specific target for therapeutic development. Unfortunately, most recent therapies targeting this inflammation suppress the entire immune response and are thereby not specific to treat SCI. To date, the only approved therapy is the anti-inflammatory immunosuppressive drug, methylprednisolone (5). Although it is the only approved clinical treatment for SCI, its use has decreased over the past years due to both its limited treatment window of eight hours and associated risks, such as gastrointestinal bleeding and wound infections (5). With currently no effective and safe treatment available and the lifetime costs of a SCI patient rising to $2.35 million, it is crucial to develop new efficient therapies (3). A strong candidate for effective therapy is immunomodulation of neuroinflammation in the injured spinal cord. Accordingly, immunomodulation becomes the main goal to replace the unspecific immunosuppression of existing therapies.

Cells that play an important role in neuroinflammation are macrophages. The inflammatory macrophage response is associated with both beneficial and detrimental effects on spinal injury. In the early period following SCI, infiltrated macrophages at the site of injury are both classically activated M1 macrophages with a pro-inflammatory phenotype and alternatively activated M2 macrophages with an anti-inflammatory phenotype (4). While M1 macrophages are considered detrimental cells which secrete pro-inflammatory factors, stimulate fibrotic scar formation, and attack axons, M2 macrophages are seen as beneficial macrophages responsible for removing debris, fighting possible pathogens, and promoting axonal regrowth (4). Although both types are present after a SCI, the pro-inflammatory macrophages outbalance the anti-inflammatory macrophages several days post-injury (4). This overabundance of M1 cells is associated with oxidative damage and further neurodegeneration contributing to further destruction of the spinal cord (4). In contrast, classical wound healing processes are characterised by a different M1:M2 balance. In normal wound healing, M1 macrophages are initially present to deal with the inflammation by phagocytosing dead cells – similar to a SCI. However, afterwards, in the proliferation and remodelling stage of the wound, the balance shifts towards the M2 macrophages. As it is shown that inflammation might be beneficial for the treatment of SCI, the focus is not on decreasing the amount of pro-inflammatory macrophages, but rather on increasing the amount of anti-inflammatory neuroprotective M2-like macrophages by macrophage switching to affect the M1:M2 ratio (6).

Macrophage switching, a popular concept in modern research, is based on the polarization of macrophages. Activation of either the pro-inflammatory or anti-inflammatory phenotype is mainly induced by interferon-regulatory factor/signal transducer and activator of transcription (IRF/STAT) signalling pathways (*Figure 1*, adapted from (7-10)) (9, 11). While IRF3, IRF5, STAT1, and STAT5 drive M1 polarization, IRF4, STAT3, and STAT6 mediate M2 polarization signals (9, 11). The pro-inflammatory M1 macrophages are activated during inflammation by Toll-like receptor ligands, like lipopolysaccharide (LPS), or Th1 cytokines such as interferon γ (IFN-γ) and tumour necrosis factor α (TNF-α) (9, 11). This eventually results in

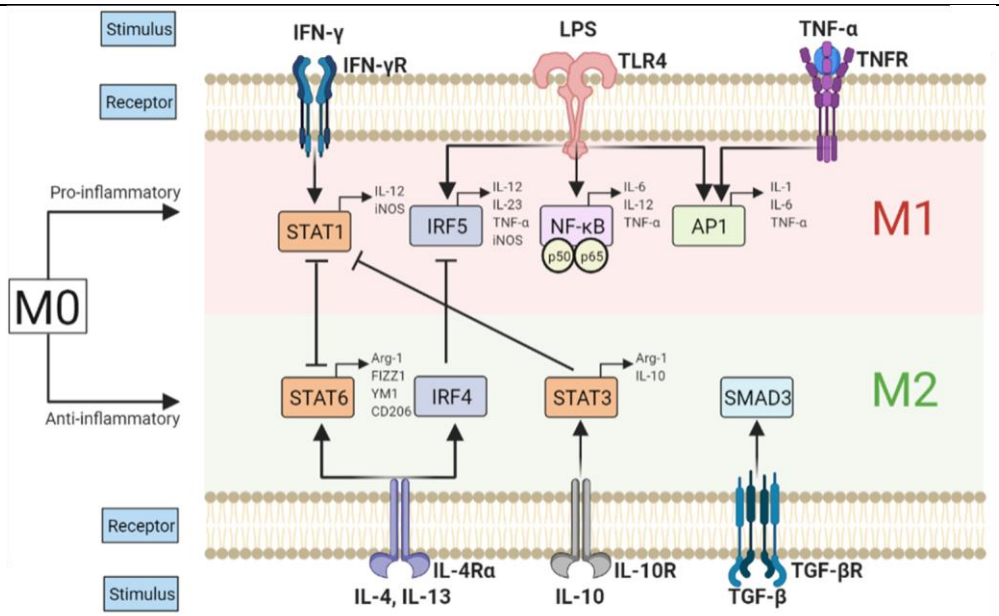


Fig. 1 – Macrophage polarization pathways. Through binding to their receptor, M1 stimuli (IFN-γ, LPS, TNF-α) and M2 stimuli (IL-4/IL-13, IL-10, TGF-β) activate transcription factors. M1 stimuli trigger IRF/STAT family members like IRF5 and STAT1, as well as NFkβ heterodimer p50-p65 and AP1. Once polarised in the M1 phenotype, M1 macrophages produce cytokines (TNF-α, IL-6, IL-12, IL-23, IFN-γ), chemokines, reactive oxygen species, nitric oxide, inducible nitric oxide (iNOS), and human leukocyte antigen-cell surface receptor. M2 stimuli trigger IRF/STAT members IRF4, STAT3, and STAT6, as well as SMAD3 resulting in the activation of M2 markers. When polarised in the M2 phenotype, M2 macrophages produce specific cytokines (IL-10), chemokines, and proteins (CD206, Arg-1, FIZZ1, YM1). LPS: lipopolysaccharide; TNF-α: tumour necrosis factor α; IFN-γ: interferon γ; TGF-β: transforming growth factor β; IRF: interferon-regulatory factor; STAT: signal transducer and activator of transcription; NFkβ: nuclear factor kappa β; AP1: activator protein 1; SMAD3: Mothers against decapentaplegic homolog 3. (Adapted from (7-10)).

enhanced production of e.g. interleukins (IL) or pro-inflammatory cytokines (IL-1β, IL-2, IL-6, IL-12, IL-23), reactive oxygen species (ROS), and nitric oxide (NO) (9, 11). Stimulating macrophages using IL-4/IL-13, IL-10, or transforming growth factor β (TGF-β) results in the M2 macrophage phenotype (9). Through activation of IRF/STAT family members, M2 macrophages get polarized and produce specific cytokines (IL-10), chemokines (e.g. CCL5, CCL17), and proteins (e.g. Arg-1, FIZZ1, YM1, CD206) (11). In SCI, levels of several pro-inflammatory cytokines such as TNF-α, IFN-γ, IL-6, and IL-1β are increased shortly after injury due to structural and cellular damage of the spinal cord (12). These classical ligands can bind to their receptor and thereby lead to activation of IRF/STAT pathways responsible for M1 polarization.

Studies have already been performed on overexpressing M2 stimuli such as IL-4/IL-13 or IL-10 through local cytokine injections in the spinal cord in order to increase the number of M2 macrophages that contribute to the recovery of SCI patients (13-15). However, spinal injection of these cytokines by themselves is not sufficient to create a stable M2 overexpression as these molecules are rapidly cleared from the injury site due to their short half-life. This indicates the need for local sustained delivery of anti-inflammatory molecules. Recent studies were able to polarize macrophages towards the M2 phenotype through overexpression with M2 stimuli, such as IL-10 and IL-4, generating expression of anti-inflammatory markers such as CD206, FIZZ1, and Arg-1, and enhancing SCI recovery (15). Stem cell delivery of IL-13 also induced improved functional recovery after SCI while simultaneously expressing M2 markers such

as Arg-1 and FIZZ1 (14). Nevertheless, overexpressing IL-13 might cause allergic reactions (16). Additionally, when using M2 stimuli to create these macrophages or stem cells, pathways - such as STAT6 - first need to be activated in order for the cells to produce anti-inflammatory M2 markers. Furthermore, it is not possible to control which macrophage markers are present after overexpressing macrophages with M2 stimuli. Another aspect that must be considered is the route of administration. So far, studies have been performed using local or intraspinal injections (14). However, these can cause perforation and thereby generate additional damage to the already injured spinal cord. A better and safer administration route would thereby be systemic administration. In this way, cells are not locally administered. To be able to migrate towards the lesion after systemic injection, macrophages would benefit from optimisation. Following injury, monocyte recruitment to the wound is regulated through a chemokine gradient. Chemokines released from the site of injury attract monocytes/macrophages via their chemokine receptors. In SCI, M1 macrophages migrate fast towards the injury via C-C motif ligand 2 (CCL2), also known as monocyte attractant protein-1 (MCP-1), through binding the C-C motif chemokine receptor 2 (CCR2) (17, 18). As it is indicated that CCL2 RNA and protein levels are increased in the acute phase after SCI, overexpressing CCR2 may stimulate macrophage migration towards the injury site (18).

To gain stable and continuous expression of M2 markers, this study aimed to create M2-like macrophages overexpressing specific M2 markers that are anti-inflammatory by nature and can possibly act as regulators of the M2-like phenotype to overcome the need for M2 stimuli such as IL-4/IL-13 and the prior activation of the STAT6 pathway. By overexpressing different M2 markers in the macrophages, a 'cocktail' of M2-like macrophages can be developed to maximize the associated anti-inflammatory effects and improve functional recovery after a SCI. The first M2 marker candidate to overexpress is arginase-1 (Arg-1). Arg-1 depletes L-arginine which impairs T cell proliferation and IFN-γ production (19). Furthermore, Arg-1 also reduces NO production, thereby decreasing inflammation and cytotoxicity (19). Other promising candidates that have not yet been reported in the literature are the anti-inflammatory markers FIZZ1 and YM1.

Found in inflammatory zone 1 or FIZZ1, also known as hypoxia-induced mitogenic factor (HIMF) or resistin-like molecule α (RELMα), is a cysteine-rich secreted protein that can be upregulated by helminth infection, IL-4, and IL-13 via the STAT6 pathway (*Figure 1*) (11). It is known that FIZZ1 is upregulated in eosinophils and epithelial cells, and thereby reduces inflammation during helminth infection (20). The finding that FIZZ1 antagonizes nerve-growth factor (NGF), and the secretion of NGF potentially amplifies the inflammatory reaction, suggests that FIZZ1 is rather an anti-inflammatory molecule (11, 20). This suggestion agrees with the finding that IFN-γ – an M1 stimulus – suppresses FIZZ1 protein expression – an M2 marker (11).

YM1 or chitinase-3-like protein (Chi3l3) is a secretory lectin with affinity to glycosaminoglycans (GAGs), including heparin and heparan sulfate. When binding to these GAGs, YM1 causes their lysis. Heparan sulfate GAGs are part of the macrophage glycocalyx and diminished sulfation of heparan sulphate can influence the macrophage function in diseases, as it was shown that reduced macrophage sulfation can cause atherosclerosis and obesity through, for instance, the conversion into foam cells – an M1 macrophage type. While decreased sulfation leads to the M1 phenotype, overexpression of heparanase – the enzyme responsible for degrading heparan sulfate – is related to increased expression of the M2-stimulating molecules like IL-10 and CCL2. This indicates the possible role of YM1 in macrophage activation through regulating heparan sulfate levels, as low heparinase levels increase YM1 accumulation in macrophages (21). YM1 also binds chitin. In this way, YM1 may have a defensive role by binding chitin-containing pathogens. However, due to a lack of chitinase activity, its precise mechanism remains unknown. Macrophages synthesise YM1 during infection or allergy. In these macrophages, YM1 can be upregulated via IL-4 and IL-13 through the binding of these stimuli to the IL-4 receptor, activating STAT6 (11). Like FIZZ1, IFN-γ suppresses YM1, suggesting its anti-inflammatory character and indicating why

sustained overexpression of these proteins might lead to an M2-like phenotype (20).

FIZZ1 and YM1 might not need the prior activation of the IL-4/STAT pathway to induce anti-inflammatory features in macrophages. This is derived from a study by Goren *et al.* which describes that YM1 is not only expressed in alternatively activated macrophages but is also present in peritoneal and bone marrow-derived neutrophils at higher concentrations (22, 23). Moreover, as YM1 contains a secretory signal peptide, it is secreted in substantial amounts from these cells (22). YM1 secreted by neutrophils at the wound site can be taken up by macrophages without the activation of the IL-4/STAT pathway (22). In that way, YM1 might contribute to anti-inflammatory changes in these macrophages as a response to the wound neutrophils (22). However, although FIZZ1 and YM1 are considered signature markers for M2 macrophages, their functional role within macrophage polarization has yet to be discovered. With this study, we want to evaluate whether the overexpression of anti-inflammatory M2 markers FIZZ1 or YM1 in naive macrophages can lead to an M2-like phenotype. We hypothesise that overexpression of M2 markers into naive macrophages can create functional anti-inflammatory M2-like macrophages.

Besides overexpressing M2 markers to increase functional recovery after SCI, increasing the number of M2 macrophages could also be achieved through a different route, based on the findings of the research team which revealed that IL-13 overexpressed mesenchymal stem cells increased the number of alternatively activated macrophages while promoting functional recovery after SCI (14). It was also found that IL-13 overexpressed macrophages could enhance this effect. However, as previously stated, local injection of these macrophages might perforate tissue and cause bleedings, while systemic injection may cause allergic reactions. Thereby, it is crucial to find an alternative administration route. Overexpressing CCR2 in combination with pre-polarization towards the M2 phenotype – using for example IL-13 recombinant treatment – might therefore be the optimal approach to induce functional recovery of SCI after systemic injection. Accordingly, genetically engineering M2 macrophages to provide sustained local administration might have beneficial effects after a SCI.

Normally, macrophages exhibit functional plasticity and the capacity to switch between M1 and M2 phenotypes depending on the microenvironment of the injury (12). In the present study, we strive towards creating stable M2-like macrophages that do not switch to the pro-inflammatory M1 phenotype when present in a pro-inflammatory environment. As no research has been performed on using these proteins to create M2-like macrophages, our study will be the first in its field to create FIZZ1 or YM1 overexpressed macrophages. Consequently, this study might provide insight into the role of FIZZ1 and YM1 in the action of macrophages and ultimately might play a role as a novel therapeutic approach for SCI patients.

EXPERIMENTAL PROCEDURES

Cell culture – HEK293T cells (HEK) or RAW264.7 cells (RAW) were cultured in high-glucose Dulbecco's Modified Eagle's medium (DMEM) (Sigma) supplemented with 10% heat-inactivated fetal calf serum (FCS) at 37°C and 5% CO_2. Cells were passaged every four days until plated out for further experiments. For HEK and RAW cells, Trypsin/EDTA or cell scraping was used for passaging, respectively.

HEK or RAW cell transfection – Cells were cultured in 6-well plates at $1.56*10^4$ cells/cm^2 (HEK) or $2.6*10^4$ cells/cm^2 (RAW) using DMEM+FCS. Cells were co-cultured with FIZZ1 or YM1 overexpression vectors. Green fluorescent protein (GFP) transfection was used as a control. Calcium phosphate transfection was performed one day after plating the cells. Briefly, 2 µg DNA was diluted in 86 µl 2xHBS and 5.1 µl of 2.5 M $CaCl_2$ per well. After 2 minutes of incubation at room temperature, the mixture was added dropwise to the cells.

RAW cell stimulation – RAW cells were stimulated towards M1 using LPS (Merck, 200 ng/ml, 24h) or towards M2 using IL-10 (Peprotech, 10/15/20 ng/ml, 3h or 24h) or IL-4 (Peprotech, 20/33/40 ng/ml, 3h or 24h).

RT-qPCR – Total RNA was extracted from the cells according to the RNeasy Mini Kit protocol (Qiagen). RNA concentration was measured using a Nanodrop® 2000. Complementary DNA (cDNA) was synthesised using reverse transcription and qScript cDNA supermix (Quanta Biosciences). SYBR Green PCR master mix (Applied Biosystems) was used for the RT-qPCR gene expression analysis. Sequences of the primers are shown in *Supplementary Table 1*.

Western blot – Protein concentrations of RAW cells transfected with FIZZ1 or YM1 or stimulated towards M1 or M2 were measured using the Pierce BCA protein assay kit (Thermo Scientific) and the iMARK microplate reader (Bio-Rad Laboratories). Protein samples (10 µg) were denatured and separated on a 7% (iNOS) or 12% (Arg-1) SDS gel and transferred at 200V for 45 minutes onto a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked in 5% non-fat powdered milk in Tris-buffered saline-Tween 20 (0.1%) (TBS-T) for 30 minutes before probing overnight at 4°C with a primary antibody: mouse anti-mouse iNOS (1/500, Sigma-Aldrich), rabbit anti-mouse Arg-1 (1/500, Santa Cruz Biotechnology), mouse anti-mouse β-actin (1/2000, Santa Cruz Biotechnology), or FIZZ1 (1/1000, Abcam). Afterwards, the membrane was washed with TBS-T and incubated with rabbit anti-mouse horseradish peroxidase (HRP)-labelled secondary antibody (1/1000, Dako). A goat anti-rabbit HRP secondary antibody was used to evaluate FIZZ1 protein expression. An ECL Plus detection kit (Thermo Scientific) was used to detect the chemiluminescent signal by a luminescent image analyzer (ImageQuant LAS 4000 mini, GE Healthcare).

Plasmid amplification – Plasmids encoding FIZZ1, YM1, or CCR2 were transformed into *Escherichia coli* (NEBiolabs) and amplified overnight at 37°C and 180 rpm in LB medium containing ampicillin (1/1000). Plasmids were isolated using the Nucleospin® Plasmid Easypure kit (Macherey-Nagel). Purification was performed according to the Nucleobond® Xtra Midi kit (Macherey-Nagel).

Bone marrow isolation and culture of bone-marrow derived macrophages (BMDMs) – Bone marrow cells were isolated from tibias and femurs of 6-8 weeks old C57BL/6 mice by flushing the marrow cavity with sterile Phosphate buffered saline (PBS). Cells were passed through an 18-gauge needle to obtain a single-cell suspension. Cells were cultured for 7 days in RPMI medium supplemented with 15% L-cell conditioned media (LCM) before plating in 24-well plates at $7.5*10^4$ cells/cm^2. BMDMs were transduced with different concentrations of CCR2 (LV5, LV10, LV20) to create CCR2 overexpression. Macrophages undergone puromycin selection (1.5 µl in 10 ml) and stimulation with either rIL-13 (Peprotech, 33 ng/ml) or LPS (Merck, 200 ng/ml) to obtain M2 and M1 controls, respectively.

ELISA CCR2 – CCR2 production was measured in the medium of M0, M1 (LPS, 200 ng/ml), M2 (IL-13, 33 ng/ml), and CCR2 stimulated BMDMs. The assay was performed using the mouse CC-chemokine receptor 2, CCR2 ELISA kit according to the manufacturer's instructions (MyBiosource).

Statistical analysis – All statistics were performed using GraphPad Prism 9.1.0 software. Data were analysed for normal distribution using D'Agostino-Pearson normality test. Differences between two groups were evaluated using the t-test for normally distributed data. Differences between multiple groups were evaluated using ANOVA or the non-parametric Kruskal-Wallis test. Data was represented as mean ±SEM. Statistical differences were considered significant at $P < 0.05$.

RESULTS

Transfecting RAW cells with FIZZ1 or YM1 plasmids results in expression of only the FIZZ1 and YM1 gene, respectively – First, the efficiency of the FIZZ1 and YM1 plasmids needed to be confirmed. Therefore, RAW cells – a monocyte/macrophage-like cell line – were transfected with either a FIZZ1 or YM1 plasmid to overexpress these anti-inflammatory genes. Initially, HEK cells were transfected as proof of principle since they are easily transfected (25). HEK cells transfected with FIZZ1 only expressed the FIZZ1 gene (*Supplementary Fig1A, B*). For YM1, this experiment yields a comparable result (*Supplementary Figure 1C*). *Figure 2* shows the same results as in the HEK control experiment but in RAW cells. After transfection of RAW cells with

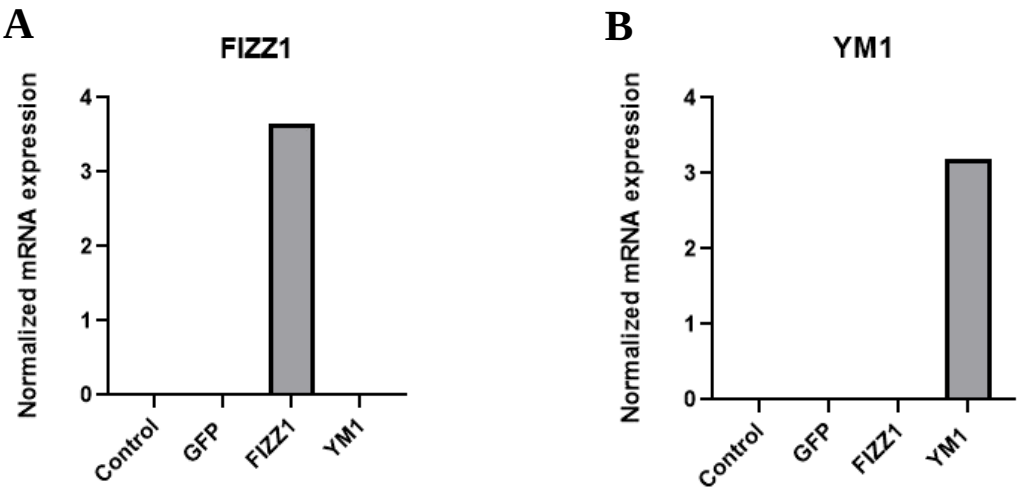
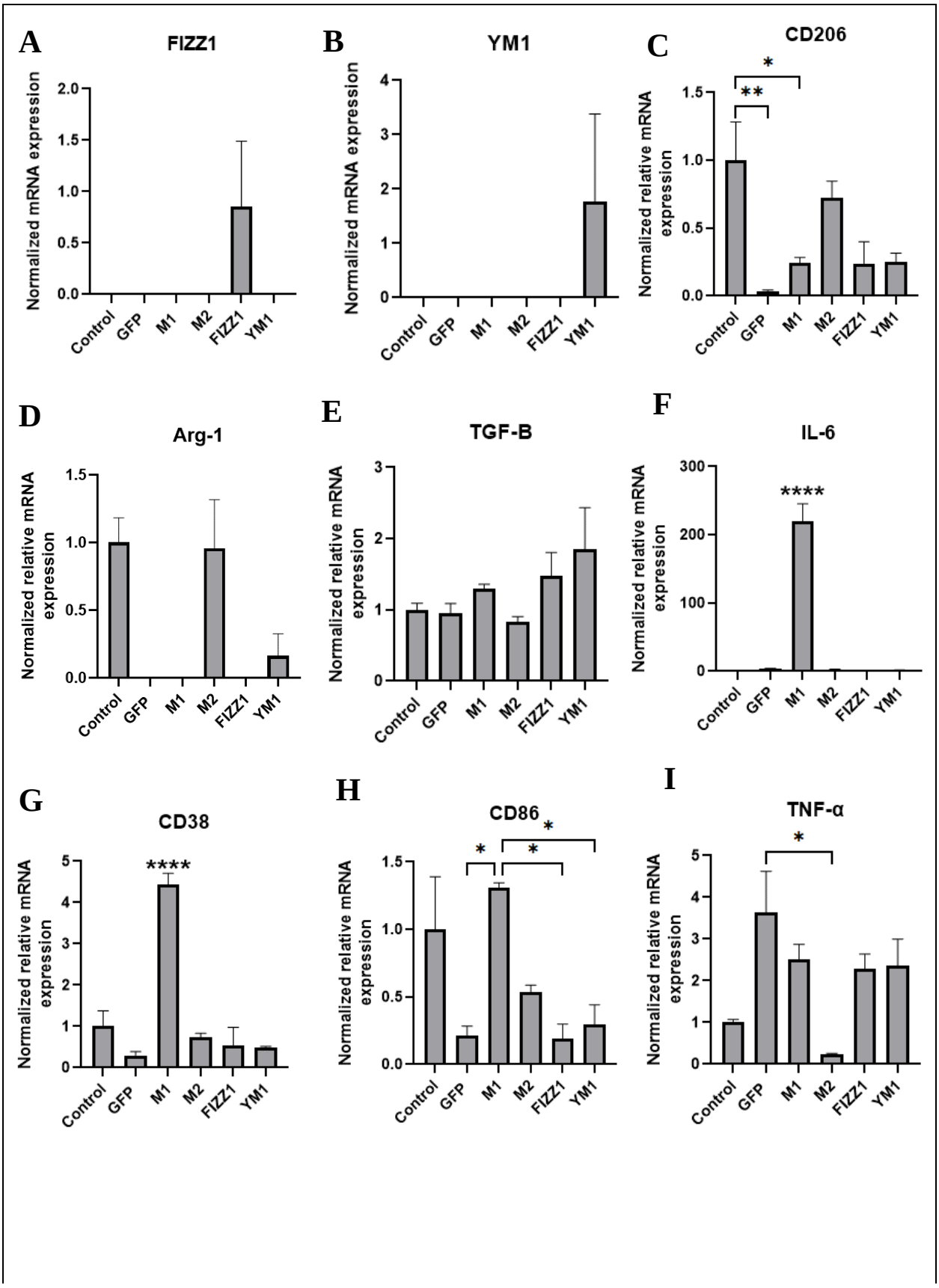


Fig. 2 – RAW264.7 cells express FIZZ1 and YM1 after transfection. An RT-qPCR was performed to evaluate the gene expression of FIZZ1 **(A)** and YM1 **(B)** after transfection with different plasmids shown on the x-axis. Control cells were not transfected. FIZZ1 **(A)** and YM1 **(B)** mRNA is only present in FIZZ1 and YM1 transfected cells, respectively. Data was normalised using housekeeping genes YWHAZ and GAPDH (n=1).

the FIZZ1 vector, only the FIZZ1 gene is expressed while no expression of YM1 could be detected (*Figure 2A*). The same conclusion was validated for the YM1 vector. Here, only YM1 expression could be detected in the transfected cells *(Figure 2B)*. As a control, non-transfected cells and cells transfected with GFP were included. GFP-transfected cells served as a visible confirmation for the transfection process. Since these are preliminary results (n=1), no statistical analysis was performed.

RAW cells do not polarise towards an M2 phenotype after transfection with FIZZ1 or YM1 – After confirming successful transfection, the next step was to evaluate whether RAW cells polarised towards an M1 or M2 phenotype after transfection with FIZZ1 or YM1. To test this, the presence of macrophage phenotype markers was investigated using RT-qPCR. The gene expression of both M1 markers (IL-6, CD38, CD86, TNF-α, iNOS) and M2 markers (FIZZ1, YM1, CD206, Arg-1, TGF-β) was evaluated. As a positive control, RAW cells were stimulated with either LPS (200 ng/ml, 24h) or IL-10 (10 ng/ml, 24h) to create an M1 or M2 phenotype, respectively. As a negative control, cells were left untreated. After transfection with M2-associated genes FIZZ1 or YM1, cells did not polarise towards an M2 phenotype (*Figure 3*). Firstly, overexpression of FIZZ1 and YM1 was confirmed (*Figure 3A, B*). Gene expression of M2 markers CD206 and Arg-1 was not increased in RAW cells transfected with FIZZ1 or YM1 (*Figure 3C, D*) However, transfected RAW cells showed increased gene expression of one examined M2 marker, TGF-β (*Figure 3E*). Nevertheless, expression of TGF-β was also detected in M1 stimulated and M2 stimulated cells (*Figure 3E*). Additionally, no M2 overexpression could be created within this experiment after stimulation with IL-10, as the expression of M2 markers CD206, Arg-1, and TGF-β was not significantly increased in these M2 stimulated cells compared to the untreated control cells. Next, no effect of transfection on the gene expression of M1 markers IL-6, CD38, and CD86 could be detected (*Figure 3F-H*). TNF-α expression was increased in both M1 stimulated cells and FIZZ1 and YM1 transfected cells (*Figure 3I*). The same is applicable for the iNOS gene expression (*Figure 3J*). To confirm these findings on the protein level, western blot was performed. *Figure 4* shows that the iNOS protein expression in RAW cells stimulated towards M1 is increased compared to M2 stimulated cells (*Figure 4A, B*). No significance was detected as iNOS expression was not measured in the M2 group. iNOS proteins could also be detected in FIZZ1 or YM1 transfected cells, however, expression was not significantly different compared to the other groups (*Figure 4B*).


A
FIZZ1
Normalized mRNA expression
Control
GFP
M1
M2
FIZZ1
YM1
B
YM1
C
CD206
Normalized relative mRNA expression
*
**
D
Arg-1
E
TGF-B
F
IL-6
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G
CD38
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CD86
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TNF-α

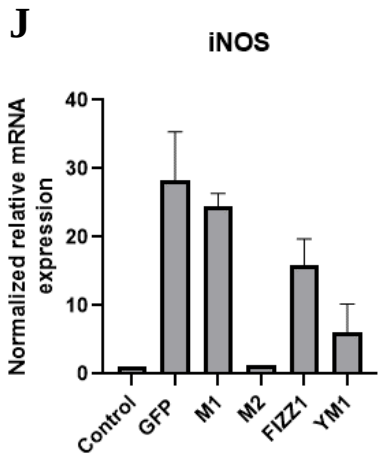


Fig. 3 – FIZZ1 and YM1 transfected RAW cells tend to show increased pro-inflammatory gene expression at passage 16. Using RT-qPCR, the presence of M2 markers FIZZ1 **(A)**, YM1 **(B)**, CD206 **(C)**, Arg-1 **(D)**, TGF-β **(E)** and M1 markers IL-6 **(F)**, CD38 **(G)**, CD86 **(H)**, TNF-α **(I)**, iNOS **(J)** was evaluated after transfection with either FIZZ1, YM1, or GFP, shown on the x-axis. To control the polarisation state of each gene, RAW cells were stimulated with LPS (200 ng/ml, 24h) or IL-10 (10 ng/ml, 24h) to generate an M1 or M2 phenotype, respectively. FIZZ1 and YM1 gene expression was exclusively detected in FIZZ1 and YM1 transfected cells, respectively **(A, B)**. Gene expression of M2 markers CD206 and Arg-1 was not significantly different from M1 stimulated cells **(C, D)**. Expression of M2 marker TGF-β was increased in M1 stimulated cells compared to M2 cells **(E)**. M1 markers IL-6, CD38, and CD86 were not detected after FIZZ1 or YM1 transfection **(F-H)**. Gene expression of TNF-α and iNOS tends to be increased after transfection **(I, J)**. Data was normalised using housekeeping genes YWHAZ, 18-S, CYPA, and HMBS. Data represent mean ±SEM (n=3-4). *p < 0.05; **p < 0.01; ****p < 0.0001.

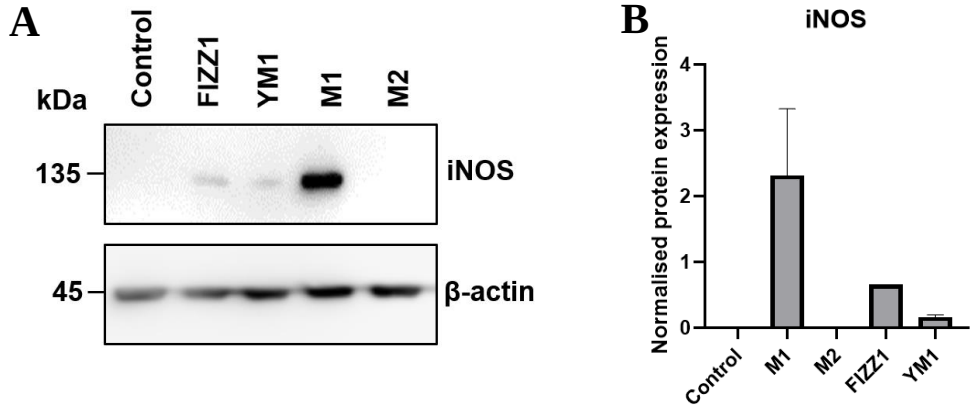
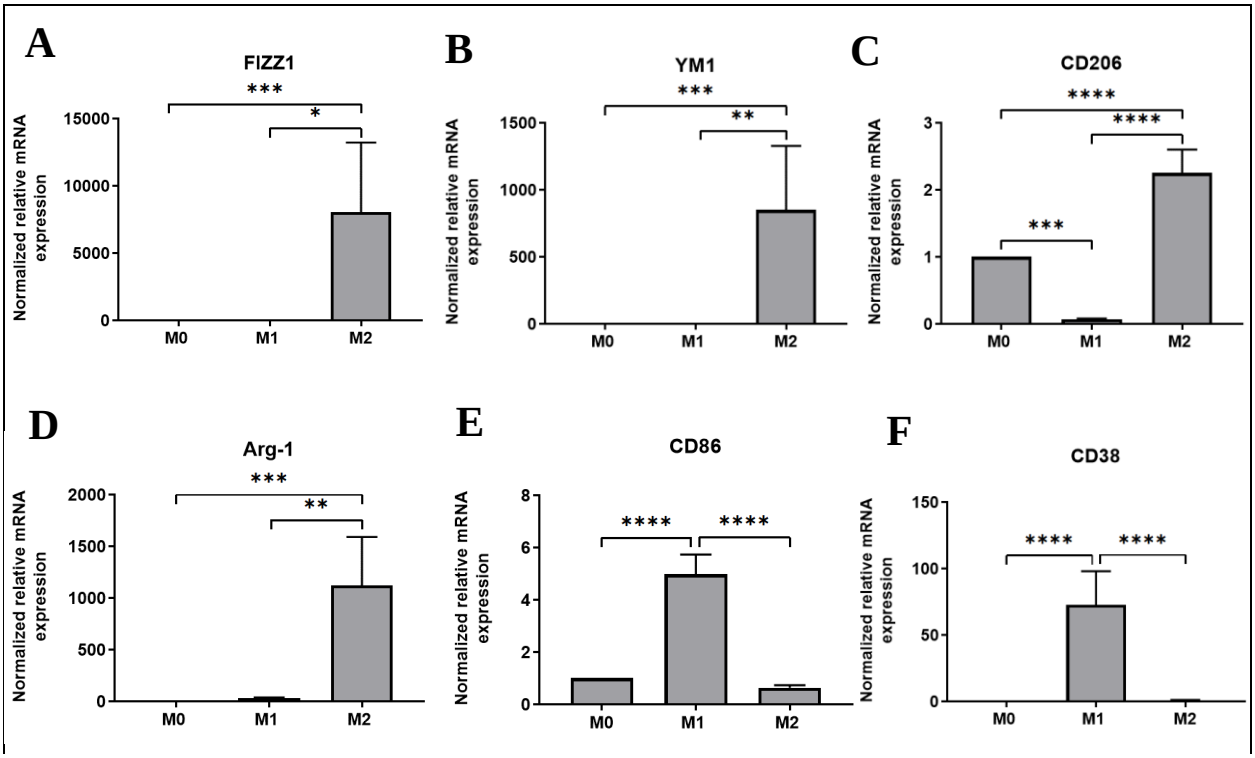


Fig. 4 –FIZZ1 or YM1 transfected cells do not significantly express the iNOS protein. A western blot was performed to evaluate the expression of the iNOS protein after transfecting RAW cells with FIZZ1 or YM1 or after stimulating the cells towards M1 or M2 with LPS (200 ng/ml, 24h) or IL-10 (10 ng/ml, 24h), respectively, as shown on the x-axis. iNOS protein expression was the highest in the positive control group, the M1-stimulated RAW cells **(A-B)**. No significant expression of iNOS is found after transfection with FIZZ1 or YM1 **(A-B)**. Data represent mean ±SEM (n=3).

IL-4 or IL-10 stimulation of RAW cells does not lead to a clear M2 phenotype – As the gene expression of CD206 and Arg-1 after M2 stimulation with IL-10 did not significantly differ from the control cells (*Figure 3*) and thereby did not result in a positive control for the experiment, an M2 optimisation experiment was performed using different concentrations and stimulation times of IL-4 or IL-10, as both cytokines are commonly used to induce M2 macrophage polarisation. While CD206 expression was present after 24h stimulation with IL-4 at a concentration of 20 ng/ml and 40 ng/ml, Arg-1 gene expression was elevated after 3h of stimulation with IL-4 at 33 ng/ml (*Supplementary Figure 2A, B*). TGF-β expression was present in all samples except for the control cells (*Supplementary Figure 2C*). While M1 markers IL-6 and TNF-α were elevated after LPS stimulation and not after IL-4 stimulation (*Supplementary Figure 2 D, F*), CD86 and iNOS were not expressed after M1 stimulation (*Supplementary Figure 2 E, G*). Following stimulation with IL-10, CD206 was not detected (*Supplementary Figure 3A*), while Arg-1 expression was increased after 3h of stimulation (*Supplementary Figure 3B*). TGF-β expression was found in all IL-10 stimulated samples (*Supplementary Figure 3C*). M1 markers IL-6, TNF-α, and iNOS were primarily identified after M1 stimulation (*Supplementary Figure 3D, F, G*), while CD86 gene expression was found in the control cells and after 24h stimulation with IL-10 (*Supplementary Figure 3E*). No statistical analysis was performed on these preliminary results (n=1).

BMDMs polarise towards an M2 phenotype after transduction with IL-13 – As no clear M2 control group could be created in RAW cells, BMDMs were used instead. IL-13, one of the main mediators of the alternatively activated phenotype in macrophages, was used to stimulate BMDMs to achieve M2 polarisation (33-35). After stimulating BMDMs with IL-13, there is a significant increase in expression of M2 markers FIZZ1, YM1, CD206, and Arg-1 (*Figure 5A-D*). Gene expression of M1 markers CD86, CD38, TNF-α, and iNOS is significantly decreased in IL-13 stimulated cells and the naïve control cells (M0) compared to the LPS stimulated M1 cells (*Figure 5 E-H*).

CCR2 transduction does not polarise BMDMs towards an M2 phenotype – To stimulate macrophage migration, CCR2 was transduced into BMDMs. RT-qPCR results revealed no polarisation towards M2, as gene expression of M2 markers CD206 and Arg-1 was not significantly increased (*Figure 6A, B*). TGF-β expression was present in all groups (Figure 6C). M1 markers IL-6



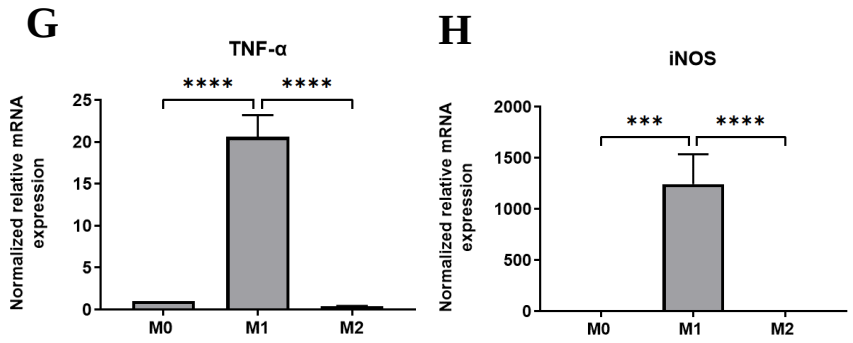
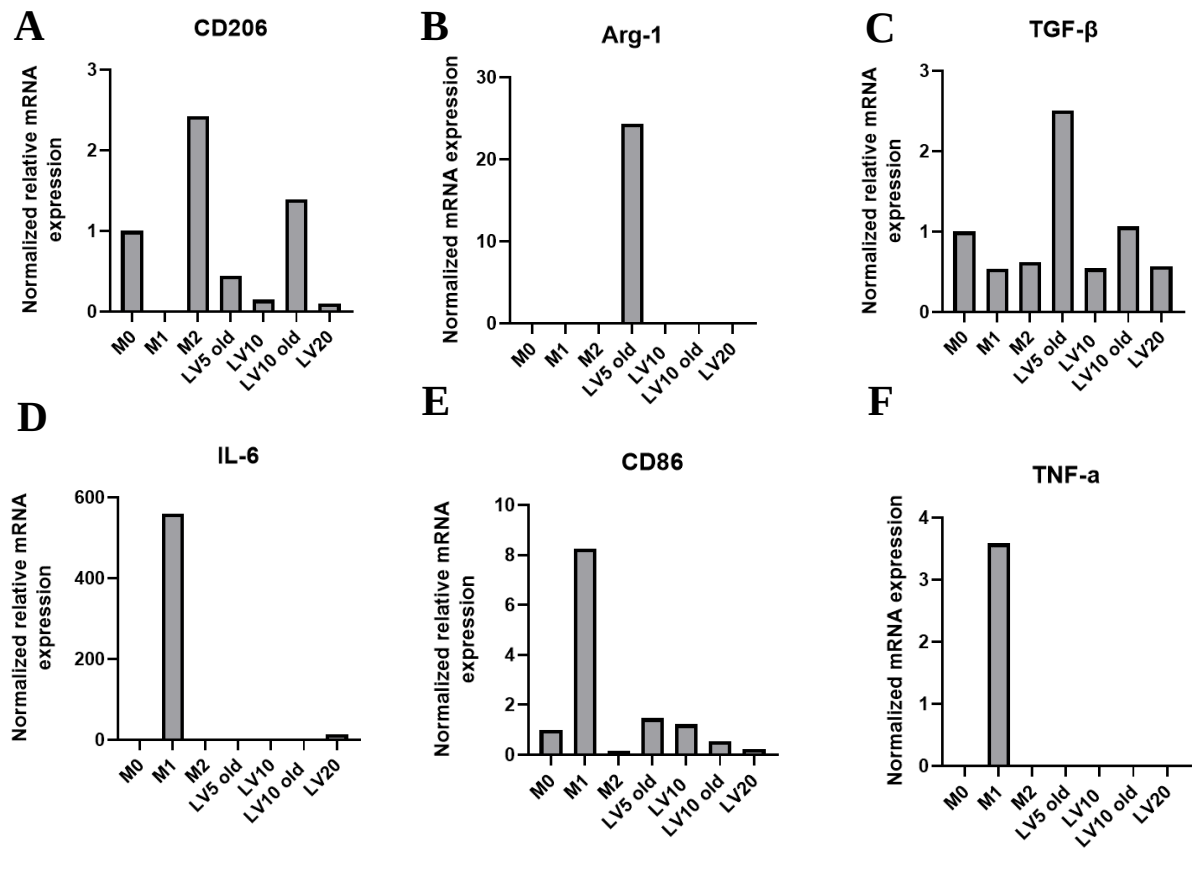


Fig. 5 – BMDMs polarise towards an M2-phenotype after transduction with IL-13. Using RT-qPCR, the presence of M2 markers FIZZ1 **(A)**, YM1 **(B)**, CD206 **(C)**, Arg-1 **(D)** and M1 markers CD86 **(E)**, CD38 **(F)**, TNF-α **(G)**, and iNOS **(H)** was evaluated after stimulating BMDMs towards an M2 phenotype using IL-13 (33 ng/ml). To control the polarisation state of each gene, BMDMs were stimulated with LPS (200 ng/ml) to generate an M1 phenotype. Gene expression of M2 markers FIZZ1 **(A)**, YM1 **(B),** CD206 **(C)**, and Arg-1 **(D)** is significantly increased in M2 stimulated cells compared to M1 stimulated cells. M1 markers CD86 **(E)**, CD38 **(F)**, TNF-α **(G)**, and iNOS **(H)** are significantly increased in M1 stimulated BMDMs compared to unstimulated (M0) and M2 stimulated cells. Data was normalised using housekeeping genes YWHAZ and HMBS. Data represent mean ±SEM (n=12-14). **p < 0.01; ***p <0.001; ****p < 0.0001.



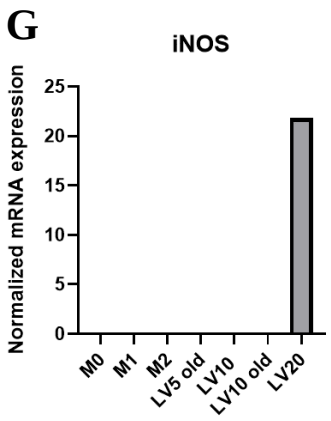


Fig. 6 – BMDMs stimulated with CCR2 do not polarise towards an M2 phenotype. Using RT-qPCR, the presence of M2 markers CD206 **(A)**, Arg-1 **(B)**, TGF-β **(C)** and M1 markers IL-6 **(D)**, CD86 **(E)**, TNF-α **(F)**, iNOS **(G)** was evaluated after stimulating BMDMs with CCR2 at different concentrations (LV, MOI). To control the polarisation state of each gene, BMDMs were stimulated with LPS (200 ng/ml) or IL-13 (33 ng/ml) to create an M1 or M2 phenotype, respectively. Expression of M2 markers CD206 and Arg-1 was decreased in the transduced cells compared to the control cells **(A-B)**. M2 marker TGF-β was expressed in the CCR2 transduced BMDMs **(C)**. Expression of M1 markers IL-6, CD86, and TNF-α was increased in the M1 control cells **(D-F)**. Increased iNOS gene expression was detected in the M1 stimulated cells and in the LV20 CCR2 group **(G)**. Data was normalised using housekeeping genes YWHAZ, 18-S, and CYPA. Data represent preliminary results (n=1). LV: lentivirus; MOI: multiplicity of infection.

CD86, TNF-α, and iNOS were all detected in M1 stimulated cells (*Figure 6D-G*). M2 and CCR2 transduced cells showed no expression of M1 markers IL-6, CD86, and TNF-α (*Figure 6 D-F*). Only iNOS expression was found in cells transduced with the highest number of lentiviral particles (*Figure 6G*).

CCR2 transduction in BMDMs does not result in CCR2 overexpression – Since the CCR2 transduction did not result in an M2 switch of the macrophage phenotype, it was evaluated whether the CCR2 transduction – and thereby overexpression – was successful itself. For this, CCR2 production was measured via ELISA. *Figure 7* shows that after CCR2 transduction, no significant increase of CCR2 production could be measured compared to the untreated (M0) and pro-inflammatory (M1) macrophages. However, CCR2 production was significantly decreased in M2 stimulated BMDMs compared to M0 cells.

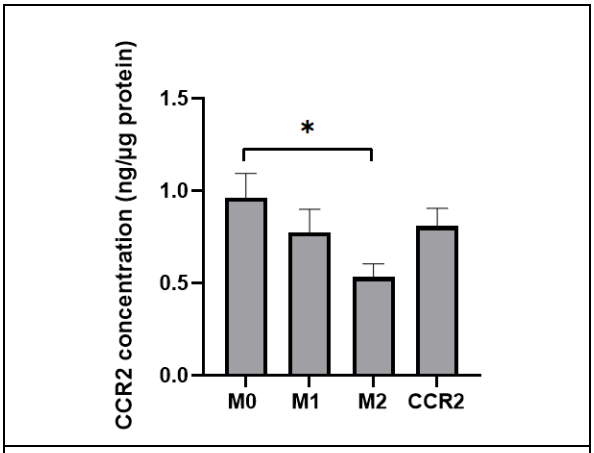


Fig. 7 – CCR2 overexpressed BMDMs have no significant increased CCR2 production compared to M0, M1, and M2 cells. Culture medium was collected to analyse CCR2 production via ELISA. No difference in CCR2 production can be detected between the CCR2 stimulated cells, the M0 cells, and the M1 cells. In IL-13 stimulated cells (M2), CCR2 production was significantly decreased compared to the M0 control cells. Data represent mean ±SEM (n=12-14).

DISCUSSION

BMDMs are more easily polarised towards an M2 phenotype compared to RAW cells – In this study, we first investigated whether the overexpression of M2 markers FIZZ1 or YM1 can polarise RAW cells towards an M2 phenotype to benefit from their anti-inflammatory characteristics after SCI. To control the polarisation state, M1 and M2 stimulated cells were developed. However, after stimulation with IL-10, no obvious polarisation towards an M2 phenotype was detected as M2 markers CD206 and Arg-1 were not significantly increased compared to the untreated control cells. Therefore, additional experiments were performed using different concentrations and time points of RAW cell stimulation with either IL-10 or IL-4, according to the literature (24-29). Nevertheless, also from these experiments, no stable M2 control could be generated. Hence, BMDMs were used instead to evaluate whether an M2 phenotypical switch could be successfully generated in these cells, as it is indicted in the study by Das *et al.* that macrophage cell lines do not express a BMDM signature (30). In fact, it is shown that M2 markers CD206, Arg-1, FIZZ1, and YM1 are significantly increased in IL-13 stimulated M2 cells whereas gene expression of M1 markers CD86, CD38, TNF-α, and iNOS is significantly decreased compared to LPS stimulated M1 cells. Consequently, polarisation towards M2 is easier generated in BMDMs compared to RAW cells.

Overexpression of M2 marker Arg-1 as a first candidate to create super macrophages – The final goal of this study is to create super macrophages by overexpressing anti-inflammatory markers or migration-mediating receptors in macrophages and creating a mixture of these different genetically engineered M2-like macrophages to improve functional repair after SCI. The first candidate to overexpress is the enzyme Arg-1. The macrophage arginine metabolism is key to define the two opposite macrophage phenotypes: M1 and M2 (31). In macrophages, arginine is the precursor of two metabolic pathways: it is either metabolised by iNOS to NO and citrulline or it is hydrolysed by arginase to ornithine and urea (31). NO production from iNOS inhibits cell proliferation, an M1 response, while ornithine produced from arginase promotes cell proliferation and the repair of tissue damage, an M2 response (32). Thereby, overexpressing Arg-1 in macrophages is a logical start to create M2-like macrophages. As a significant increase of Arg-1 could already be acquired through IL-13 overexpression, this study wanted to focus on the overexpression of other M2 markers, namely FIZZ1 and YM1 (14).

FIZZ1, YM1, and CCR2 as overexpression candidates –Although FIZZ1 and YM1 are both claimed to be anti-inflammatory markers that are expressed after stimulation of the STAT6 pathway, they were not previously used to create M2-like macrophages. Besides having the anti-inflammatory characteristics from the genetically engineered macrophages, it is equally crucial for the cells to reach the lesion site. Therefore, overexpression of CCR2 – the CCL2 receptor – was added to the mixture of macrophages as CCL2 production is significantly increased at injury (18). In that way, cells with a CCR2 overexpression can – in theory – easily migrate towards the lesion size (33). However, since no CCR2 plasmid was available at the time, only transfection with FIZZ1 and YM1 was executed in RAW cells, whilst CCR2 overexpression was performed in BMDMs. FIZZ1 and YM1 overexpression could not be tested in BMDMs yet due to the unavailability of their lentiviral vectors which could not be created within the time frame of this study.

No clear macrophage phenotype after transfecting RAW cells with FIZZ1 or YM1 – Contrary to the hypothesis, our findings indicate that after transfecting RAW cells with FIZZ1 or YM1, gene expression of M2 markers CD206 and Arg-1 is not increased compared to the untreated controls. The passage number might be a possible explanation for this. As Taciak *et al.* reported in their study, gene expression in RAW cells is highly dependent on the passage number of the cells (34). Consequently, it is possible that RAW cells with passage number 16 are more activated and consequently have a higher baseline gene expression. Taciac *et al.* indicated that gene expression of both the M1 marker CD86 and the M2 marker CD206 is indeed increased in RAW cells with higher passage numbers (34). An increased gene expression of these specific macrophage markers in untreated control cells was also visible in the RT-qPCR of this study. When looking at the other M2 marker, TGF-β, its

expression was increased after transfection but was also increased in the M1 stimulated cells. This is in line with the results of a study by Hald *et al.* where RAW cells were stimulated with LPS after which TGF-β expression levels did not differ compared to vehicle stimulated cells (35). Therefore, TGF-β might not be the ideal M2 marker to test M2 polarisation in RAW cells. The results of the M1 markers were more robust. Gene expression of M1 markers IL-6, CD38, and CD86 was significantly decreased in cells transfected with FIZZ1 or YM1 compared to the M1 control group. However, gene expression of other M1 markers, TNF-α and iNOS, was not significantly different between FIZZ1 and YM1 transfected cells and M1 controls. This indicates that RAW cells might have gained a slight pro-inflammatory phenotype after transfection with either FIZZ1 or YM1.

Calcium phosphate transfection is a variable method to introduce DNA into RAW cells – Not only was TNF-α and iNOS mRNA expression increased after FIZZ1 and YM1 transfection, but the same was evident after GFP transfection, suggesting that the transfection procedure itself might influence the polarisation state of the RAW cells. Thompson *et al.* already detected that calcium phosphate transfection showed the greatest transfection variation in RAW cells compared to other transfection methods such as electroporation and lipofectamine (36). Additionally, several studies recommend using electroporation as it has the greatest inducibility of genes in RAW cells (36, 37). Also lipofectamine and Fugene are commonly used to transfect plasmid DNA into RAW cells (38, 39). Calcium phosphate transfection is not commonly used in RAW cells, but rather in HEK cells. Additionally, the calcium phosphate transfection protocol was optimised within our research team specifically for HEK cells. In conclusion, other transfection methods besides calcium phosphate transfection might provide a more stable introduction of the DNA into the RAW cells and should therefore be considered for future RAW cell experiments.

The anti-inflammatory markers FIZZ1 and YM1 also have pro-inflammatory characteristics – Although FIZZ1 is a well-known marker for the anti-inflammatory M2 macrophages, it also plays a significant role in inflammatory pathways in the lungs (40). There, FIZZ1 upregulates pro-inflammatory mediators like MCP-1 and vascular endothelial growth factor (VEGF) while recruiting pro-inflammatory macrophages and producing ROS (41). As a result, FIZZ1 is responsible for pulmonary vascular remodelling, which is characterised by thickening of the pulmonary arterial wall, leading to increased pulmonary vascular resistance (41). Thereby, inhibition of FIZZ1 in the lungs is a possible route to reduce lung inflammation. Considering this, the pro/anti-inflammatory characteristics of FIZZ1 might be location-dependent. Additionally, YM1 might also contribute to lung inflammation as it is associated with epithelial damage in the lungs (11, 42).

Are the used M1 and M2 markers optimal for RAW cells? – The fact that both FIZZ1 and YM1 might also have pro-inflammatory characteristics is in line with the gene expression found in this study which shows that RAW cells transfected with FIZZ1 or YM1 also express pro-inflammatory M1 markers such as TNF-α, while this marker could not be found in M2 stimulated RAW cells. These results can be confirmed through the results from Boehler *et al.* who treated RAW cells with a lentivirus containing IL-10 (13). Boehler *et al.* found that TNF-α expression was highest in the M1 stimulated group and lowest in the IL-10 treated group (13). Consequently, it can be concluded that TNF-α is a true M1 marker for RAW cells. Besides TNF-α being an appropriate M1 marker to check polarisation in RAW cells, also iNOS can be considered adequate as iNOS gene expression could not be detected in the untreated control group and the M2 stimulated cells. This low baseline iNOS expression lies in line with the study of Taciak *et al.* (34). There, it was shown that iNOS expression was stable at a low concentration throughout different passages (34). In this study, iNOS gene expression was increased in M1 stimulated cells throughout all performed experiments, making it a true M1 marker in RAW cells. Additionally, M1 markers IL-6 and CD38 were only significantly increased in the LPS stimulated RAW cells and thereby can be considered valid M1 markers. Only CD86 also had an increased expression in the control cells and thereby might be less adequate according to our study. However, it is a commonly used M1 marker for M1 polarisation of RAW cells (43-45).

Concerning the M2 markers, also CD206 and Arg-1 were expressed in the control cells, which might be explained by their high baseline expression depending on the passage number (34). Therefore, future studies potentially need to consider additional M2 markers when polarising RAW cells, such as CD163. Another solution is to change the primer sequence for the M2 markers used for determining gene expression levels to make them more specific for RAW cells. For example, in a study by de Campos *et al.*, Arg-1, FIZZ1, and YM1 protein expression was evaluated in RAW cells using RT-qPCR, but different primers sets were used compared to the ones used in this study (46). However, a study performed by Liu *et al.* did use the same primers as this study for FIZZ1 but also used a different Arg-1 primer, which is also different from the one used in the study of de Campos *et al.* (46, 47).

CCR2 overexpression does not polarise BMDMs towards an M2 phenotype – Besides overexpressing M2 markers with the goal to benefit from their anti-inflammatory characteristics after SCI, this study was also interested in increasing the migration potential of genetically engineered BMDMs. Therefore, BMDMs were transduced with the chemokine receptor CCR2 since its ligand CCL2 is increased at the spinal injury site. Consequently, a higher expression of CCR2 would – theoretically – result in an increased migration towards the lesion. However, no overexpression of CCR2 could be detected via ELISA. The CCR2 lentiviral vector used in this study did not contain a fluorescent marker such as GFP or a fluorescent protein such as mCherry. Thereby, future studies will benefit from adding a fluorescent marker as transduction efficiency can be checked earlier during the experiment by measuring the fluorescent-expressing cells as a percentage of the total number of cells. Additionally, no clear polarisation was observed as gene expression of both M1 and M2 markers could not be detected, possibly because CCR2 could not be overexpressed in BMDMs. As these were only preliminary results (n=1), repetition is still needed. However, as inhibiting CCR2 reduces M2 phenotype polarisation in BMDMs, overexpression is expected to have the opposite effect: a more prominent M2 phenotype (48).

BMDMs might be better cells to induce FIZZ1 or YM1 overexpression – Since M2 polarisation could not be acquired in RAW cells, M2 overexpression was investigated in BMDMs. In BMDMs, overexpression of M2 markers FIZZ1, YM1, CD206, and Arg-1 could be detected after stimulation with IL-13. This indicates that the BMDMs might be more adequate cells as both M1 and M2 controls can be made more effectively. Additionally, since RAW cells are a murine cell line, their immaturity might limit experimental conclusions (49). As BMDMs are isolated from mice, experiments on these cells are better extrapolated to *in vivo* macrophage functions. However, as also experienced by our own research team, BMDMs are more difficult cells to work with as they need to grow for around ten days prior to use (50, 51). Additionally, as transduction is able to create a more sustained and stable overexpression, this method is should also be considered for future experiments in RAW cells besides changing the transfection method (52).

CONCLUSION

In summary, our work reveals no increased expression of M2 markers CD206 and Arg-1 after transfecting RAW264.7 cells with M2-associated genes FIZZ1 or YM1. The other M2 marker, TGF-β, cannot be considered a true M2 marker as its expression was also detected in both M1 stimulated cells and control cells. As gene expression of M1 markers TNF-α and iNOS was increased after overexpression with FIZZ1 or YM1, this indicates a slight pro-inflammatory phenotype after transfection. However, this might also be due to the calcium phosphate transfection procedure. Therefore, forthcoming RAW cell experiments may consider other transfection methods or transduction instead. As an M2 phenotype could be achieved in BMDMs but not in RAW cells, BMDMs are preferred for future experiments. When overexpression of M2 markers is successful in BMDMs and induces a true M2 phenotype, research might be one step closer to improving functional recovery after SCI.

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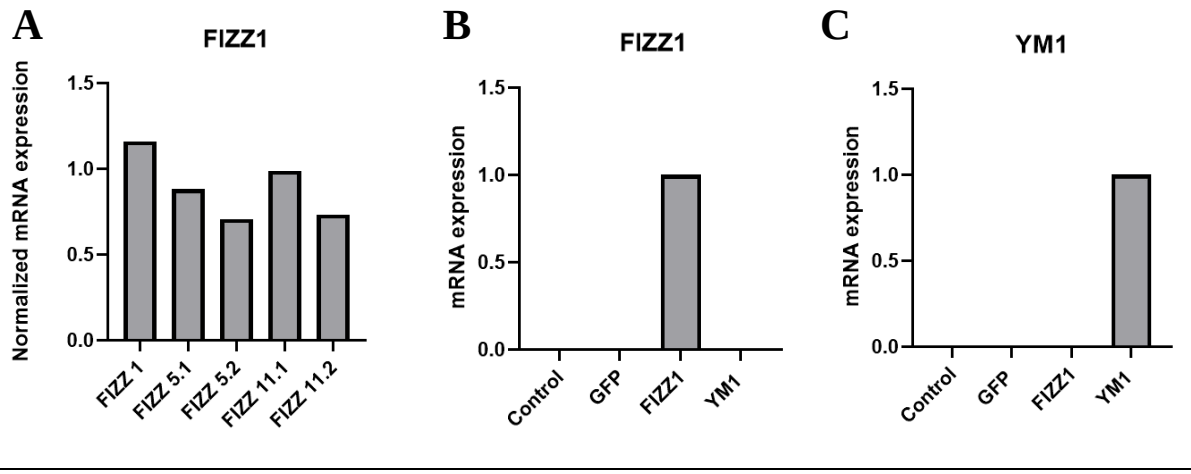
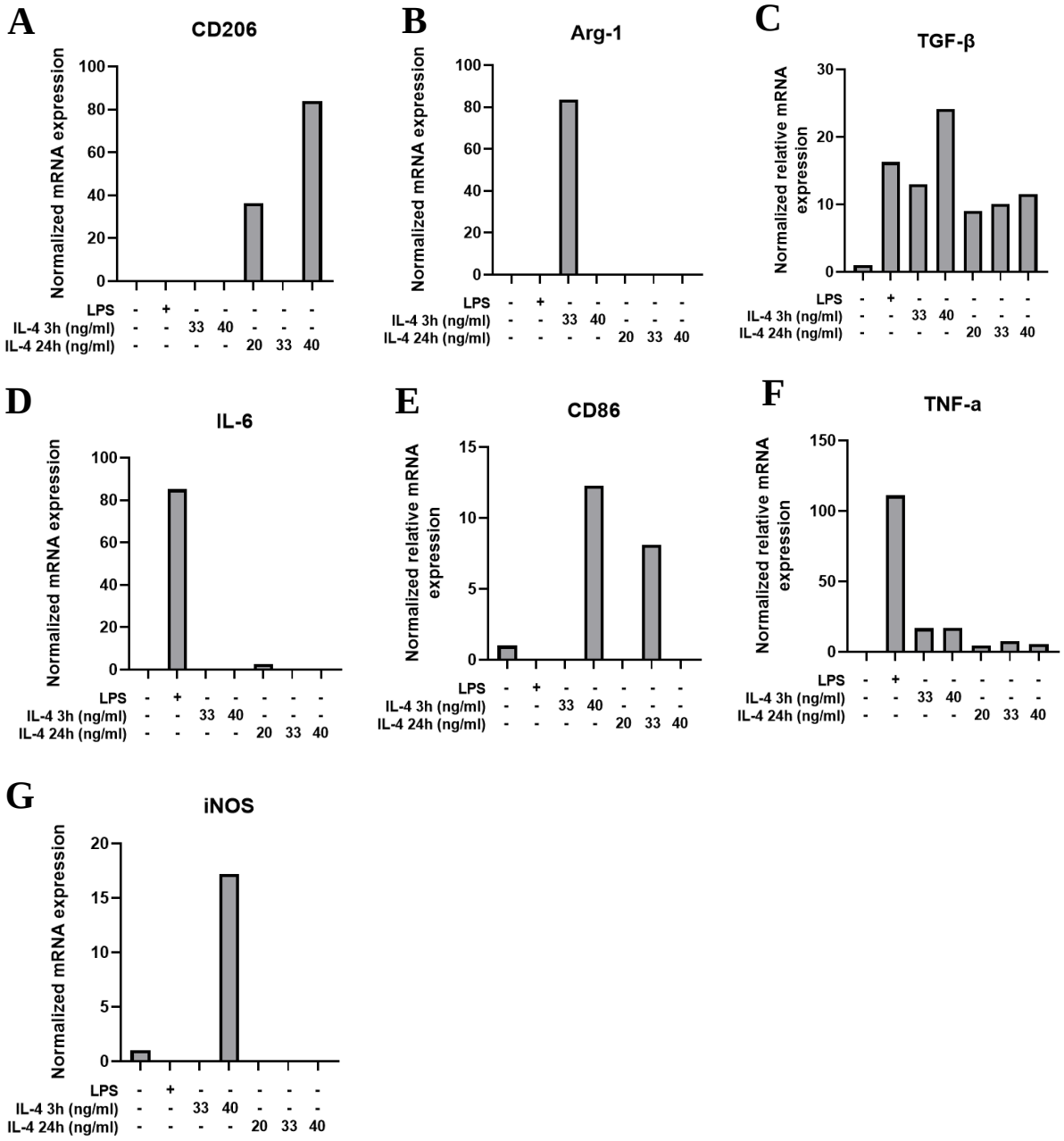
SUPPLEMENTARY



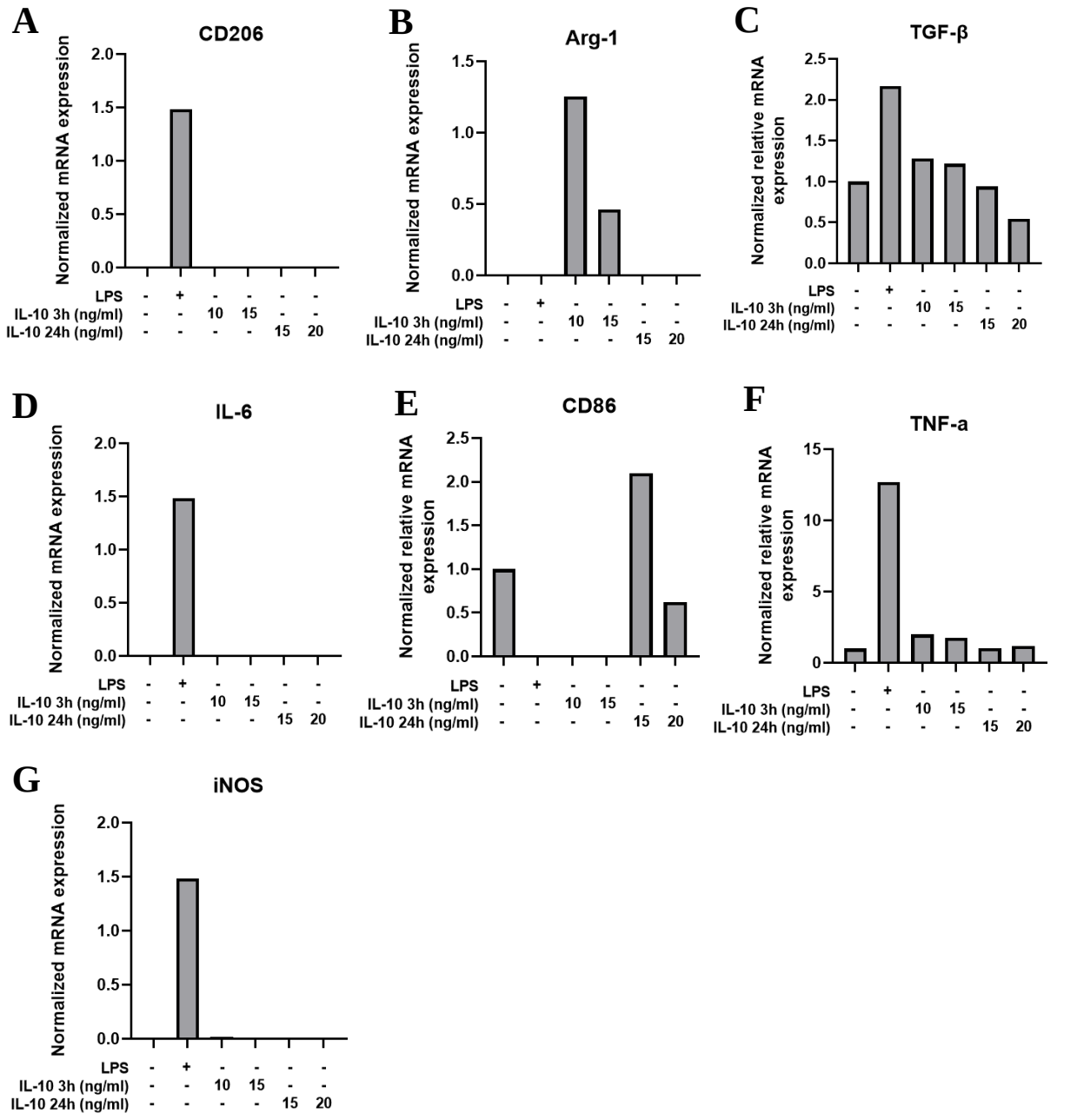
Supplementary Fig. 1 – Vectors FIZZ1 and YM1 express FIZZ1 and YM1 gene, respectively after HEK293T cell transfection. An RT-qPCR was performed to evaluate the gene expression of FIZZ1 **(A, B)** and YM1 **(C)** genes in their respective vectors. Different FIZZ1 types showed the presence of the FIZZ1 gene after transfection **(A)**. Data was normalised using housekeeping genes CYCA and YWHAZ (n=1). After FIZZ1 **(B)** and YM1 **(C)** transfection, only FIZZ1 and YM1 genes were present, respectively, while expression of other genes was not detected (n=2).

Supplementary Table 1 – Sequences of qPCR primers (mus musculus)

Target gene	Gene name	Primer sequence (5' → 3')	Primer size (bp)
iNOS	Inducible nitric oxide synthase	**F:** CCCTTCAATGGTTGGTACATGG **R:** ACATTGATCTCCGTGACAGCC	158
TNF-α	Tumor necrosis factor alpha	**F:** GTCCCCAAAGGGATGAGAAGT **R:** TTTGCTACGACGTGGGCTAC	124
CD86	Cluster of differentiation 86	**F:** GAGCGGGATAGTAACGCTGA **R:** GGCTCTCACTGCCTTCACTC	101
CD206	Cluster of differentiation 206	**F:** CTTCGGGCCTTTGGAATAAT **R:** TAGAAGAGCCCTTGGGTTGA	150
Arg-1	Arginase-1	**F:** GTGAAGAACCCACGGTCTGT **R:** GCCAGAGATGCTTCCAACTG	132
IL-6	Interleukin-6	**F:** TACCACTTCACAAGTCGGAGGC **R:** CTGCAAGTGCATCATCGTTGTTC	116
IFN-γ	Interferon gamma	**F:** TGAGGTCAACAACCCACAGGT **R:** GACTCCTTTTCCGCTTCCTGAG	/
IL-10	Interleukin-10	**F:** AATAACTGCACCCACTTCCCA **R:** CAGCTGGTCCTTTGTTTGAAAG	102
TGF-β	Transforming growth factor beta	**F:** GGGCTACCATGCCAACTTCTG **R:** GAGGGCAAGGACCTTGCTGTA	82
FIZZ1	Found in inflammatory zone-1	**F:** TCCAGCTAACTATCCCTCCACTGT **R:** GGCCCATCTGTTCATAGTCTTGA	73
YM1	Chitinase-like 3	**F:** GGGCATACCTTTATCCTGAG **R:** CCACTGAAGTCATCCATGTC	305
CD38	Cluster of differentiation 38	**F:** ACTGGAGAGCCTACCACGAA **R:** TGGGCCAGGTGTTTGGATTT	251
CD163	Cluster of differentiation 163	**F:** TCAGCCTCAGAGACATGAACTCGG **R:** GCTAGACGAAGTCATCTGCACTGG	/
Housekeeping genes			
YWHAZ	14-3-3 protein zeta/delta	**F:** GCAACGATGTACTGTCTCTTTTGG **R:** GTCCACAATTCCTTTCTTGTCATC	/
GAPDH	Glyceraldehyde 3 phosphate dehydrogenase	**F:** GGCCTTCCGTGTTCCTAC **R:** TGTCATCATATCTGGCAGGTT	/
18-S	18S ribosomal RNA	**F:** ACGGACCAGAGCGAAAGCAT **R:** TGTCAATCCTGTCCGTGTCC	/
HMBS	Hydroxymethylbilane synthase	**F:** GATGGGCAACTGTACCTGACTG **R:** CTGGGCTCCTCTTGGAATG	/
CYPA	Cyclophilin A	**F:** GCGTCTCCTTCGAGCTGTT **R:** AAGTCACCACCCTGGCA	/



Supplementary Fig. 2 – RAW cells stimulated with IL-4 do not polarise towards an M2 phenotype. Using RT-qPCR, the presence of M2 markers CD206 **(A)**, Arg-1 **(B)**, TGF-β **(C)** and M1 markers IL-6 **(D)**, CD86 **(E)**, TNF-α **(F)**, iNOS **(G)** was evaluated after stimulating RAW cells with IL-4 at different time points and concentrations. To control the polarisation state of each gene, RAW cells were stimulated with LPS (200 ng/ml) to obtain an M1 phenotype. Expression of M2 marker CD206 was only detected after 24h stimulation with IL-4 while Arg-1 could only be detected after 3h of stimulation **(A, B)**. TGF-β gene expression was present in all groups except the M0 control **(C)**. M1 markers IL-6 and TNF-α were detected specifically in the LPS stimulated cells (M1) **(D, F)**. Gene expression of M1 markers CD86 and iNOS could not be measured in the M1 controls but were detected after IL-4 stimulation **(E, G)**. Data was normalised using housekeeping genes YWHAZ, 18-S, and CYPA. Data represent preliminary results (n=1).



Supplementary Fig. 3 – RAW cells stimulated with IL-10 do not polarise towards an M2 phenotype. Using RT-qPCR, the presence of M2 markers CD206 **(A)**, Arg-1 **(B)**, TGF-β **(C)**, CD163 **(D)** and M1 markers IL-6 **(D)**, CD86 **(E)**, TNF-α **(F)**, iNOS **(G)** was evaluated after stimulating RAW cells with IL-10 at different time points and concentrations. To control the polarisation state of each gene, RAW cells were stimulated with LPS (200 ng/ml) to create an M1 phenotype. Gene expression of M2 marker CD206 was only detected in the M1 control group **(A)**. M2 marker Arg-1 was detected after 3h of stimulation with IL-10 while TGF-β expression was present in all groups **(B, C)**. M1 markers IL-6, TNF-α, and iNOS were mainly expressed in LPS stimulated M1 cells **(D, F, G)**. CD86 gene expression was present in both the control group (M0) as well as after 24h stimulation with IL-10 **(E)**. Data was normalised using housekeeping genes YWHAZ, 18-S, and CYPA. Data represent preliminary results (n=1).