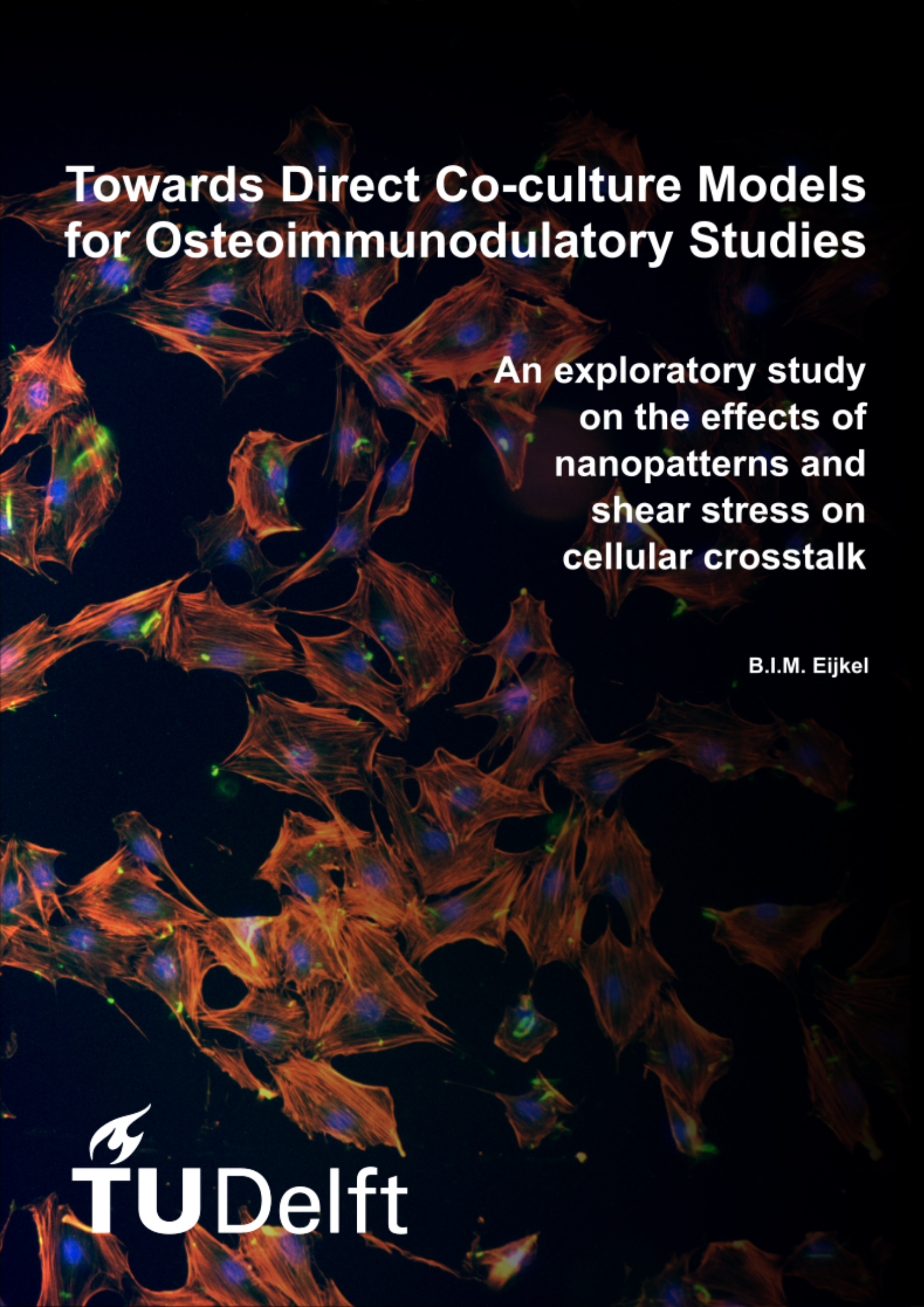

Towards Direct Co-culture Models for Osteoimmunodulatory Studies

An exploratory study on the effects of nanopatterns and shear stress on cellular crosstalk

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Towards Direct Co-culture Models for Osteoimmunomodulatory Studies

An exploratory study on the effects of nanopatterns and shear stress on cellular crosstalk

By

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"If you think you are too small to make a difference,
try sleeping with a mosquito."
- The Dalai Lama

Contents

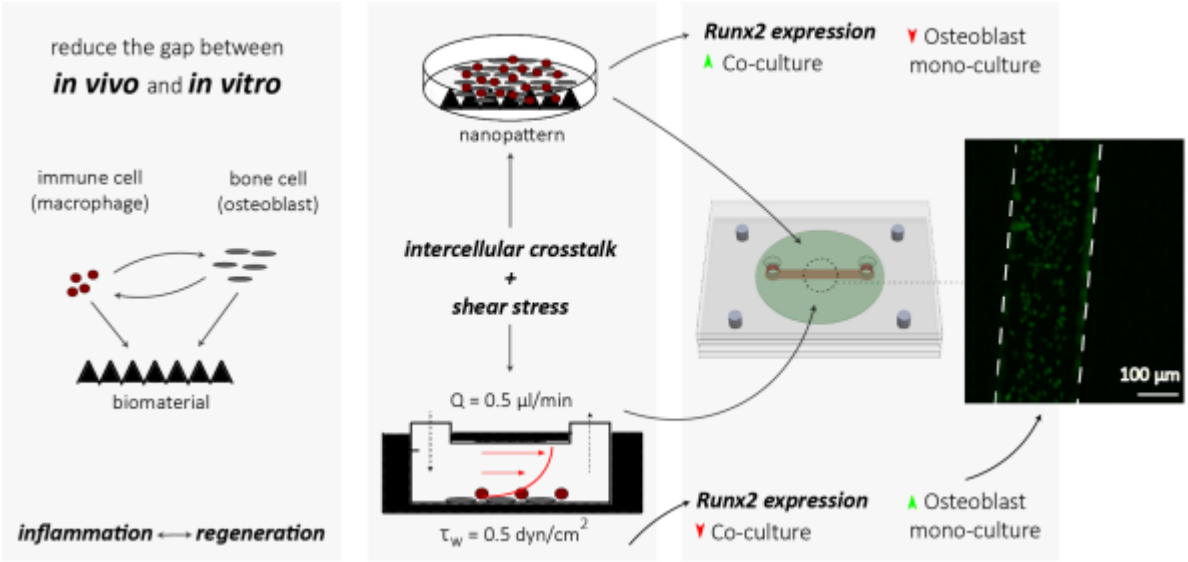
Abstract

In the last two decades, it has been generally accepted that the interaction between bone and immune cells is critical in the process of bone healing and regeneration. Steering this immune response with the development of biomaterials could lead to implant acceptance in the long term. The understanding of osteoimmunological responses is of high importance in the development of new biomaterials. In order to validate these biomaterials, better *in vitro* techniques are required that could mimic this inflammatory response immediately after implantation in more detail. This study investigated the effect of a direct co-culture model on a prototype nanopattern. In addition the effect of microflows, that are normally present *in vivo*, induced inside a microfluidic chip, on mono and co-cultures was investigated in order to improve *in vitro* research.

Static mono- and co-cultures with a 1:1 (CO11) and 1:2 (CO12) ratio of murine pre-osteoblasts MC3T3-E1s (OBs) and M1 stimulated murine Macrophages J774A.1 (+ LPS & IFN-γ) were cultured under static conditions for 4 weeks to see the effect of co-culturing on inflammatory markers (TNF-α, PGE2, and IL-10) and osteogenic markers (ALP, Runx2 & Alizarin Red Staining). It was found that the use of a co-culture did not have a positive effect on OB differentiation. The introduction of a prototype nanopattern ($500\ nm$ height and $300\ nm$ width) that induces a macrophage polarization shift towards an anti-inflammatory phenotype, did have a positive effect on Runx2 secretion in the co-cultures. Furthermore the effect of a dynamic flow, initiated 2 days after seeding, on osteogenic differentiation of OB and CO12 was investigated. After 7 days of dynamic culture, Runx2 levels of the mono-cultures were significantly higher than the static cultures. OBs were positively affected by forming dense cell layers throughout the entire microfluidic channel. For dynamic co-cultures, no to little cells were present inside the channel after 7 days. Finally we attempted to fabricate a microfluidic device that could "sandwich" the patterned waver inside, so the effect of both the nanopattern and microflow on mono- and co-cultures could be investigated. Cells were seeded inside the channel after 1 day, but unfortunately forces on the PMMA layer of this chip resulted in cracks and failure of this system. Our findings in the design and fabrication of the chip can help in future research towards the osteoimmunodulatory properties of biomaterials.

The study on a direct co-culture model, presented in this work, opens possibilities for future research towards co-cultures inside microfluidic devices. It discusses the possibilities and the drawbacks of both the use of co-cultures as the development of microfluidic devices.

Graphical Abstract



Nomenclature

β-GL	β-glycerolphosphate	
η	Absolute viscosity	(kg/ms)
ρ	Density	(kg/m^3)
σ	Wall shear rate	(s^{-1})
τ_w	Wall shear stress	(Pa)
h_0	Height of rectangular channel	(M)
Q	Volumetric flow rate	(m^3/s)
r	Radius of cylindrical channel	(M)
Re	Reynold's number	$(-)$
w_0	Width of rectangular channel	(M)
A.A.	Ascorbic-acid	
ALP	Alkaline phosphatase	
ARS	Alizarin Red S	
BMP	Bone morphogenetic protein	
CM	Conditioned medium	
COX2	Cyclooxygenase	
Dexa	Dexamethasone	
ECM	Extracellular matrix	
FBR	Foreign body response	
FBS	Fetal bovine serum	

FEM Finite element method

IFN-γ Interferon γ

IL- Interleukin-

LPS Lipopolysacharide

MΦ Macrophage

M1 Pro-inflammatory M1 macrophage

M2 Anti-inflammatory M2 macrophage

MMP Matrix metalloproteinase

MSC Mesenchymal Stem/Stromal Cell

OB Osteoblast

OC Osteoclast

OCTs Osteocytes

OPG Osteoprotegerin

pen-strep Penicillin-Streptomycin

PGE2 Prostaglandin 2

TGF-β Transforming growth factor-β

TJRs Total joint replacements

TNF-α Tumor necrosis factor α

VEGF Vascular endothelial growth factor

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CHAPTER 1

Introduction

Bones are one of the most important organs inside the human body. They provide support, protect our organs and are important for the storage of minerals, growth factors and fat. On top of that, bones are also responsible for the production of blood cells and hormones [1]. Under normal circumstances bones are able to renew themselves by removing and building bone tissue over time, this process is called remodelling and is triggered by hormones and physical stress [1]. When a bone is severely fractured or lost its function due to sickness, an implant can be introduced to support the bone or replace this part of the body. Bone implants can be as big as total joint replacements (TJRs) or as small as dental implants. However, one major drawback still remains implant rejection by the body, caused by a foreign body reaction (FBR) immediately or some time after implantation [1]. This reaction could result in infections or even in aseptic loosening of the implant.

Approximately 10 % of the TJRs in the Netherlands need to be replaced due to implant loosening, caused by the FBR or stress related issues, each year [2]. An overview can be seen in Figure 1.1. This not only increases the costs in health care, but also increases the risk of infections each time a surgical intervention takes place. An implant should therefore not only give support, but promote bone formation and reduce infections at the same time, so it will provide a healthy bone remodelling environment around the implant [3].

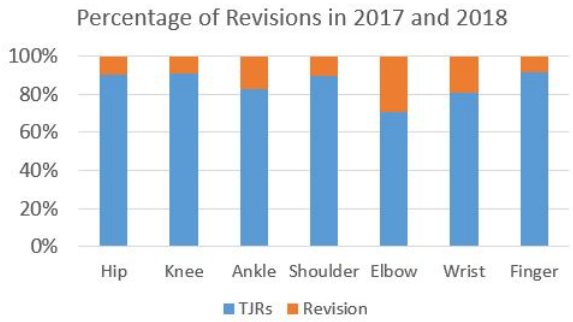

Figure 1.1: Percentage of TJR Revisions in the Netherlands in 2017 and 2018. [2]

In order to reduce the amount of implant rejections and promote long-term stability, research is focused on implant improvement, with respect to better design choices and biomaterials with enhanced bio-functionalities. These factors influence implant acceptation or rejection by the body in the short and long-term [4]. In the last two decades, it has been generally accepted that the interaction between bone cells and immune cells is critical in the process of bone healing and regeneration [5]. Implant acceptation or rejection starts immediately after implantation when acute inflammation occurs and both bone cells and immune cells are attracted to the injury site to start the healing process [6, 7]. The study and understanding of the interactions between

these cells can provide new insights into implant-bone integration, osseointegration and the performance of biomaterials over time. This new research area is called "Osteoimmunology" and the biomaterials able to modulate the response of these cells have osteoimmunomodulatory properties [4].

Macrophages (MΦs) are one of the earliest immune cells present around the implant after surgery and it is important to understand their effect on the behavior of osteoblasts (OBs), and bone regeneration in general [8]. There are two types of MΦs in the human body, resident and inflammatory MΦs [9]. Resident MΦs are normally present in most tissues. Inflammatory MΦs are derived from Monocytes which are drawn from the bloodstream under the influence of inflammatory factors. When Monocytes leave a blood vessel, they are automatically differentiated into inflammatory MΦs.

Inflammatory MΦs are so-called plastic cells, their phenotype changes in response to certain markers present in their surroundings [10, 11]. There are two main types of inflammatory MΦs, pro-inflammatory M1-MΦs (M1) and anti-inflammatory M2-MΦs (M2). M1s are mainly activated by Interferon-γ (IFN-γ), which is a cytokine secreted by T-cells as a reaction to cell debris in the fracture site, and Lipopolysacharide (LPS), a molecule found on the membrane of bacteria [11]. The main purpose of M1s is to perform phagocytosis and clean the site in which they are activated [12]. M1s induce a pro-inflammatory response and secrete different pro-inflammatory cytokines, such as Tumor Necrosis Factor α (TNF-α), Interleukin-1β (IL-1β) and IL-6.

Under the influence of IL-4 and IL-13, secreted by T cells, pro-inflammatory M1s polarize into anti-inflammatory M2s [10, 13]. Typically, this polarization appears 3-4 days after injury. This process is important because a longer inflammation period has a negative effect on tissue healing processes and can damage the surrounding tissue severely [4]. M2s are critical for the initiation of bone healing and secrete a range of cytokines and factors that enhances tissue regeneration [14]. Factors that are of high importance for bone regeneration are bone morphogenetic protein (BMP) and transforming growth factor -β (TGF-β) which promote the proliferation of OBs and induce bone growth [15]. Vascular endothelial growth factor (VEGF) stimulates angiogenesis, which enhances the migration of cells and nutrients towards the tissue site [1]. Matrix metalloproteinase (MMP) stimulates the bone remodelling process [10].

Other than triggering the immune system, pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) also promote the migration of Mesenchymal Stem Cells (MSCs) towards the fracture site. The differentiation of MSCs into OBs is also promoted by TNF-α and IL-1β [15]. MSCs and OBs are of high importance for the regeneration and healing of the tissue. Another response to the pro-inflammatory cytokines is that MSCs and OBs start to induce M1-M2 polarization by the secretion of PGE2. A bigger initial inflammatory response results in a faster recruitment of MSCs and OBs and thus in a faster polarization of MΦ towards anti-inflammatory M2 Macrophages [16, 17]. On the one hand, M1s increase the inflammatory response, causing swelling, pain, redness and fever, which can have a negative effect in the long term. On the other hand, this increase in pro-inflammatory factors has an immediate positive effect on the anti-inflammatory response and tissue healing process, by the migration and differentiation of tissue healing cells, e.g. MSCs, towards the trauma site.

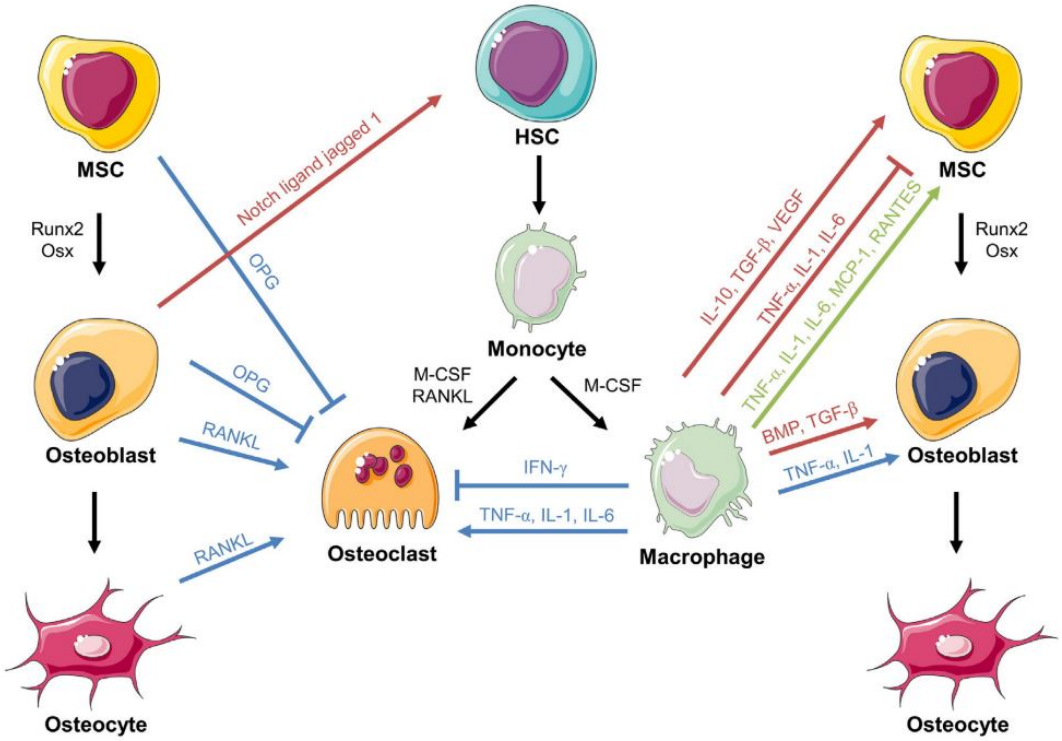

Figure 1.2: Crosstalk between Inflammatory - and Bone cells. The blue, red and green lines/arrows indicate respectively the inhibition/promotion of differentiation, proliferation and migration. [15]

Above mentioned processes are only one small part of these highly complex immunological relationships between cells during bone repair. Greater knowledge in these interactions is of high importance in osteoimmunological research, since it could better predict (un)successful biomaterial stability in the short and long-term. It is known that cells are influenced by their micro-environment. Changing the chemical, or physical, properties of a biomaterial, (e.g., adding drugs or cytokines to a biomaterial) has a direct influence on the cells present in the cell-implant niche after surgery [4]. The initial inflammatory response, initiated by the immune cells upon implantation, can be altered by these factors. In addition, the bone cells are also influenced by the properties of the biomaterial and a bad choice of properties could lead to a decreased amount of bone formation on the implant, leading to implant loosening and failure [18, 19]. Therefore, the development of a perfect implant should be based on a biomaterial that positively promotes both, or even multiple cell types.

In order to investigate what biomaterials-related factors will promote osseointegration, these have to be firstly investigated outside a living system, i.e. *in vitro*. An ideal *in vitro* model would mimic the conditions of a biomaterial within the human body (*in vivo*) perfectly. However, the imitation of the physiological and cellular environment in a living being is far more complex than a petri-dish inside an incubator. Incorrect *in vitro* models lead to false results, and eventually in higher costs, unnecessary sacrificing of animals, no final publication of the results and thus no new improved implants [20]. This is the reason why new *in vitro* models are needed that could better simulate the cell-niche environment and implant interaction inside the body.

One example of improving *in vitro* models and the effect of biomaterials on the osteoimmunological response, is the implementation of multiple cell types (co-culture) instead of merely one cell type (mono-culture), to fully apprehend all the effects a certain biomaterial has on the body [21]. Co-culture methods can be divided into direct and indirect models [21]. Direct co-culture is the most straight forward method, in which cells are mixed at a certain ratio and seeded simultaneously or in sequence onto the substrate. It is suggested that direct co-culture of MΦs and OBs provide a better environment for OB differentiation and bone formation, which is suspected to be related to close cell-cell contact and thus both physical and paracrine interactions during cell culture [22, 23, 24]. However, this culture model has some challenges, such as distinguishing two cell types from one another. This can be difficult and requires the need of specific cell markers [21].

Indirect co-culture models can be divided into methods that involve the transfer of Conditioned Medium (CM) from the assistant cells to the target cells or methods that use a barrier that physically divides the cells but allow for paracrine signalling interaction (transwell) [21]. Transwell models have been proven to be efficient and are able to test the osteogenic properties of a biomaterial in great detail [17, 25, 26]. Identification of two cell types will not be an issue, since these can be investigated separately. However, there is no direct physical contact of at least one cell type on the surface of the biomaterial and cellular crosstalk is limited to paracrine signals only.

In addition to the implementation of a co- instead of a mono-culture model, *in vitro* models can also be improved by mimicking the physical *in vivo* conditions in more detail. Instead of a static *in vitro* culture, a flow can be introduced that imitates the natural microflows present inside the body. This dynamic system not only increases the exchange of waste, oxygen and nutrients, but also introduces shear stresses, which have a positive effect on cells in terms of proliferation, differentiation and viability [20]. These microfluidics systems (or Lab-on-a-chips) have the potential to minimize the gap between *in vitro* and *in vivo* experiments [20]. Lab-on-a-chips involving co-cultures that could mimic the implant-bone environment directly after implantation are limited. One bone-on-a-chip was successfully developed by Hao et al. [27]. One major drawback was the long culture time needed (720 hours) for the development of one chip before experimentation could start. Chips found that mimic the inflammatory response could be a great example of ways to mimic the inflammatory response during bone repair. Biglari et al. [28] and Ai et al. [29] both developed a chip in order to investigate the inflammatory response during wound repair. These chips consisted of three parallel channels, interconnected with microgrooves, thus making an indirect co-culture system. These systems proved to enable real time monitoring of the cell response during a FBR. Shear stresses have been shown to improve the osteoblastic response in monocultures [30, 31, 32]. It would be interesting to see the effect of shear stress on a co-culture involving both MΦs and OBs.

As mentioned before, this thesis in involved with the improvement of *in vitro* models for the validation of a certain biomaterial. Changing the surface of a biomaterial, by the addition of micro or nanopillars, has the potential to enhance bone tissue regeneration. The introduction of such patterns on a biomaterial has proven to positively affect osteoimmunomodulation in *in vitro* and *in vivo* studies [33, 26, 19]. In our research a newly developed nanopattern will be investigated, which has shown, in a preliminary study, to

actively induce M1-M2 polarization of J774A.1 MΦs . A direct co-culture system will be established to investigate the effect of this pattern on the immune cells but also on the bone cells.

To summarize, the main goal of this thesis is to improve the validation of a biomaterial by using *in vitro* osteoimmonological culture models that will mimic the FBR inside the human body. This exploratory study consists of four parts. Each discusses different aspects that will be necessary for the understanding and improvement of *in vitro* research of a newly developed nanopattern. First, a co-culture model of both OBs and MΦs will be established and the interaction between the cells will be looked upon in detail. Secondly, the effect of a nanopattern on osteoimmunological behaviour of co-culture and mono-culture models will be compared. Furthermore, the cellular response will be looked upon after the introduction of a dynamic microflow and shear stress in terms of osteogenic differentiation in both mono- and co-cultures. Finally, the design, fabrication and validation of a microfluidic device is presented that could have the potential to investigate the effect of a co-culture on a nanopattern during a continuous dynamic flow. This lab-on-a-chip device has the potential to include both dynamic and co-culture conditions for the investigation of our biomaterial.

CHAPTER 2

Materials & Methods

2.1 Pre-culture

2.1.1 Murine macrophage culture and polarization

Frozen J774.A1 Murine Macrophages (MΦs) (Merck KGaA, Germany) (p15) were thawed and plated in 75 cm^2 culture flasks (10 000 $cells/cm^2$) containing 10 ml Dulbecco's modified Eagle's medium (DMEM; ThermoFisher Scientific, US) supplemented with 10 $\%$ Fetal Bovine Serum (FBS; ThermoFisher Scientific, US) and 1 $\%$ Penicillin-Streptomycin (pen-strep; ThermoFisher Scientific, US). They were incubated at 37 ^oC and 5 $\%$ CO_2. Culture medium was refreshed every 2-3 days until confluence (Figure 2.1 A).

MΦs were polarized according to established protocols [34]. In short, confluent MΦs, at a density of approximately 100 000 $cells/cm^2$, were stimulated towards pro-inflammatory M1 MΦs (M1s) by the addition of 100 ng/ml LPS (Sigma-Aldrich, Germany) and 10 ng/ml IFN-γ (Sigma-Aldrich, Germany) for 3 days. Anti-inflammatory M2 MΦs (M2s) polarization was stimulated by the addition of 10 ng/ml IL-4 (Sigma-Aldrich, Germany) for 3 days. One group of MΦs was used as a control and left unstimulated (M0) (Figure 2.1 A).

MΦ phenotype was confirmed by extracting 200 μl supernatant from the wells after polarization, unused supernatant was extracted and stored at -80 ^oC. It is important to identify the particular expressions for each MΦ polarization state since this could give more information about the polarization of MΦs in MΦ:OB co-cultures, and could help identify the polarization switch over time. Pro-inflammatory M1 profiles were measured using Griess assay (Abcam, UK), TNF-α ELISA (ThermoFisher Scientific, US), and PGE2 ELISA (ThermoFisher Scientifc, US). Anti-inflammatory M2 profiles were measured with IL-10 ELISA (ThermoFisher Scientifc, US). The absorbance of each assay was read with the VICTOR X3 Multimode Plate Reader (Perkin Elmer, USA), under assay specific wavelengths. The intensity of the wells is proportional (Griess, IL-10, TNF-α) or inversely proportional (PGE2) to the amount of cytokines captured in the wells. A standard curve was used in combination with a 4-parameter curve fit in MATLAB (2017), to calculate the cytokine concentrations for each sample. Culture Medium with 10 $\%$ FBS was used as a control and the mean intensity was subtracted from the samples. This was neccesary because serum tends to contain cytokines that can result in high background signals. The sensitivities of the

Griess, TNF-α, PGE2 and IL-10 ELISAs were respectively, 1 nmol/well, 0.75 pg/ml, 8.26 pg/ml, and 19.80 pg/ml.

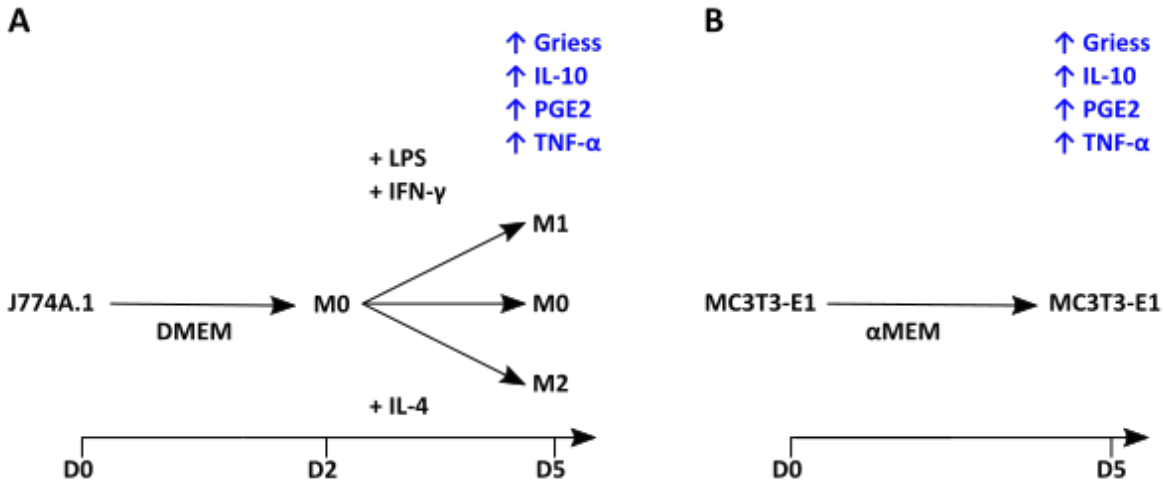

Figure 2.1: **A)** Timeline of J774A.1 (p15) pre-culture in DMEM supplemented with 10% FBS and 1% pen-strep. J774A.1 were cultured until confluent (D2). From day 2 LPS and IFN-γ was added to stimulate M1 polarization. IL-4 was supplemented to stimulate M2 polarization, and one group was left unstimulated (M0). Supernatant was stored at $-80\ ^oC$ on day 5, in order to measure Griess assay, IL-10, PGE2 and TNF-α. **B)** MC3T3-E1 pre-osteoblasts (p13) were cultured until confluent in αMEM supplemented with 10% FBS and 1% pen-strep prior to each experiment. Supernatant was stored at $-80\ ^oC$ on day 5, in order to measure Griess assay, IL-10, PGE2 and TNF-α

2.1.2 Murine pre-osteoblast culture

Frozen murine MC3T3-E1 pre-osteoblasts (p13) (OBs) were thawed and plated in a 75 cm^2 culture flask (5 000cells/cm^2) in Minimum Essential Medium alpha (αMEM; ThermoFisher Scientific, US) without ascorbic acid (A.A.), supplemented with 10% FBS and 1% pen-strep, and incubated at 37 ^oC and 5% CO_2. Culture Medium was changed every 2-3 days until confluency was reached after 5 days (Figure 2.1 B). Supernatant was extracted and used for a Griess assay (Abcam, UK), TNF-α ELISA (ThermoFisher Scientific, US), IL-10 ELISA (ThermoFisher Scientifc, US) and PGE2 ELISA (ThermoFisher Scientifc, US), unused supernatant was stored at $-80\ ^oC$. These assays were performed for OBs as a negative control. Furthermore, Culture Medium with 10 % FBS was used as a control and these values were subtracted from the samples. The absorbance of each assay was read with the VICTOR X3 Multimode Plate Reader (Perkin Elmer, USA), under assay specific wavelengths.

2.2 Co-culture

Two direct co-culture models, OB:M1 ratios 1:1 and 1:2, were compared to M1 and OB mono-culture. Both inflammatory and osteogenic response were measured. Prior to co-culture, MΦs were polarized towards M0, M1 and M2 as described above. For CO11 co-cultures, M1s (5 000 cells/cm^2) were mixed with OBs (5 000 cells/cm^2) and seeded simultaneously in a 48 well plate in mixed medium, αMEM:DMEM (1:1), + 10% FBS, and 1% pen-strep, and incubated at 37 ^oC and 5% CO2. For the CO12 co-cultures, twice the amount of M1s (10 000 cells/cm^2) were mixed with OBs (5 000 cells/cm^2)

and cultured under the same conditions. As a control, M0, M1, M2 and OBs (5 000 cells/cm^2) were plated in mixed medium. After 2 days, the mixed medium was supplemented with 50 $\mu g/ml$ Ascorbic Acid (A.A; Sigma-Aldrich, Germany) and 4 mM β-Glycerolphosphate(β-GL; Sigma-Aldrich, Germany) to induce osteogenic differentiation (Figure 2.2). Culture medium was refreshed every 2-3 days until 28 days after seeding.

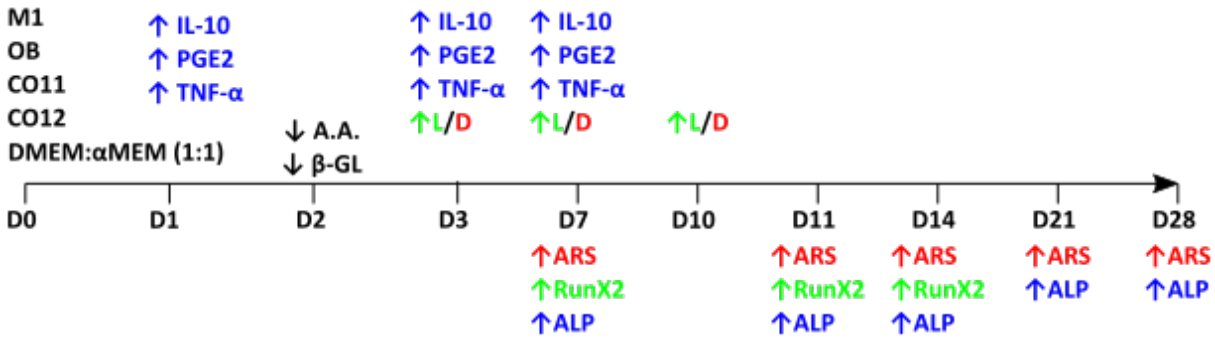

Figure 2.2: Timeline of co-culture experiment with M1, OB, CO11, CO12 cultured in mixed medium (αMEM:DMEM (1:1) supplemented with 10% FBS and 1% pen-strep). From day 2 A.A. and β-GL were added to stimulate osteogenic differentiation. Supernatant was stored at $-80\ ^oC$ for the detection of IL-10, PGE2, TNF-α and ALP. On days 7, 11 14, 21 and 28 plates were fixed with 4% PFA for Runx2 and Alizarin Red Staining. Live/Dead staining took place on days 3, 7 and 10

2.2.1 Cell viability and proliferation

Brightfield images were taken with the ZOE Fluorescent Cell Imager (BioRad, US) at days 1, 3, 7, 10 and 14 after seeding (Figure 2.2). In addition LIVE/DEAD™ Viability/Cytotoxicity Kit for Mammalian Cells (ThermoFisher Scientific, US) assay was performed on days 3, 7, 10 and 14. This assay was performed to look into the survival rate of the cells of the mono-cultures as co-cultures over time. Live/Dead staining consists out of Calcein AM and Ethidium homodimer (EthD) [36]. Calcein Am is non-fluorescent and able to travel through the cell membrane, after arrival inside a living cells, the active esterases present remove the AM group, leaving Calcein, which is a green fluorescent dye. EhtD binds to the DNA inside the cell, after binding it becomes highly fluorescent (Red). EthD, however is cell membrane impermeable, and can therefore only bind to the DNA of dead cells.

In short, 48-well plates containing mono- and co-cultures were retrieved from the incubator and washed with 10x DPBS (ThermoFisher Scientific, US) and 1x DPBS. Next, a live/dead solution of 2 $\mu M/ml$ Calcein AM and 3 $\mu M/ml$ EthD diluted in PBS was pipetted into OB, CO11 and CO12, these concentrations were obtained by optimization of the protocol (Appendix D.2). For M0, M1 and M2 a live/dead solution of 0.8 $\mu M/ml$ Calcein AM and 3 $\mu M/mL$ EthD was prepared. The samples were placed in the incubator for 30 minutes. Afterwards, The solution was discarded and replaced with 1 x DPBS. Fluorescent images were taken with the ZOE Fluorescent Cell Imager (BioRad, US) at 5 random locations. For each condition, four wells were examined. Image processing took place using open-source software FIJI[37]. In brief, living cells were selected by thresholding the green fluorescent images. The threshold was altered to fit all the green stained cells present. The remaining picture was then segmented using the "watershed" function. Finally, all the separate segmented cells were counted. The dead cells

were counted by using this same method on the red fluorescent image. The percentage of living cells in the samples was calculated using the following equation,

$$\% \text{ living cells} = \frac{\text{Green}}{\text{Green + Red}} \times 100 \tag{2.1}$$

where "Green" is equal to the amount of counted living cells, and "Red" to the amount of dead cells.

2.2.2 Inflammatory response

The inflammatory response of M1s, OBs, CO11 and CO12 was also inspected to look into the changes over time. $200\ \mu l$ of cell supernatant was taken from the wells on days 1, 3 and 7 after seeding, and stored at $-80\ ^oC$. Pro-inflammatory profiles were measured using TNF-α ELISA (ThermoFisher Scientific, US), and PGE2 ELISA (ThermoFisher Scientifc, US). and anti-inflammatory were measured with IL-10 ELISA (ThermoFisher Scientifc, US) (Figure 2.2). Mixed culture medium was used as a control. The absorbance of each assay was read with the VICTOR X3 Multimode Plate Reader (Perkin Elmer, USA), under assay specific wavelengths.

2.2.3 Osteogenic response

To see the effect of co-culture on osteogenic activity, $200\ \mu l$ culture supernatant was extracted on day 7, 11, 14, 21 and 28, and stored at $-80\ ^oC$, for the measurement of ALP activity (Abcam, UK) (Figure 2.2). In addition the samples were fixated and stained with fluorescent marker Runx2 (Abcam, UK) or Alizarin Red dye (Sigma-Aldrich, Germany). In short, after washing the wells twice with 1x DPBS, they were fixed with 4% paraformaldehyde (PFA; Sigma-Aldrich, Germany) for 15 minutes at room temperature. Wells were washed with 1x DPBS twice and stored at $4\ ^oC$ up to two weeks until staining took place. Runx2

For Runx2 staining, the Cells were permeabilized with $0.5\ \%$ Triton/DPBS (Sigma-Aldrich, Germany) for 5 minutes at $4\ ^oC$, followed by blocking with $1\ \%$ bovine serum albumin in PBS (BSA/DPBS; Sigma-Aldrich, Germany) for 5 minutes at $37\ ^oC$. Primary Rabbit Runx2 antibody was diluted in BSA/DPBS (1:250) and added to the wells. After an incubation period at $37\ ^oC$ of 1 hour, cells were washed 3 times with $0.5\ \%$ Tween/DPBS (Sigma-Aldrich, Germany). Alexa 488 donkey anti-rabbit (ThermoFisher Scientific, US) was added to the wells as a secondary antibody in 1% BSA/DPBS (1:200) and incubated for 1 hour at room temperature. Cells were washed 3 times with $0.5\ \%$ Tween/DPBS (Sigma-Aldrich, Germany). The samples were washed once with 1x DPBS and mounted on a glass slide using Prolong gold antifade reagent with DAPI (ThermoFisher Scientific, US).

Fluorescent images were taken with the ZOE Fluorescent Cell Imager (BioRad, US) at 5 random locations. Image processing took place using open-source software FIJI [37]. Runx2 expression was measured as the sum of the intensity of green fluorescence of all the cells present in a selected area. For all 10 images the background noise was measured, and the mean of all background noise was subtracted from all the images before measuring the intensity. Thereafter, the cells were selected using the same threshold method as for live/dead image processing mentioned above. Secondly, the intensity density of

the green marker was measured within each cell. Finally, the intensity densities of all the cells present in the selected area were summed up to identify the amount of Runx2 expression of all the cells in this area. The mean of the summed intensity density and standard deviation of all 5 images was then taken to obtain the final values for 1 well. For each condition, 2 wells were evaluated.

Alizarin Red Staining (ARS) was done on fixated samples. The protocol has been obtained from literature [38]. After fixation, the cells were washed twice with distilled water. A $2\ \%$ Alizarin Red S/PBS solution was added to the wells and left on the Thermal shake Lite plate shaker (VWR, USA), at 300 rpm for 20 minutes in the dark. After incubation, the wells were washed 5 times on a plate shaker at 300 rpm for 5 minutes. After the removal of all demiwater, the samples were imaged with the Leica DM500 Brightfield (Leica Microsystems, Germany) microscope at five random locations for each sample, 10 images in total. The average amount of red inside each area was measured in four wells total. FIJI [37] was used to measure the total % of Red staining in each obtained image. Images were also taken with the WIFI digital microscope (Rotek, China).

2.3 Co-culture on nanopattern

2.3.1 Pattern Fabrication

Nanoscribe's Photonic Professional GT laser lithography system (Nanoscribe, Germany) was used for the fabrication of the nanopatterns. A curable acrylate-based photosensitive resin, IP-L780, was polymerized under influence of the laser and nanopatterns of $500\ nm$ height and $300\ nm$ width were fabricated. The area with nanopillars was $1\ mm\ \times\ 1\ mm$. Preliminary research has shown that these patterns had a positive effect on the polarization of pro-inflammatory M1s towards anti-inflammatory M2s. In this research the effect of co-cultures and patterns on the osteogenic activity of OBs was assessed by direct co-culture on the patterns.

2.3.2 Co-culture

Prior to seeding, the wavers were sterilized with a 70% ethanol wash and washed twice with 1X DPBS. OBs and CO12 co-cultures were obtained as described before and carefully seeded on the sterile wavers with nanopatterns (OBp and CO12p) and without patterns (OB and CO12) inside a 6-well plate. In short, $5\ 000$ cells/cm^2 OBs were seeded in the OB and OBp wells, and $5\ 000$ cells/cm^2 OBs and $10\ 000$ cells/cm^2 M1s in the CO12 and CO12p wells. The cells were cultured in mixed medium and osteogenic supplements (A.A. and β-GL) were added two days after seeding (Figure 2.3). Media was changed every 2/3 days.

2.3.3 Morphology

Brightfield images were taken with the ZOE Fluorescent Cell Imager (BioRad, US) to asses the morphology and behavior of the cells with and without the pattern on days 1, 5, 7 and 9. The contrast of the images was enhanced using FIJI [37].

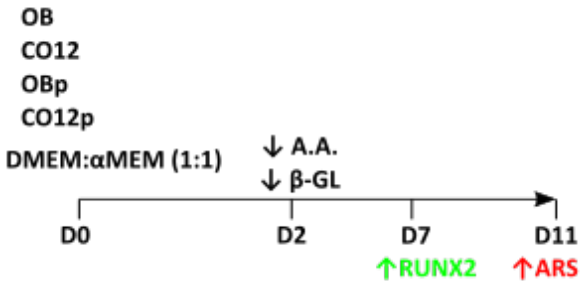

Figure 2.3: Timeline co-culture experiment seeded on the sterile wavers with (OBp and CO12p) and without (OB and CO12) a pattern inside a 6-well plate. Cultured in mixed medium (αMEM:DMEM (1:1) supplemented with 10% FBS and 1% pen-strep). From day 2 A.A. and β-GL were added to stimulate osteogenic differentiation. On day 7, 4 wavers were fixed with 4% PFA for Runx2 stainng. On day 11, 2 wavers were fixed for ARS staining.

2.3.4 Osteogenic response

Cells were fixated on days 7 and 11 for 15 minutes with 4% PFA for Runx2 (n=4) and ARS (n=2) respectively. Extra care was taken to minimize the effect of the fluorescent behavior of the patterns in the green spectrum ($488\ nm$). The image processing in FIJI was again similar to the co-culture experiment. In addition, the same background signal was subtracted from each image to minimize the green fluorescent signal coming from the pattern itself. Cells were stained with Runx2 and ARS under the same conditions as the static co-culture experiment above.

2.4 Dynamic co-culture

One promising solution as *in vitro* technique is a microfluidic-sytem. A Microfluidic system has the potential to mimic *in vivo* conditions in more detail. Instead of a static *in vitro* culture, a flow can be introduced that imitates the natural microflows present inside the body. This increases the exchange of waste, oxygen and nutrients, and introduces shear stresses, which all have a positive effect on cells in terms of proliferation, differentiation and viability [20]. This next section describes a dynamic co-culture system that was used to investigate the effect of shear stress on OB and CO12.

2.4.1 Shear stress

Shear stress, τ_w, that is induced by a microfluidic flow enhances osteoblastic response [30]. A typical amount of induced shear stress by the normally present fluidic microflows inside human bone is in the range of $6-30\ dyn/cm^2$, $1\ dyn/cm^2$ is equal to $10\ Pa$ [30]. Leclerc already showed in 2006 that the ALP activity of $2x10^6/ml$ MC3T3-E1s was enhanced $3-7.5$ fold at shear stresses of $0.05-0.7\ dyn/cm^2$ compared to flat static cultures. Perfusion dynamic culture up to 16 days showed a 6-fold increase of calcium deposition compared to static conditions [31]. In these cultures, the shear stress was as low as $0.1\ dyn/cm^2$. Jang et al. also found an increased ALP activity of MC3T3-E1 cells cultured for 10 days, with a shear stress of $0.07\ dyn/cm^2$ [32].

Wall shear stress, τ_w, can be calculated using following formula [39],

$$\tau_w = \eta \times \sigma \tag{2.2}$$

where η is the absolute viscosity of the fluid, (kg/ms), and σ the wall shear rate, (s^{-1}). The wall shear rate is the velocity profile of the fluid. The wall shear rate under laminar flow conditions ($Re < 2000$) for cylindrical and rectangular channels can easily be calculated using the next equations,

$$\begin{aligned} \sigma_{\text{cylindrical}} &= \frac{3Q}{\pi r^3} \\ \sigma_{\text{rectangular}} &= \frac{3Q}{2(h_0/2)^2 w_0} \end{aligned} \tag{2.3}$$

where Q is the volumetric flow rate (m^3/s), r the radius of the cylindrical channel (m), and h_0 and w_0 the height and width of the rectangular channel (m). In addition, the Reynold numbers are important to calculate, to ensure the laminar flow conditions are met ($Re < 2000$),

$$\begin{aligned} \text{Re}_{\text{cylindrical}} &= \frac{Q\rho}{\pi r \eta} \\ \text{Re}_{\text{rectangular}} &= \frac{Q\rho}{(w_0 + h_0)\eta} \end{aligned} \tag{2.4}$$

where ρ is equal to the fluid density (kg/m^3).

2.4.2 COMSOL model

As mentioned before, the effect and implementation of shear stresses on a CO12 co-culture has the potential to enhance in-vitro research. Before the effect of shear stresses on cells seeded on top of nanopatterns can be assessed, we shall look at mono- and co-cultures only, so the outcome is only dependent on the shear stresses and not on the integrated pattern.

A microfluidic chip was bought from Darwin Microfluidics (see Figure 2.4 A). The chip consisted out of a Poly Carbonate (PC) toplayer and a PDMS bottom layer, in which a channel of 200 $\mu m \times 50\ \mu m \times 10\ 000\ \mu m$ (W x H x L) was made (Darwin microfluidics, France). This PDMS layer allows for free gas exchange between the co-culture and the environment, and allows for observation and imaging [27].

As mentioned above, shear stresses of $0.03 - 0.7$ dyne/cm^2 have a positive effect on OBs over time. A Finite Element method (FEM) was used in COMSOL Multiphysics v. 5.4. (COMSOL, Sweden) to simulate the shear stress through the channel under different flow rates. Following a similar model, properties of water at $37^o\ C$ was used to imitate the mixed medium [40]. PDMS was assumed to be an impermeable wall with a no-slip boundary condition. A simple rectangular of 200 $\mu m\ x\ 50\ \mu m\ x\ 10\ 000\ \mu m$ (W x H x L) was made to imitate the channel. The flow rates were introduced at one side of the channel (inlet) and the pressure on the outlet was equal to the atmospheric pressure.

Several runs were made to optimize the mesh of the channel and minimize changes as follows. 7 meshes, beginning at aproximately $40\ 000$ degrees of freedom (DOFs), ending at $5\ 000\ 000$ DOFs, were created. The mean shear stress at the bottom of the channel, caused by a fluid flow of $Q\ =\ 1\ \mu l/\text{min}$ was calculated. The results were plotted until the $\%$ of change between each measurement was $<\ 1\ \%$. The Mesh that qualified and had the lowest calculation time was then used to look at the mean, minimum and maximum shear stress induced on the bottom of the channel. Flow rates ranging from $Q\ =\ 0.1 - 1\ \mu l/\text{min}$ were introduced at the inlet of the channel. The bottom was chosen, because this would be the place cells would attach and grow initially [40]. The optimal flow would than be calculated by choosing the flow that would not exceed the range of $0.03 - 0.7\ \text{dyn}/\text{cm}^2$.

2.4.3 Dynamic co-culture

Prior to seeding, the channels were coated with Fibronectin (FN; Sigma-Aldrich, Germany) to promote cell adhesion in the channels [28]. The channels were flushed with mixed medium twice by filling the opposite inlets with respectively $70\ \mu l$ and $50\ \mu l$. This height difference introduces a flow through the channel. Next, the chips were placed in the incubator for 30 minutes to ensure that the channels were fully hydrated. The medium was aspirated carefully from the inlets and a Luer inlet connector (Darwin Microfluidics, France) that fitted a pipette tip was used to carefully pipet $25\ \mu l$ FN ($50\ \mu g/ml$) into the channel. After 30-45 minutes inside the incubator the channels were flushed once with mixed medium, again using a channel inlet to push the medium through the channel, this proved necessary to entirely unclog the system.

For the seeding of the channels, again a height difference was used to introduce a flow through the channels. In short, the opposite inlets were filled with $50\ \mu l$ and $30\ \mu l$ mixed medium. Next, $20\ \mu l$ of cell solution was inserted in the opposite inlets, with a waiting period of 2 minutes in-between. The cell concentration seeded in the channels was for OBs 1×10^6 cells/ml and for CO12 1×10^6 cells/ml:2×10^6 cells/ml (OB:M1). These concentrations were found in a preliminary study in which different seeding densities ranging from 0.5, 1 and 1.5 million OBs$/ml$ were seeded in the channels. We found that the highest cell density resulted in a widely distributed cell layer in the entire channel even after two days. Taking in mind that the total volume of mixed medium in the channel is $120\ \mu l$ and $40\ \mu l$ of culture medium was seeded, approximately $13\ 000$ and $13\ 000 :\ 26\ 000$ cells were seeded in the channels to ensure an equal distribution of cells throughout the entire channel. After seeding, the channels were imaged to check for adhesion, the chips were then placed in the incubator at $37\ ^oC$ and $5\ \%$ CO2 and left overnight. The next morning the channels were refreshed by carefully extracting the medium from the inlets and by filling the opposite sides with $70\ \mu l$ and $50\mu l$ of mixed medium. Per static condition, $100\mu l$ supernatant was extracted and stored at $-80\ ^oC$. Two days after seeding, the mixed medium was supplemented with $50\ \mu g/ml$ A.A. (Sigma-Aldrich, Germany) and $4\ mM\ \beta$-GL (Sigma-Aldrich, Germany).

In addition, on day 2 a flow was applied to the channels under dynamic conditions (Figure 2.4 B, C & D). First, 2 syringes and tubing were filled with osteogenic mixed medium. A luer connector that would fit into the channel inlets was connected to PTFE tubing. The syringes were placed on the push position on the Legato 270 syringe pump (KD Scientific, USA) and the tubing was inserted through the back of

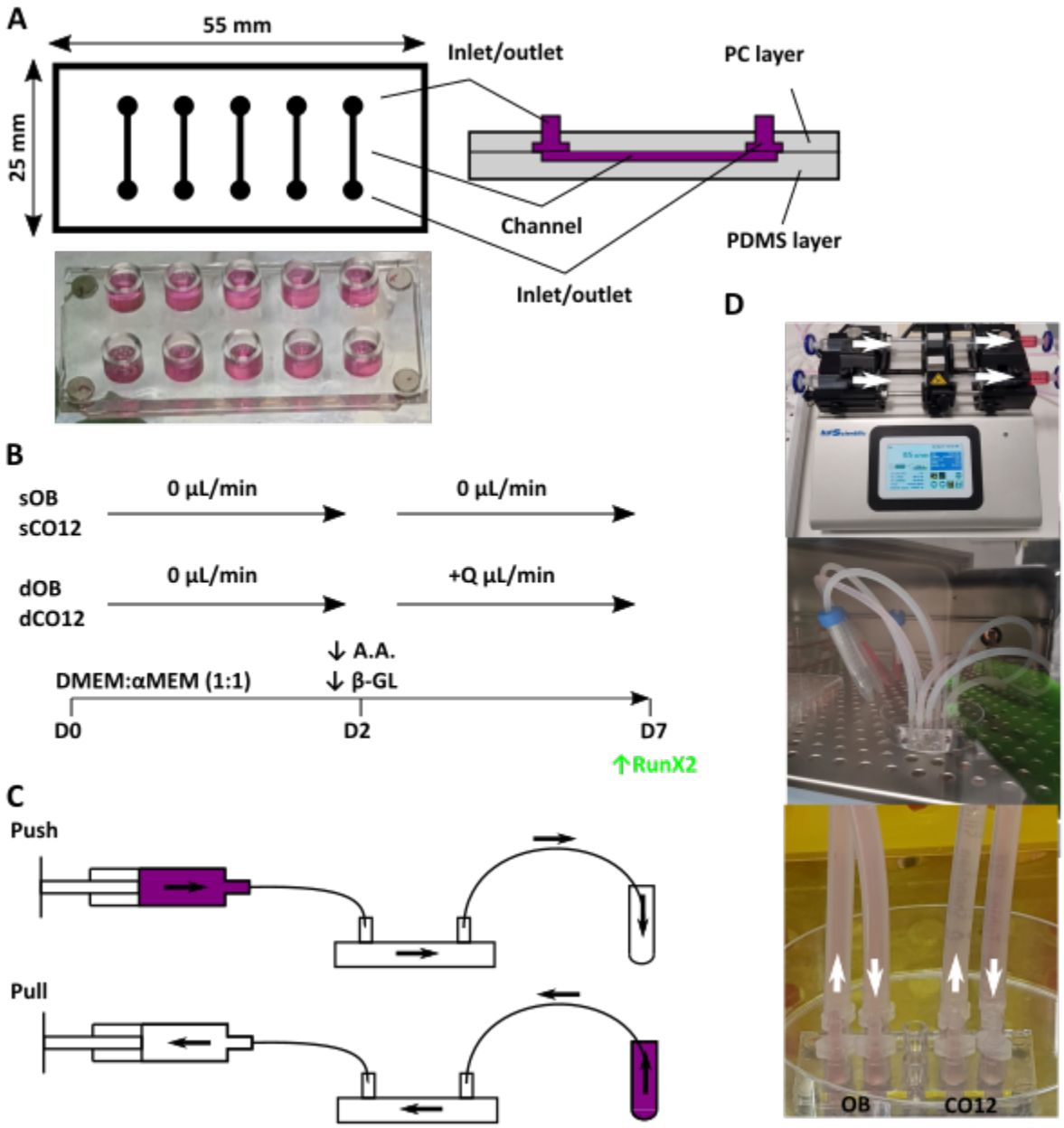

Figure 2.4: **A)** Microfluidic chip from Darwin Microfluidics with five channels of 200 μm × 50 μm × 10 000 μm (W x H x L). **B)** Timeline of dynamic direct co-culture experiment seeded inside the microfluidic channels and Cultured in mixed medium (αMEM:DMEM (1:1) supplemented with 10% FBS and 1% pen-strep). From day 2 A.A. and β-GL were added to stimulate osteogenic differentiation. A flow was introduced on day 2 in two channels with OBs (dOB) and two channels with CO12 (dCO12). Medium was refreshed for the channels under static conditions (sOB and sCO12) each day. **C)** For each condition flow was introduced inside the channel by pushing the medium from a syringe or pulling the medium from a tube. **D)** Setup of the dynamic system from top to bottom. Syringe pump pushes or pulls the medium into the chip. The arrows indicate the flow directions through the channels.

the incubator. The tubings were fit into one dOB and one dCO12 inlet. The outlets opposite from these channels were connected to additional tubing and an empty 15 ml conical tube. Secondly 2 additional syringes and tubing wer filled with 1X DPBS, except for the outer end of the tubing, which was filled with

osteogenic mixed medium. These 2 syringes were placed on the pull position on the syringe pump and the PTFE tubing was inserted through the back of the incubator. A 15 ml conical tube and PTFE tubing was filled with mixed medium, and attached to the outlet opposite from the pull inlets, medium could be pulled through the channel at the same time, as it will be pushed through the other two channels (Figure 2.4 C). This way, 4 channels can be used on the chip for each experiment, instead of 2. Enough medium was available for 5 days of dynamic culture. The static chip was refreshed every day with osteogenic mixed medium.

2.4.4 Morphological response

Brightfield pictures were taken to see the cell growth and proliferation of sOB and sCO12 over time. For the dynamic cultures, dOB and dCO12, only images were taken on days 2 and 7. I choose not to detach the dynamic chips from the system during culture, since this would increase the risk of contamination, and loosening of the cell layers, caused by pressure differences when attaching and detaching the Luer inlets. This resulted in no images of cells inside the dynamic channels for days 3 to 6.

2.4.5 Inflammatory response

On days 2 and 7 $100\mu l$ of supernatant was extracted from the static and dynamic channels and stored at $-80\ ^oC$. PGE2 ELISA (ThermoFisher Scientifc, US) secretion was measured for these two time points to gain insight in both osteogenic as inflammatory response due to cellular cross-talk and shear stress.

2.4.6 Osteogenic response

Runx2 activity was measured on day 7 only. The reason for this, is that during the first dynamic experiment, the FN coating was flushed out of the channel, after washing the channels with culture medium twice. By reducing this to only one wash, cells were found to attach to the channels immediately after seeding. With only 2 chips left, we decided to reduce Runx2 staining to only one day, day 7, instead of two days, days 4 and 7. In short, staining was performed following the same protocol as before, except that channels were washed and filled by filling opposite inlets with $70\ \mu l$ and $50\ \mu l$ of fluid. Fluorescent intensity was measured using FIJI [37], by selection of the cells using the threshold function and then taking the mean intensity density of all the cells within the seleted area. For each channel 10 images were taken. In addition, the channels were stained with $1\ \mu g/ml$ Hoechst 33392 (ThermoFisher Scientific, US), in order to visualize all the cells present. After Hoechst staining, the channels were washed with 1x PBS three times and imaged.

One channel of dOBs was so full of cells that no flow could go through the channel which resulted in no staining inside this channel. Runx2 and Hoechst staining both were not able to flow through the channel. In addition, one inlet of a dCO12 channel was detached during the night, which resulted in dead cells within this channel. Multiple images were taken from the two available channels of the dynamic conditions dOB and dCO12 and the mean intensity density of every cell was taken into account for the determination of the relative expression of Runx2 with sOB as the control. For each static condition, sOB and sCO12, two channels were available for Runx2 imaging.

2.5 Statistical Analysis

One-way ANOVA with Sidak's multiple comparisons test in GraphPad Prism version 8.0.1 for Windows (GraphPad Software, USA) was assessed to analyze the differences among mono and co-cultures. For tests with multiple conditions, at multiple days, two-way ANOVA was used followed by Sidak's multiple comparisons test. Data are expressed as mean $\pm$ standard deviation of the mean (SD), where $(ns) = P > 0.05$,$(*) = P < 0.05$,$(**) = P < 0.01$, $(***) = P < 0.001$. Probability values of $P < 0.05$ were considered significant. Outliers in Elisa results were removed using ROUT test, with $Q = 1\%$.

2.6 Dynamic co-culture on a nanopattern (POC)

Following section describes the design and fabrication process of a prototype fluidic device in which the nanopattern could be inserted in order to introduce a dynamic flow on the pattern that mimics *in-vivo* microflows inside the bone niche.

2.6.1 Design

The dynamic chip had to met particular criteria to be used for the integration of a fluidic flow on a patterned waver. First of all, the waver needed to be press fit inside a two part "sandwich" chip. This was necessary, because the wavers are very thin, and fragile, removing a channel layer that was integrated with the waver for analysis could result in the destruction of the waver. Secondly, the chip has to provide for an insert in which the waver with a diameter of $D = 31mm$ should fit. Furthermore, the design of the channel, in which the fluidic flow will be introduced, should induce a suitable shear stress within the range of $0.03 - 0.7$ dyn/cm^2. Finally, the chip should be made out of biocompatible materials, should allow for free gas exchange of the cells within the incubator environment, and it should be able to sterilize the chip prior to cell culture.

Using these design criteria three microfluidic chips were produced in Solidworks (Dassault Systèmes, France), two direct co-culture and one in-direct co-culture chip. Since this thesis focused on direct co-culture models, the direct co-culture chips were fabricated and tested. Details about the fabrication and validation of the chips will be discussed later. After testing and validating the first design, Finite Element method (FEM) models were used to check whether sufficient fluidic flows were induced inside the channels. Using the results from the FEM models and test results, a final chip design was developed.

2.6.2 Modelling and optimal parameters

A Finite Element method (FEM), similar to the model used before, was used in COMSOL Multiphysics v. 5.4. (COMSOL, Sweden) to simulate the shear stress through channels with different dimensions under the same flow rate. However, prior to these simulations, the wall shear stress τ_w was calculated using equations 2.2 and 2.3 to find a suitable range of dimensions and flow rates in which the FEM models should be made. The variables were the height of the channel, $h_0 = [50\ 100\ 500\ 1000]\ \mu m$, the width of the channel, $w_0 = [1\ 3]\ mm$, and the flow rate, $Q = [1\ 10\ 100\ 1000]\ \mu l/min$.

2.6.3 Fabrication

Fabrication of the chip consisted out of three main parts: Fabrication & Post-processing of the mould, PDMS polymerization inside the mould, and assembly of all the parts, i.e. PDMS, PMMA, & magnets.

The chips consisted out of 2 main parts, the upper and lower part, and these consisted out of 2 layers, PMMA and PDMS. The lower part would provide a chamber in which the waver could fit and the upper part a channel through which the cells could be seeded on top of the waver. The chamber and channels were made in PDMS. The PDMS layers were attached to the PMMA layers using uncured PDMS as a glue. In addition magnets were placed in both PDMS layers, to ensure the outline of the channel and waver was perfect. A clamp was also made out of stainless steel to ensure the chip would not leak during culturing.

First of all the PDMS layers were fabricated by molding a negative replicate of the COMSOL design using the SLA printer Form 3 (Formlabs, USA). Grey resin (Formlabs, USA) was used to print the mould. The resolution of the print was set on "adaptive", ranging from $25 - 160\ \mu m$. This setting resulted in lower printing time, since the supports were printed at a higher resolution than the channels. After printing, the mould was firstly washed in dirty isopropanol (IPA; Sigma Aldrich, Germany) for 10 minutes, followed by a wash in fresh IPA for another 10 minutes to fully remove all the uncured resin. Thereafter the mould was placed under UV light at $60\ ^oC$ for one hour. In addition, to fully cure the entire mold, an additional $60\ ^oC$ overnight bake was introduced. A final wash with dish soap and water using a toothbrush afterwards removed the last layer of uncured resin from the mould.

Before pouring PDMS into the mould, the mould was cleaned by sonicating it for 5 minutes with IPA, rinsing it with demiwater and airdry it after. Sylgard 184 PDMS (Sigma Aldrich, Germany) at a 1:10 ratio was poured in a petri dish and stirred for 2 minutes. This petri dish was degassed for 30 minutes until all bubbles were removed. Degassed PDMS was poured carefully in the mould, followed by degassing for another 30 minutes. PDMS was cured for 4 hrs at $60\ ^oC$. After curing, the mould and PDMS were cooled down at room temperature and the PDMS layer was carefully retrieved with sterile tweezers and a scalpel inside a flow cabinet.

PMMA layers were cut using the laser cutter from the student workshop at 3mE. For the upper section of the chip, holes with a diameter of $3.1\ mm$ were cut out to fit luer plugs. The PMMA layers were cleaned with IPA, air dryed and coated with a thin layer of PDMS using the spin coater for 5 minutes at $1000\ rpm$. The PDMS layer and PMMA were placed on top of each other and clamps were used to apply pressure to the PDMS and PMMA layer [41]. the PMMA-PDMS construction was baked for 2 hours at $70\ ^oC$. After cooling down, 4 magnets were inserted and glued with uncured PDMS. This final PMMA-PDMS-magnet assembly was baked another hour at $70\ ^oC$ and cooled down at room temperature afterwards.

2.6.4 Validation

First of all, the chip was attached to the syringe pump, and a flow rate, twice as large as necessary for a suitable shear stress was introduced to check for leakage after 2 days of continuous flow. Luer connectors

were press-fit into the inlets and a flow was introduced through the channel

Secondly the effect of UV sterilization and 1x PBS rinsing on the integrity of the chip was tested. UV sterilization is essential for the decontamination of the chip. A suitable sterilization protocol was obtained that had no negative effects on the polimerized PDMS material and PMMA layer, since bonds can be broken under influence of the UV light [42]. In short, the outer PMMA parts, of the chip were cleaned with 70 % ethanol. The inner PDMS layer was rinsed twice with 1x PBS, and airdried under a UV light for 30 minutes. All the tubing and connectors were rinsed with 1x PBS and sterilized using a autoclave before use. The stainless steel clamp was also washed with 70 % ethanol. After sterilization the chip was tested for leakage inside the incubator for 1 day under a continuous flow.

2.6.5 Dynamic culture

OBs ($5\ 000$cells$/$cm^2) were seeded inside the channel ($\pm 0.9 cm^2$) in mixed medium, and incubated at $37\ ^oC$ and 5% CO2. After 24 hours, mixed medium was refreshed and a fluidic flow was introduced. This process was similar to the setup of the pre-fabricated microfluidic device from Darwin Microfluidics. After 1 day, cell adhesion. and proliferation was tested with DAPI staining.

CHAPTER 3

Results

3.1 Macrophage polarization

To confirm successful MΦ polarization, M1 and M2 MΦ phenotype was validated using Brightfield imaging and cytokine profiles, i.e. Griess, TNF-α, PGE-2 and IL-10. J774A.1 MΦs were polarized towards M1 and M2 in DMEM +10% FBS + 1% pen-strep supplemented with LPS and IFN-γ or IL-4, for M1 and M2 respectively, for 3 days (Figure 2.1 A). Several Morphological differences were observed between M0, M1, and M2s (Figure 3.1 A). On first sight, M0s and M2s seemed to be very much alike, however more elongated cells were present in M2 stimulated cultures overall.M0s and M1s both have a more round shape. Where M0 and M2s are rather small (25 μm) M1s could become twice as large and adapt an amoeboid morphology. In figure 3.1 A, an example of one M1 can be seen that has grown up to 150 μm long. All the three polarization states tend to grow in colonies.

The cytokine profiles matches the polarization states of the MΦs. NO secretion (Griess assay) was significantly higher for M1 (51.17 $nmol/ml \pm 2.02$) with respect to M0 (10.32 $nmol/ml \pm 0.33$) and M2 (10.99 $nmol/ml \pm 2.13$). In addition TNF-α secretion was also significantly higher for M1s (313.96 $pg/ml \pm 3.99$) compared to M0s (62.62 $pg/ml \pm 61.49$) and M2s (172.08 $pg/ml \pm 65.41$). PGE2 secretion was also significantly higher for M1 (1868.06 $pg/ml \pm 138.52$) with respect to M0 (80.75 $pg/ml \pm 38.50$) and M2 (120.30 $pg/ml \pm 0.35$). In contrast, the anti-inflammatory marker IL-10 was significantly higher for M2s (727.26 $pg/ml \pm 133.35$) in comparison to M0 (163.31 $pg/ml \pm 33.06$) and M1s (120.01 $pg/ml \pm 38.758$).

The results found showed macrophage polarization towards M1 resulted in amoeboid-like morphology and a significant increase in pro-inflammatory cytokines, TNF-α, PGE2 and NO, with respect to unpolarized M0s and M2s. M2s were found to secrete more of the anti-inflammatory cytokine, IL-10, however, comparison of M2 and M0 morphology resulted in minimal differences with respect to the amount of elongated cells present in the cultures.

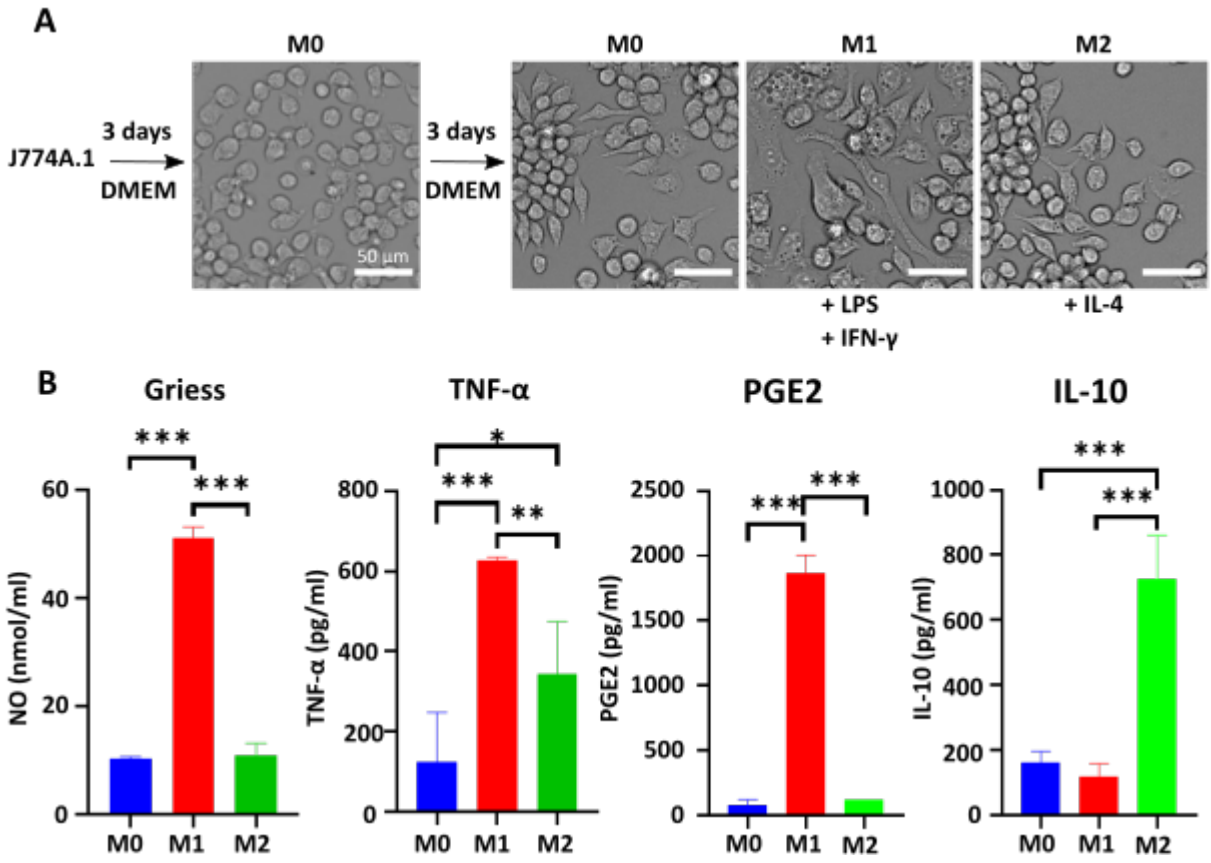

Figure 3.1: **A** Brightfield images of J774A.1 MΦs before and after stimlution. M0s (DMEM), M1 (DMEM +IFN-γ & LPS) and M2s (DMEM + IL-4), taken with ZOE microscope, showing their morphological features. **B)** NO, TNF-α, PGE2 and IL-10 secretion in cell culture supernatant measured with ELISA (mean ± SD). Ordinary One-way ANOVA was assessed. $(ns) = P > 0.05$, $(*) = P < 0.05$, $(**) = P < 0.01$, $(***) = P < 0.001$

3.2 Co-culture

In M1-MSC co-culture studies, a higher M1 ratio has been shown to have a positive effect on OB differentiation and even on M1-M2 polarization [43, 17]. Here we investigated whether a higher M1-OB ratio would lead to a similar effect. We looked at OB-M1 co-cultures with the ratios 1:1 (CO11) and 1:2 (CO12), and see the effect in inflammatory and osteogenic response over time of these co-cultures in comparison to the mono-cultures of OBs and M1s.

3.2.1 Cell viability

First of all, we confirmed that mixed medium was suitable in terms of viability for the mono- and co-culture of M0, M1, M2 and OBs after 3, 7, 10 and 14 days using Live/dead staining. On day 3 the dilution of live staining used did not result in the staining of MC3T3-E1s, therefore an optimization experiment with 2.0 $\mu M/ml$ instead of 0.8 $\mu M/ml$ Calcein AM was performed. The higher amount of Calcein AM resulted in perfectly stained living cells (Appendix D.2). Live/dead assay showed that the percentage of living cells over the total number of both living as dead cells was not significant over time for all the experimental conditions (Figure 3.2 A). The highest differences were observed in M1s from day 7 to day 10 ($P < 0.15$) and CO11 from day 10 to day 14 ($P < 0.09$). On the long term there is no significant

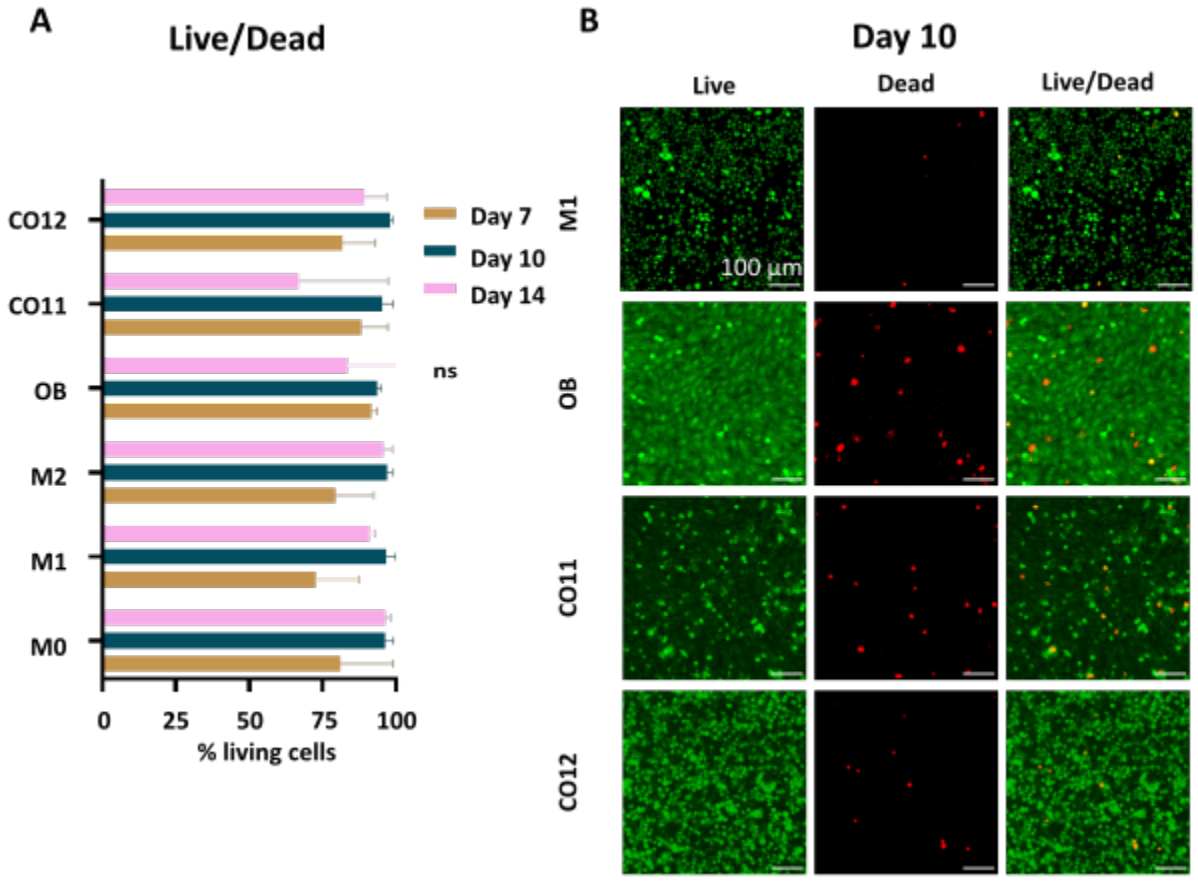

Figure 3.2: **A)** % of living cells (mean ± SD) for days 7, 10 and 14 of mono-cultures M0, M1, M2, and OB and co-cultures CO11 and CO12. One way Anova with Tukey multiple comparison test was assessed. No significant ($P > 0.05$) differences were found. **B)** Live/Dead (green/red) staining fluorescent images for M1, OB, CO11 and CO12 on day 10. "Find Edges" feature of ImageJ was used on the "Live" images to enhance the cell outline.

difference in dead cells compared to living cells, which indicates that the 1:1 mixed medium is appropriate to be used during our experiments.

3.2.2 Morphology

To analyze the proliferation and differentiation of all mono- and co-cultures, Brightfield images have been made on days 1, 3, 7, 10 and 14 after seeding (Figure 3.3). On day 1, M1 morphology is similar to the morphology 3 days after LPS and IFN-γ stimulation (Figure 3.1). The cells still have an amoebic like morphology. From day 3, more cell clusters have been formed and more elongated cells all ready seemed to grow in the wells. At the same time, the amount of Macrophages with a M1-like morphology seem to reduce and a mixture of M0, M1 and M2 phenotype grows inside the wells.

OBs are hard to image with a brightfield camera, since they are more flatly spread on the well plate. The enhancement of contrast shows the largeness of the cells, and how a mono-layer has been formed on day 7 already. After day 7 there seems to be no noticeable changes over time, since there already exists a tight mono-layer. Filtering out separate cells is already difficult after one week.

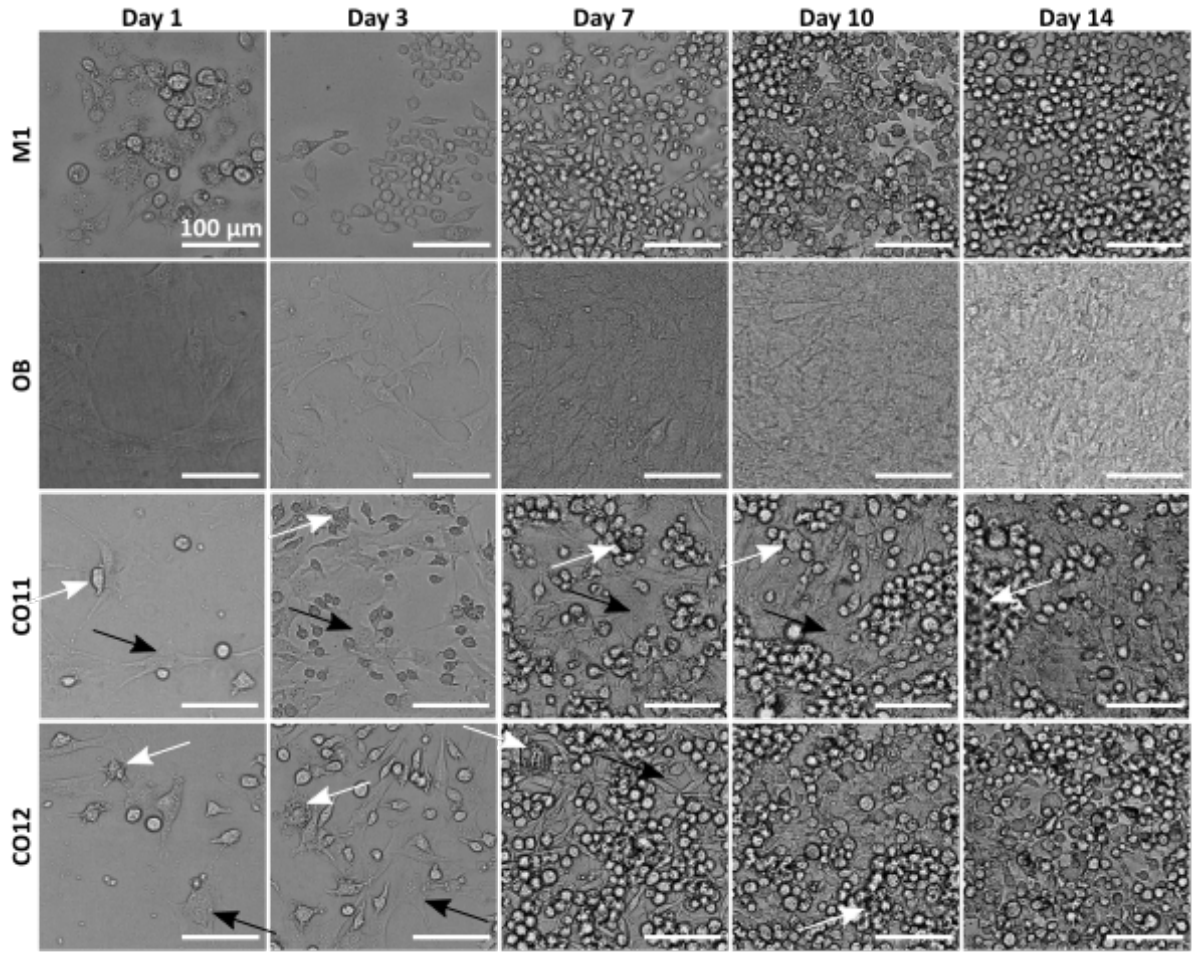

Figure 3.3: Brightfield images of M1, OB, CO11 and CO12 cultures, on days 1, 3, 7, 10 and 14 after seeding. White and Black arrows indicate M1 and OB cells respectively. Image contrast is enhanced with FIJI [37]

CO11 is at day 1 less crowded than CO12. It looks like the mono-layer formed by OBs is located beneath MΦs, however, this observation can also be caused by the fact that MΦs are more easily in focus. This mono-layer seems to be less dense than OB mono-culture. It looks like the MΦs and OBs grow tightly together after one week of culture.

3.2.3 Inflammatory response

The secretion of both pro- and anti-inflammatory markers were measured over time to gain more insight about the polarization state of the MΦs during co-culture. For all co- and mono-cultures TNF-α secretion rates rise for days 1, 3 and 7 (Figure 3.4 A). M1, CO11 and CO12 TNF-α levels are significantly higher on day 7 compared to day 1, and 3. Furthermore, both co-cultures, CO11 and CO12, secrete significantly more TNF-α on day 7 compared to M1. Between days 1 and 3, there are no significant difference between the supernatants and medium. The indication of a possible pro-inflammatory response at day 7 is also visible in the PGE2 secretion rates. As mentioned before, PGE2 secretion by OBs is proportional to TNF-α [44]. For OB mono-culture, PGE2 secretion starts very high, and decreases significantly from day

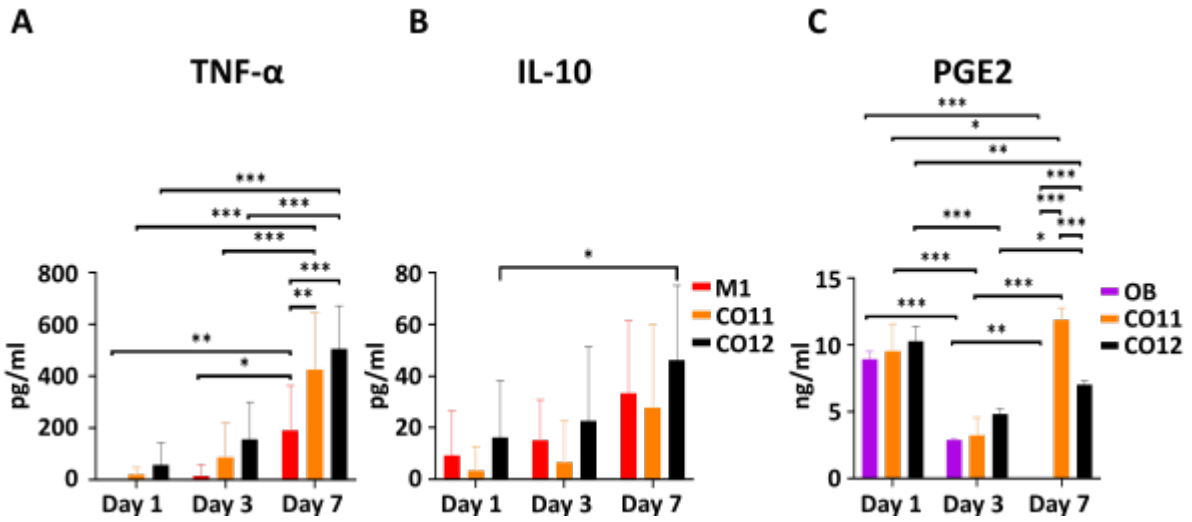

Figure 3.4: **A)** pro-inflammatory TNF-α secretion values of M1, CO11 and CO12 measured with ELISA from cell culture supernatant at days, 1, 3 and 7 after seeding (mean ± SD). **B)** anti-inflammatory IL-10 secretion values of M1, CO11 and CO12 measured with ELISA from cell culture supernatant at days, 1, 3 and 7 after seeding (mean ± SD). , **C)** OB, CO11 and CO12 PGE2 secretion measured with ELISA at days 1, 3 and 7 after seeding. Ordinary Two-way ANOVA was assessed. $(ns) = P > 0.05$, $(*) = P < 0.05$, $(**) = P < 0.01$, $(***) = P < 0.001$. N.D. = Not Detectable

1 to 3 and 3 to 7. CO11 and CO12 also show a high amount of PGE2 on the first day after seeding. This amount decreases up to day 3, and rises again on day 7. CO11 secretion on day 7 is significantly higher than both OB and CO12. PGE2 secretion for M1s has not been shown here, because this was near to zero and therefore undetectable in comparison to OB, CO11 and CO12.

Anti-inflammatory cytokine IL-10 was measured to see a possible M1-M2 polarization shift of the macrophages over time. On days 1 and 3 IL-10 values between M1, CO11, CO12 and Culture Medium are not significantly different (Figure 3.4 B). On day 7 the levels seem to rise, and the difference in IL-10 between day 1 and 7 is only significant for CO12. Generally, the amount of secreted IL-10 in the wells is limited for all cultures.

3.2.4 Osteogenic response

Measuring the osteogenic activity of both mono- and co-cultures gives more insight about which co-culture ratio is the best in the long term. Runx2 is an early osteogenic marker, it is necessary for pre-osteoblast differentiation into osteoblasts (Figure 1.2) [45]. A lack of Runx2 inhibits osteoblast differentation. Runx2 antibody staining seems to stain M1s as well as OBs (3.5 B). However, this intensity was filtered out by subtracting the same background from all the images. The relative Runx2 expression is shown in figure 3.5 A, with OB mono-culture at day 7 as the control group. Relevant Runx2 expression of CO12 was compared to OBs, since CO12 secreted most anti-inflammatory cytokine, IL-10, and therefore it was believed that this would have the greatest improvement in early osteogenic activity. At day 3 Runx2 expression is so low, that there exists no relevant differences between the groups. At day 7 the difference between M1 and OB and CO11 is significant ($P < 0.001$). Furthermore, CO12 is also significantly lower than OB ($P < 0.001$) and CO11 ($P < 0.001$).

Alkaline phosphatase-, mediated calcification (ALP) is a by-product of osteoblastic activity and can determine early osteogenic activity [15]. No differences per group were observed in ALP expression for days 7 and 11 (Figure 3.5 C). On day 21, OB ($1.79\ mU/ml\ \pm\ 0.13$ expression is higher than CO11 ($1.20\ mU/ml\ \pm\ 0.74$). On day 28 ALP secretion by OBs ($2.26\ mU/ml\ \pm\ 0.23$ is significantly higher than both co-cultures CO11 ($1.48\ mU/ml\ \pm\ 0.026$) and CO12 ($0.96\ mU/ml\ \pm\ 0.70$).

Alizarin red is also a common method to test the calcium deposit, or mineralization, induced by OB activity [15]. Alizarin staining binds to the calcium present in osteocytes (OCTs), and the extracellular matrix (ECM) resulting in a red colour. This red colour relates to the amount of formed bone tissue. Alizarin Red Staining (ARS) was performed to compare the mineralization, and thus long-term osteogenic response of mono and co-cultures. ARS resulted in almost no noticeable "red"/calcium staining for both co-cultures on day 28 (Figure 3.5 D). OB mono-culture did have an increase in redness up to day 28 after seeding.

In short, in this section we tried to show the effects of two different ratios of M1:OB co-culture and the effect on osteoimmunological cross-talk in terms of pro- and anti-inflammatory cytokines, and osteogenic differentiation shown as Runx2 staining, ALP and ARS. First, we showed that cell viability was not significantly reduced for all culture conditions. Three days after seeding M1 morphology is reduced or not detectable and co-cultures are both densely packed with cells. The pro-inflammatory response, TNF-α and PGE2, increased over time for all cultures, except OB mono-culture, which only showed an increase in PGE2 on the first day. No significant results were found that would indicate an anti-inflammatory response (IL-10). Measurements towards osteogenic activity, Runx2, ALP and ARS, showed a reduction of OB differentiation and activity after 28 days for both co-cultures.

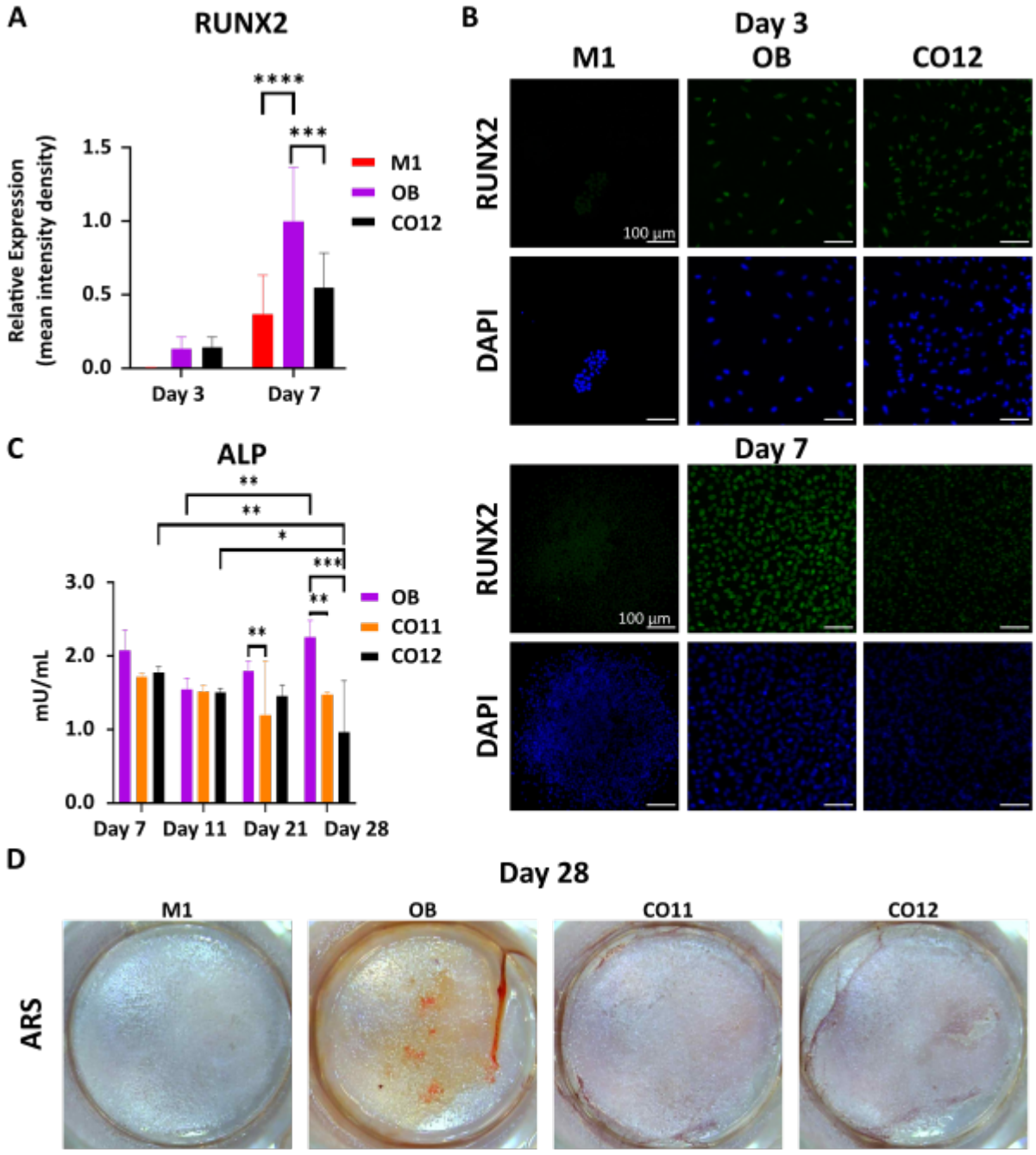

Figure 3.5: **A)** Relative Runx2 expression measured as the sum of the integrated Runx2 expressed density. OB at day 7 is the control (mean ± SD). Ordinary One-way ANOVA was assessed. **B)** Runx2 & DAPI images of OB, CO12 and M1. **C)** OB, CO11 and CO12 ALP response measured with ELISA from cell culture supernatant at days, 11, 14, 21 and 28 after seeding (mean ± SD). Ordinary Two-way ANOVA was assessed. **D)** Alizarin Red Staining (ARS) of M1, OB, CO11 and CO12 at day 28. $(ns) = P > 0.05$, $(*) = P < 0.05$, $(**) = P < 0.01$, $(***) = P < 0.001$

3.3 Co-culture on nanopattern

3.3.1 Morphology

Differences in OB morphology with and without the presence of a nanopattern were not observable in brightfield images (Figure 3.6 A). At Day 7, the monolayer of OBs have enveloped the whole pattern, in the same way OBs behave without the pattern. M1s in co-culture, tend to migrate toward the pattern, the intersection between pattern and slide, can easily be seen, since M1s outline this part of the pattern. In general OBs grow on the pattern, and M1s seem to grow on top of the OBs. From day 7 it is more difficult to see the pattern outline since a monolayer of both OBs as M1s has formed on the entire well. In short, their seems to be no immediate effects of the pattern on OB and CO12 morphological behavior over time.

3.3.2 Osteogenic response

Runx2 staining was assessed to see the difference in osteogenic response 7 days after seeding. Relative Runx2 expression was not significantly different between all groups with and without a pattern. There is however a trend in elevated Runx2 expression for the co-culture on the pattern with respect to co-culture without the pattern. The difference in relative expression between OB and CO12 is almost similar to the Runx2 expression for the previous direct co-culture study. It was found that there was no significant increase in Runx2 for mono-cultures. However, the addition of the patterns in the co-culture had a p-value of 0.085. The pattern itself was fluorescent, but by reducing the gain of the microscope and subtracting the same background value to all images this interaction was minimized. Subtraction of the background value also minimized the background intensity of the M1 macrophages.

In addition ARS staining was performed on day 11. Mineralization occurs mainly at a later stage, however we were wondering whether some minerals already had been formed at this day. The results can be seen in appendix E.1. No differences between groups were found and no immediate effect of the patterns on mineralization was observed. The OB monocultures with and without the pattern both expressed some red staining, however this was not present on the pattern, but at random places inside the wells. In addition it was hard to find the pattern surface using the Leica DM500 Brightfield (Leica Microsystems, Germany) microscope, since the pattern was covered under a densely packed cell layer.

Briefly, the addition of nanopatterns on the osteoimmunological response of M1s and OBs have been shown in this section. Their seems to be no immediate effects of the pattern on OB and CO12 morphological behavior over time. In terms of osteogenic activity a trend in Runx2 activity for the co-culture seeded on top of the pattern has been found.

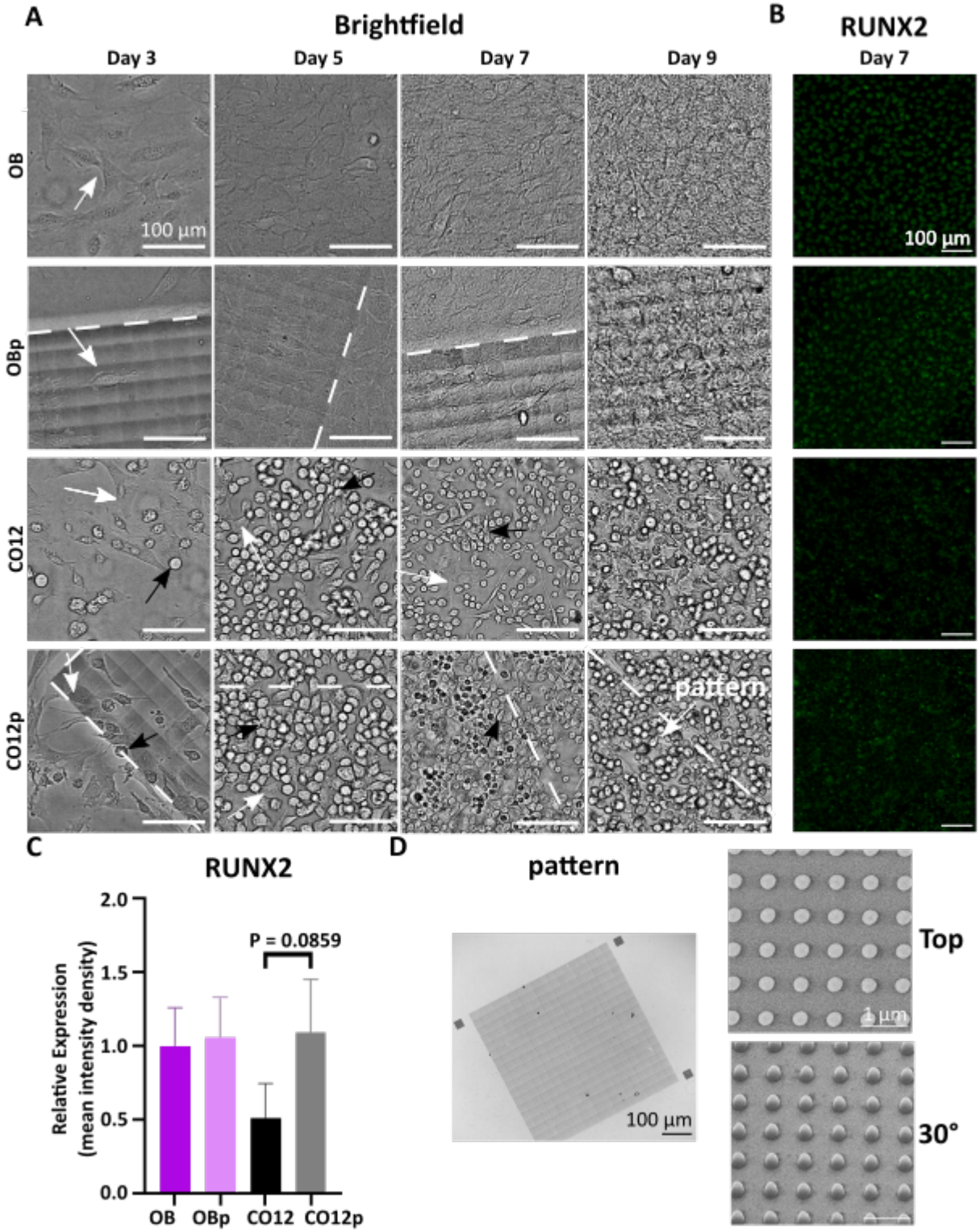

Figure 3.6: **A)** Brightfield images of OB, OBp, CO12 and CO12p on days, 3, 7 and 9 after seeding. "Black" and "white" arrows indicate M1 and OB cells respectively. The pattern borders have been indicated with a white line. Image contrast have been enhanced using FIJI [37] **B)** Runx2 images of OB, OBp, CO12 and CO12p. **C)** Relative Runx2 expression measured as the sum of the integrated Runx2 expressed density. OB without the pattern is the control. Mean intensity density (mean ± SD). Ordinary One-way ANOVA was assessed.**D)** Brightfield and SEM images from the p pattern. $(ns) = P > 0.05$, $(*) = P < 0.05$, $(**) = P < 0.01$, $(***) = P < 0.001$

3.4 Dynamic co-culture

In this section the effect of shear stress on both OB mono- and CO12 co-cultures will be shown. First the shear stress profiles and resulting microfluidic flow rate, which was implemented during dynamic co-culture, will be shown. Thereafter the effect of shear stress on cellular behavior will be looked upon in terms of morphological behavior, Runx2 staining and PGE2 measurements on day 7.

3.4.1 Shear stress

COMSOL was assessed to define appropriate shear stresses on the cells within the microfluidic channel. The results have been shown in Figure 3.7. The results of the convergence study showed that the use of a mesh with $\pm\ 2\ \times\ 10^6$ DOFs would be sufficient for the simulation of the flow inside the channel (3.7 A). The change in shear stress was less than 1%, and therefore these mesh parameters were optimal in terms of calculation time and mesh convergence. A flow of $0.5\ \mu l/min$ resulted in a shear stress profile within the range of $0.03 - 0.7$ dyn/cm^2 (Figure 3.7 B, C & D). The mean shear stress calculated on the bottom layer of the channel was 0.501 dyn/cm^2. The minimum and maximum shear stresses on the bottom of the channel were 0.046 and 0.585 dyn/cm^2 respectively. The wall shear stress was also calculated by hand using formula 2.2 as a sanity check (Figure 3.7 D). No leaking was found after testing this flow rate of $0.5\ \mu l/min$ for two days within the microfluidic chip.

3.4.2 Morphology

Brightfield images were taken on day 7 to check the differences between OBs and CO12 under static and dynamic conditions (3.8 A). An increased risk of contamination existed if the dynamic co-culture systems had to be detached every time for imaging. Therefore, only images for day 7 are available. For all conditions, the highest amount of cells were present near the inlets, especially for the static cultures. dOBs under dynamic conditions resulted in a highly dense layer of OBs through the channel, one channel was so densely covered that a height difference of fluid between the opposite inlets was not sufficient to flush the channel (Figure 3.8 E). dCO12 had little to no cells present in the midsections of the channel. The cells, mainly M1s, that were present were mainly located at the side of the channel. During the washing steps before fixation, it was noticeable that detached/dead cells were flowing through the channel at high velocities. Most cells were present near the inlets.

3.4.3 Osteoimmunological response

Runx2 expression of dOB was significantly higher than sOB and sCO12. There was no difference between sCO12 and dCO12 (Figure 3.8 B & C). PGE2 expression was measured on D7 with supernatant extracted from the inlets after detachment of the tubing and luer plugs. PGE2 expression was significantly highest for sCO12 ($1950.00\ pg/ml\ \pm\ 71.00$) compared to sOB ($900.34\ pg/ml\ \pm\ 65.59$), dOB ($160.95\ pg/ml\ \pm\ 122.70$) and dCO12 ($544.57\ pg/ml\ \pm\ 103.89$) (Figure 3.8 D). In contrast, PGE2 expression of dOBs was significantly lower than sOBs, sCO12 and dCO12 ($P\ <\ 0.05$) at day 7.

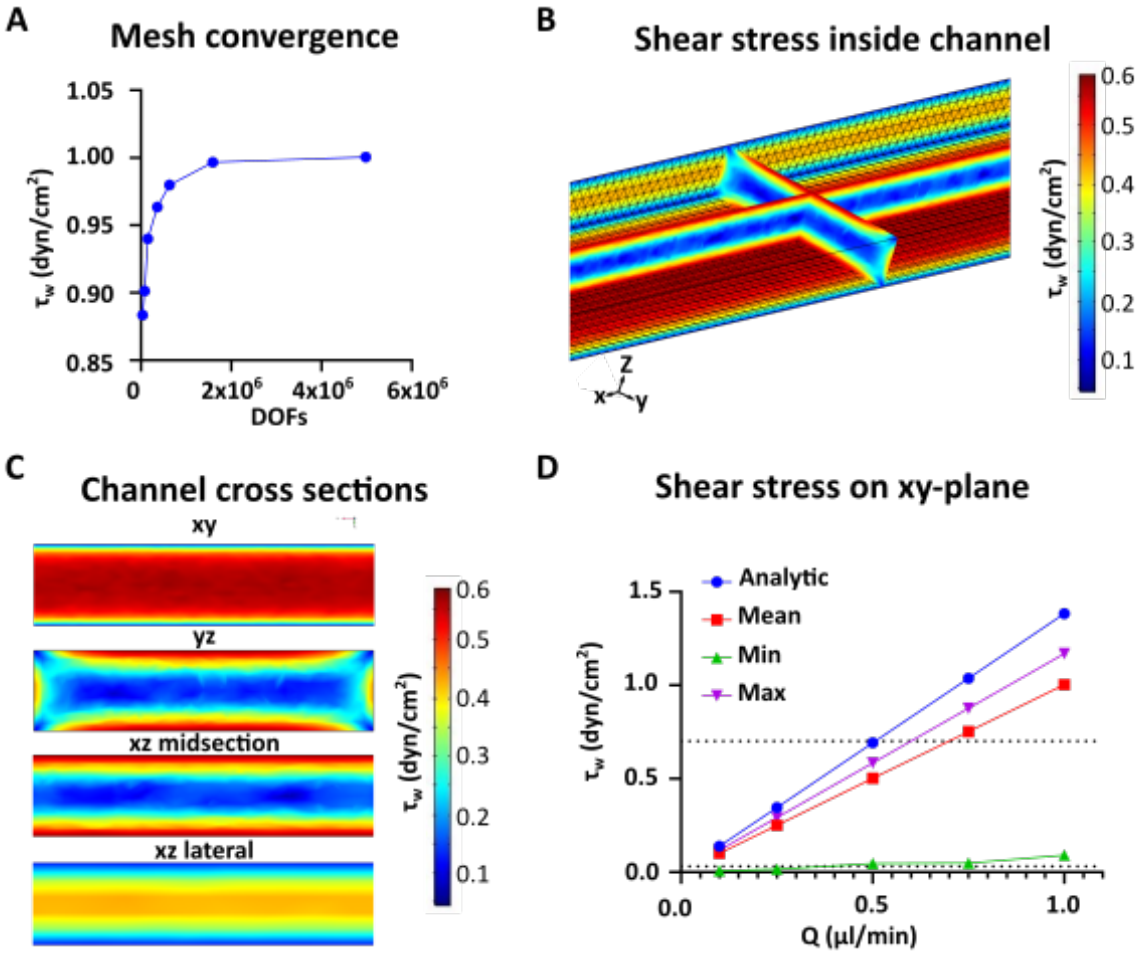

Figure 3.7: **A)** The change of the mean wall shear stress, τ_w, using different mesh sizes, DOFs. **B)** Wall shear stress, τ_w, in the middle of the channel. **C** Wall shear stress, τ_w, of 4 different cross sections of the channel. **D** The analytical calculated, mean, minimum and maximum wall shear stress, τ_w, induced on the bottom of the channel at different flow rates Q.

To sum up this section, a flow of $0.5\ \mu l/min$ resulted in a shear stress profile within the range of $0.03 - 0.7$ dyn/cm^2. This shear stress significantly enhanced Runx2 expression and decreased PGE2 secretion on day 7 in OB mono-culture.

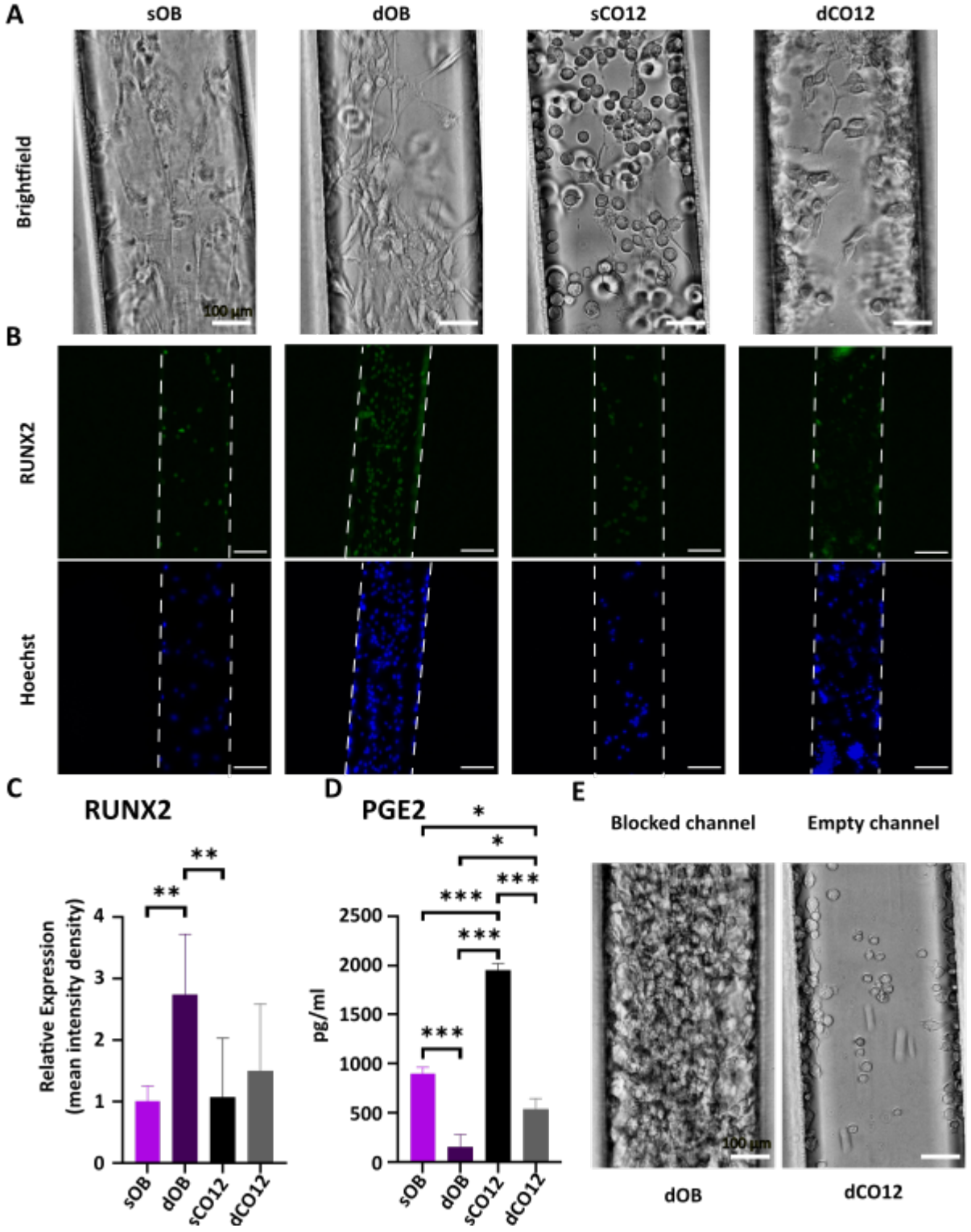

Figure 3.8: **A)** Brightfield images of static (sOB & sCO12) and dynamic (dOB & dCO12) channels at day 7. **B)** Runx2 and Hoechst 33342 images of sOB, dOB, sCO12 and dCO12 **C)** Relative Runx2 expression measured as the mean of the integrated Runx2 expressed density with respect to sOB (mean ± SD). Ordinary One-way ANOVA was assessed. **D)** PGE2 secretion at day 7 (mean ± SD). Ordinary One-way ANOVA was assessed. **E)** (left) Blocked channel overgrown with OBs in dynamic condition. (right) Empty channel in dynamic CO12 channel. $(ns) = P > 0.05$, $(*) = P < 0.05$, $(**) = P < 0.01$, $(***) = P < 0.001$

3.5 Dynamic co-culture on pattern

This final part is involved with the development of a lab-on-a-chip system that could be used to establish a dynamic flow on the nanopattern during the co-culture of OBs and M1s. This way, the effect of a dynamic microflow on a nanopattern could be assessed, and most importantly the induced osteoimmunological response of a co-culture seeded on top of the pattern could be investigated.

3.5.1 Design

Initially three designs were made using Solidworks (Dassault Systèmes, France), two direct co-culture and one indirect co-culture chip (Figure 3.9 A). For the direct co-culture chips, one was made out of a round chamber with the same diameter as the nanopattern waver ($D = 31\ mm$). The other was made out of a channel. The third design was a channel, consisting of one central culture chamber, that would precisely fit on the pattern, with a porous wall placed in the middle. Two additional channels were placed perpendicular to the fluidic flow channel through which cells can be seeded but still would be separate to make a in-direct co-culture fluidic system. The porous wall had openings of $25 \times 25\ \mu m$, through which cytokines could flow freely but cells would not be able to cross [29], preferably the channels would be smaller than $25\mu m$, however the SLA printer could not print smaller, so therefore I choose for $25\ \mu m$.

3.5.2 Shear stress

First of all, rough estimations for the induced shear stress were calculated to find a suitable range for the FEM models (Appendix table E.1). Relevant channel height, width and flow rates were defined to be in the range of $h_0 = 50 - 150\ \mu m$, $w_0 = 1 - 3\ mm$ and $Q = 1\mu l/min$. Next, COMSOL was assessed using these different parameters to define the final design and flow rate of the channel (Figure 3.9 B). The cell culture surface of a channel with a width of $3\ mm$ and a length of $\pm 3\ cm$ is approximately equal to the culture surface of one well in a 48 well-plate ($1\ cm^2$). This would mean that it is more easy to compare static cultures in a 48 well plate with dynamic cultures inside the microfluidic chip. Therefore we choose to develop a channel with $w_0 = 3\ mm$ and $h_0 = 100\ \mu m$ under a fluidic flow of $20\ \mu l/min$, which would induce a shear stress of approximately $\tau_w = 0.3$ dyn$/cm^2$.

3.5.3 Fabrication

The fabrication process is shown in figure 3.10. By designing, fabricating, testing and solving problems, a final design of the chip was made (Figure 3.9 C). One important difference between the initial designs and the final concept is the placement of the inlets. During testing, medium preferred to flow underneath the waver instead of through the channel. By changing the place of the inlets on the waver this problem was easily overcome.

3.5.4 Validation

The chip was tested outside the flowhood to check for leakage, several different assemblies were made to prevent leaking, in the end it was decided to use cyanoacrylate (biocompatible) glue around the inlet and outlet luer plugs of the channels. This ensured that the chip stayed leak free after 2 days of continuous flow up to $50\ \mu l/min$. In figure 3.10 B an example of two different luer plugs that were glued on the top

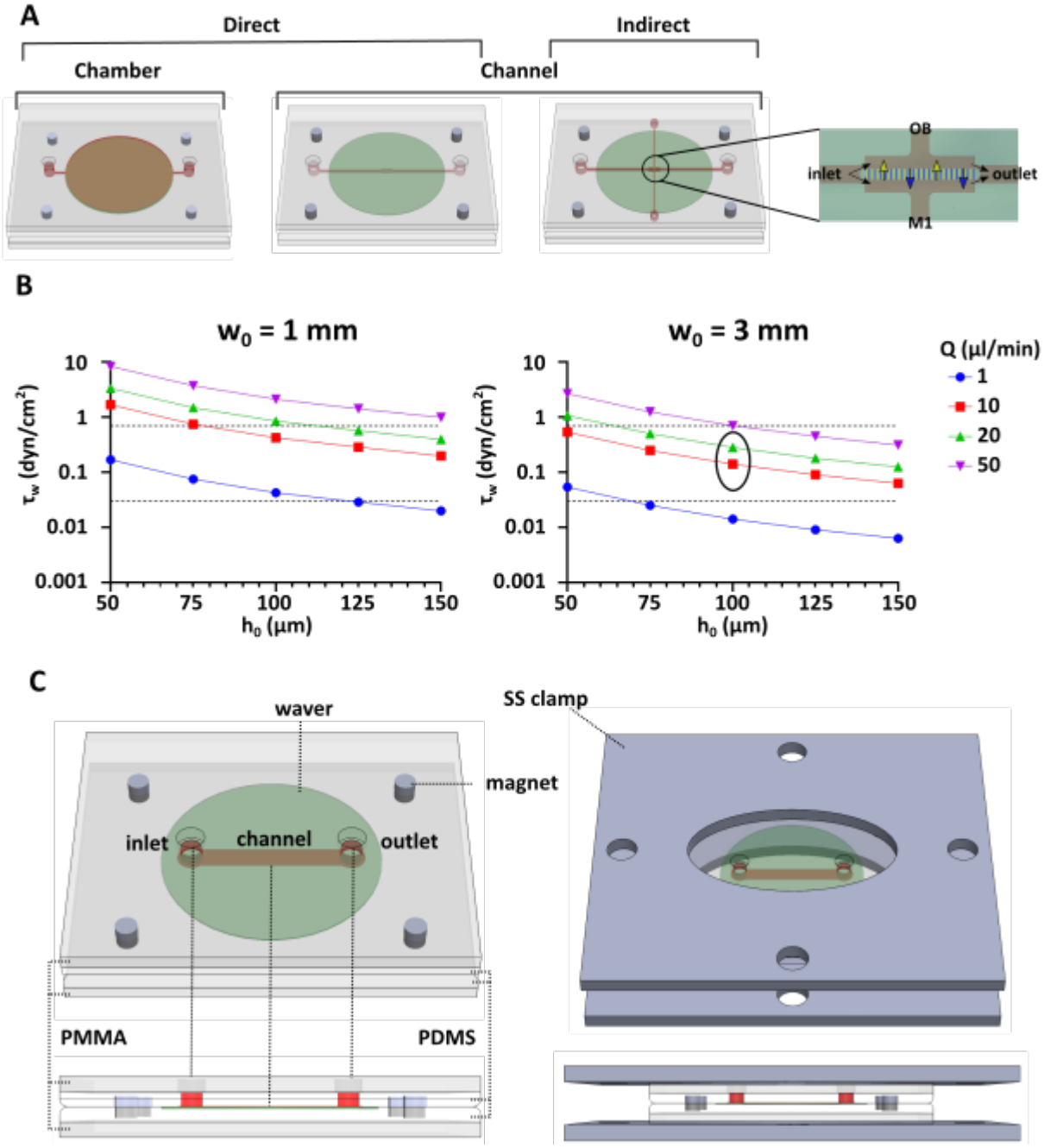

Figure 3.9: **A)** Initial microfluidic chip designs **B)** The resulting mean Wall shear stress, τ_w at different flow rates, Q, in $w_0 = 1\ mm$ and $w_0 = 3\ mm$ rectangular channels with different heights, h_0. **C)** Final design without and with clamp

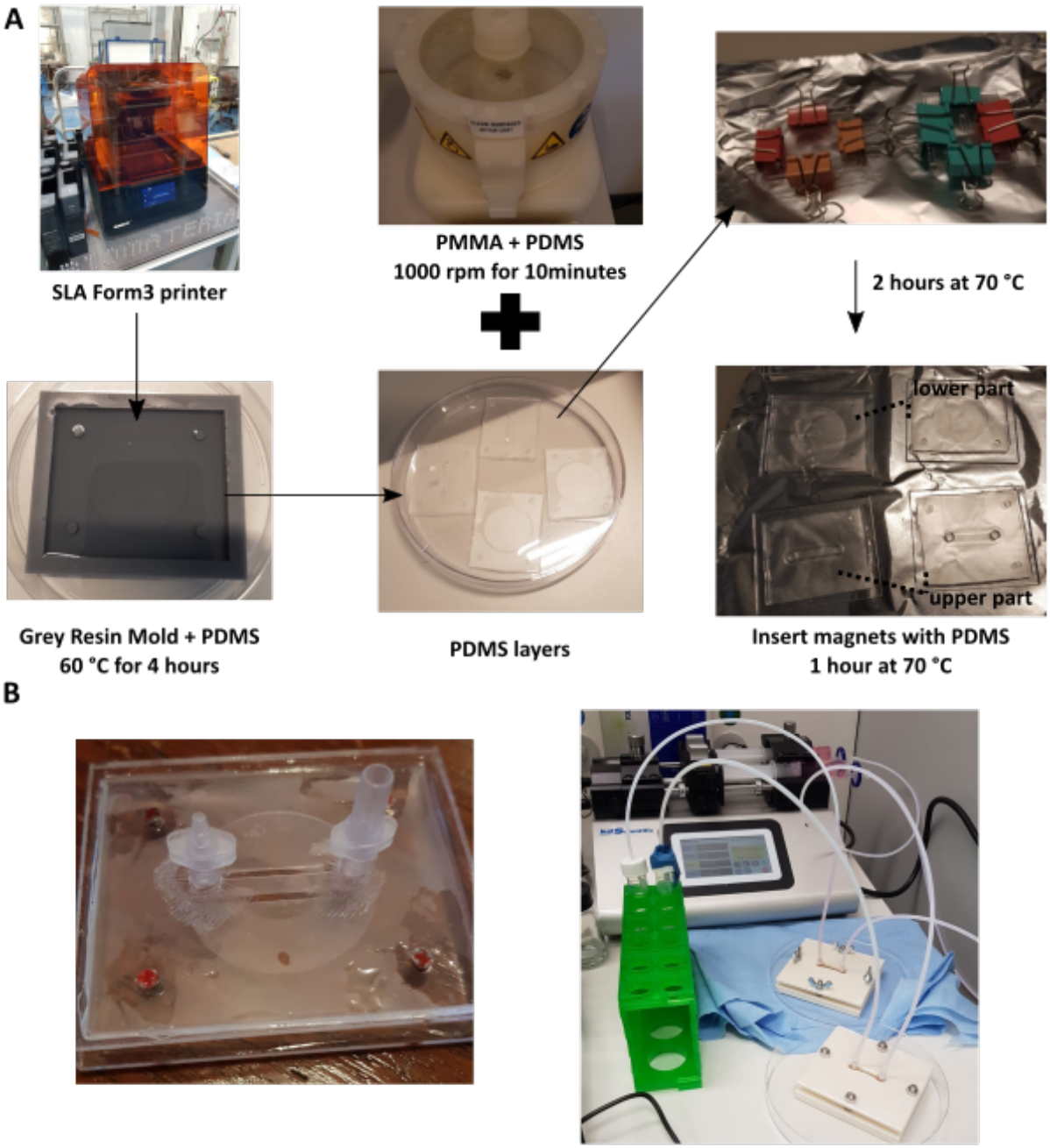

Figure 3.10: **A)** Microfluidic chip fabrication steps **B)** (left) Final chip design with two different luer connectors. (right) Two microfluidic chips attached to the Legato 270 syringe pump

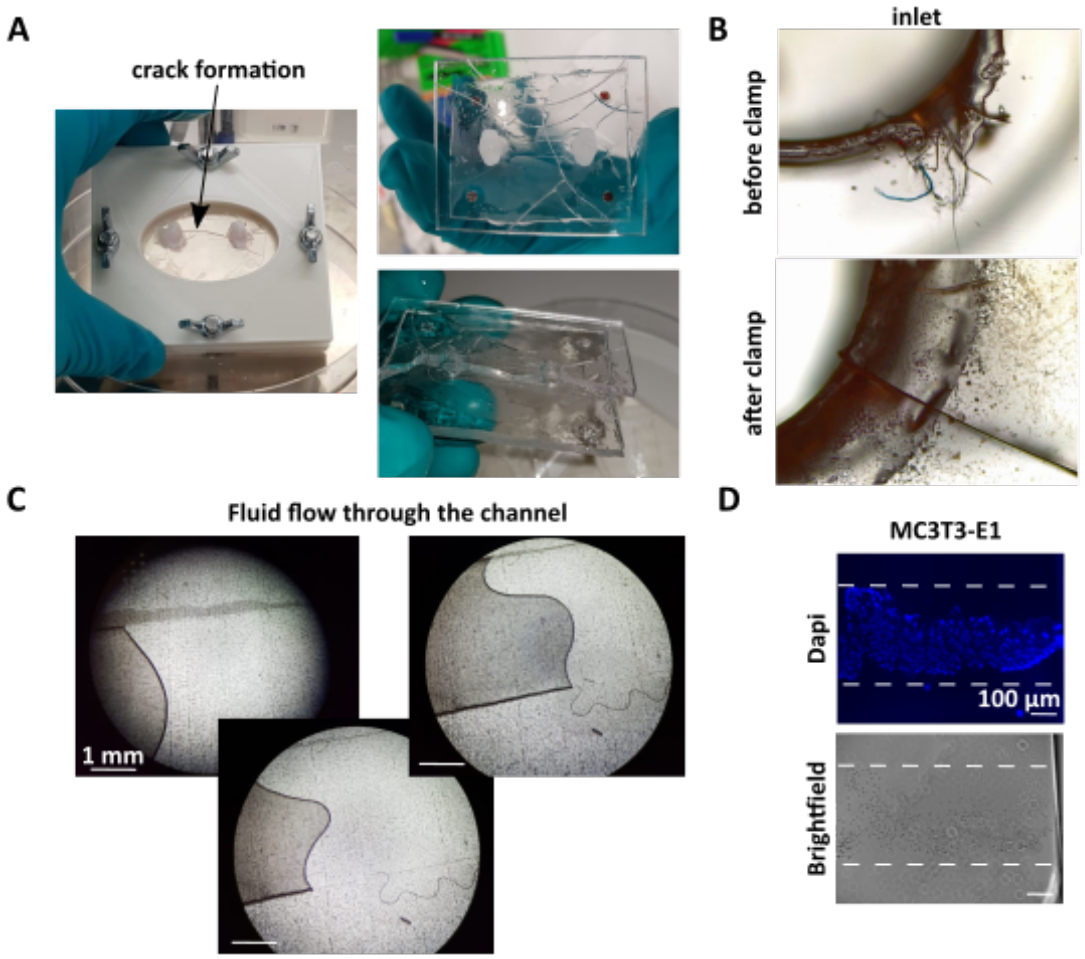

Figure 3.11: **A)** Crack formation around the inlets of the dynamic chip. **B)** Closer look at the crack formation before and after usage of a clamp, images were taken with the LEICA DM 590. **C)** Fluid flow through the channel inside the chip images were taken with the LEICA DM 590. **D)** Dapi staining of the waver, after MC3T3-E1 cell seeding inside the dynamic chip.

PMMA layer of the chip is shown.

Secondly the effect of UV sterilization and PBS rinsing on the integrity of the chip was tested. Unfortunately, from this stage, 3 out of 5 of the developed chips started to form cracks by the time they were inserted in the clamp (Figure 3.11 A). The cracks originated from the inlets. The Leica DM 500 microscope was assessed to look closer around the lasercut inlets. Images taken from freshly lasercut PMMA slides already had small cracks around the inlets (Figure 3.11 B before clamp). After fitting the waver inside the chip and the clamp, more cracks were formed, originating from the earlier small cracks. We did manage to seed some cells inside the channels, using DAPI, it can be seen that cells were able to adhere and grow on the waver inside the channel (Figure 3.11 D).

In this section, I have attempted to design and fabricate a lab-on-a-chip device that would give us the ability to see the effect of a dynamic microflow on a co-culture seeded on top of the nanopattern. Finally a design was made consisting out of a channel with $w_0 = 3\ mm$ and $h_0 = 100\ \mu m$, which would be able to induce induce a shear stress of approximately $\tau_w = 0.3$ dyn$/cm^2$, under a fluidic flow of 20 $\mu l/min$. In the end, the induced force of the clamp used to ensure no leakage, resulted in cracks throughout the

entire chip, which originated from the inlets. This ultimately lead to the failure of the lab-on-a-chips.

CHAPTER 4

Discussion

In this thesis we attempted to establish an *in vitro* osteoimmunological co-culture system for the evaluation of a prototype nanopattern. First of all, the morphological, inflammatory and osteogenic differences between static mono- and co-culture of OBs and M1s were investigated. Thereafter, the influence of CO12 co-culture on the pattern was assessed. Thirdly, the effect of a dynamic microflow and the resulting shear stress was looked upon in terms of osteogenic differentiation in both mono- and co-cultures. Finally, the design, fabrication and validation of a microfluidic device was presented that could have the potential to investigate the effect of a co-culture on a nanopattern during a continuous dynamic flow, and thus include both dynamic and co-culture conditions on the investigation of a nanopattern.

4.1 Static co-culture models and intercellular crosstalk

The morphological differences between M0, M1, and M2s after stimulations are comparable to other studies from different species [46, 33, 47]. The M2 elongated morphology is somewhat less present than the typical M1 shapes. In addition, the high amount of NO, PGE2 and TNF-α for M1, verify the pro-inflammatory polarization state of the M1s after stimulation [48, 49, 50, 15, 44]. Finally, the significantly higher amounts of IL-10 correspond to a anti-inflammatory phenotype for M2s after stimulation [15].

Choices in co-culture models also include the important choice between direct or in-direct co-culturing. Direct cell-cell contact could result in different outcomes compared to in-direct studies [24]. A study by Cordova et al. showed that in-direct transwell co-culturing of MC3T3-E1 and donor murine MΦs resulted in a reduced effect of overall bone formation in the transwell setting, compared to direct co-culturing. They suggested that this was likely caused by the important role of adhesion molecules and the need for intercellular contact. This is the reason we also choose for a direct co-culture system. Another advantage of a direct co-culture system is that the design and fabrication of a prototype microfluidic chip would be more easy, since there is no need for a microporous barrier that would divide the cells. Such a barrier also increases the chance to damaging the fragile nanopatterns.

With the validation of the stimulation protocol we started to optimize static direct co-culture conditions, beginning with the co-culture medium. Several culture-media used for the direct co-culture of MΦs and OBs where found in literature (Table D.1). It can be seen that every study uses a different medium composition. No medium is perfect, and it differs strongly between laboratories and studies

which medium is being used. We decided to use a mixed medium made out of 1:1 ratio mix OB and MΦ medium. The Live/dead staining results showed no significant decrease in cell viability over time, therefore we decided that our mixed medium was suitable for this co-culture study.

The TNF-α peak on day 7 for both co-cultures is significantly higher than M1 mono-culture and the control. The amount of secreted TNF-α for static co-cultures is almost similar to the amount three days after M1 stimulation. This would indicate that an inflammatory reaction is taking place on day 7 [15]. This pro-inflammatory response is also visible in the PGE2 secretion rates. As mentioned before, PGE2 secretion by OBs is proportional to TNF-α [44]. At high concentrations, i.e. the initial inflammatory response, PGE2 stimulates OC activity, and enhances the cleaning of the injury site [51]. In contrast, unlike the low amounts of TNF-α on day 1, PGE2 secretion is very high on the first day compared to TNF-α. This is most likely caused by the harvesting procedure of OBs using trypsin, since this has been shown to be a stimulator of PG synthesis [52].

The trend in a rise of anti-inflammatory marker IL-10 seems to indicate a polarization shift of M1-M2 MΦs [10, 13]. M2s are critical for the initiation of bone healing and are responsible for the secretion of cytokines, such as IL-10 [14]. However, both pro and anti-inflammatory cytokines rise from day 1 to day 7. On top of that, IL-10 values are not significantly higher than the control. So this does not indicate a polarization shift at all. It could also mean that more cells, especially macrophages, have been growing in the well, which in turn produce more cytokines over time.

Malheiro et al. showed that the harvesting of macrophages could have a negative effect on the phenotype, polarization state and cell viability directly after harvesting. Furthermore, they showed that without additional stimulation factors such as LPS, IFN-γ and IL-4 MΦs tend to fall back into their dormant M0 phenotype over time [15, 53]. This could also explain the decrease of M1 phenotype towards a mixture of M0, M1, and M2 morphologies over time that were observed in the brightfield images. [15] showed that M1:MC3T3-E1 co-culture enhanced osteogenic ability when IL-4 was administered 72 hours after seeding, this was to allow for a M1-M2 transition and mimic the in-vivo inflammatory response. In this study no additional IL-4 was added to the co-cultures, this could have lead to an increased and long inflammatory response with a new peak of inflammation on day 7.

Unlike other studies, the use of a direct co-culture model did not result in a decrease of the inflammatory response and an enhancement of MC3T3s osteogenic activities [23]. RUNX2, ALP and ARS expression were all lower with respect to OB monoculture. This could also be seen in the PGE2 secretion rates of OBs compared to the co-cultures. PGE2 secretion was chosen to be measured because this would give more insight into the cellular crosstalk of OBs and MΦs. PGE2 secretion by MSCs promotes MΦ polarization towards M2 [54]. By knocking down cyclooxygenase-2 (COX2), the enzyme responsible for the production of prostaglandins, TNF-α secretion by LPS stimulated monocytes was significantly reduced, indicating that PGE2 has a a noticeable effect on TNF-α secretion [55]. This PGE2/TNF-α relationship has even been found to be proportional between MG-63 OBs (H) and U937 MΦs (H) [44]. The positive or negative effect on osteogenesis of PGE2 is directly related to the TNF-α concentration present. At high concentrations, i.e. the initial inflammatory response, PGE2 stimulates OC activity, and

enhances the cleaning of the injury site. At low concentrations, i.e. the anti-inflammatory response, PGE2 has a positive effect on OB proliferation [51]. OB mono-culture have lower PGE2 secretion rates than the co-cultures. This would indicate that osteogenic activity is attenuated for both co-cultures. This was also seen for all osteogenic markers.

4.2 Effect of nanopatterns on co-culture

Sadowska et al. [56] cultured OBs on the nano surface and submerged them with conditioned medium from MΦ, which were also cultured on the nano surface. These OBs differentiated more early than OBs that were cultured in normal wells. This effect was only limited, however it shows that OBs are influenced by the nanopatterns as well. Since OBs are also present on the implant surface *in vivo*, it is more than logic that they are also influenced by the biomaterial, just like MΦs. Therefore, for both OBs as for MΦs the cell-surface interaction should not be denied during co-culture and a method that includes the cell-surface and cell-cell interaction for all the cells has the potential to mimic the *in vivo* environment in more detail. In our direct co-culture we tested the effect of the nanopattern on both OB mono-culture as CO12 co-culture. Only for the co-culture a rising trend in Runx2 expression was found. This suggests that this altered expression is caused by the polarisation effect of the nanopatterns on M1s. Our nanopattern has the potential to induce M1-M2 polarisation and thus its presence will result in as anti-inflammatory environment. As expected, the co-culture on the nanopattern show a trend in early osteogenic activity, most likely caused by the anti-inflammatory M2 MΦs. However, the osteogenic response of the co-culture was not greater than for the mono-culture.

The effect of a nanopattern that likely induces M1-M2 polarization over time on osteogenic activity remains complex. It would seem more than logic that a nanopattern that inhibits the pro-inflammatory response and promotes a tissue regenerative environment would result in better osteogenic differentiation over time. Chen et al. performed a similar research towards nanopatterns that inhibited the pro-inflammatory response, and promoted an anti-inflammatory M2 phenotype. They suggested that this anti-inflammatory environment would be beneficial for osteogenesis, in contrast, they found that those patterns did not increase the formation of mineralization nodules (ARS). The patterns that did not inhibit an inflammatory response were in fact the patterns that promoted osteogenic tissue regeneration. They suggested that this could be related to IL-6 expression during the initial inflammatory stage, and the importance of the cross-talk between MΦs and MSCs for the bone formation. The importance of this initial inflammatory response was also stated by Loi et al. [15]. To fully apprehend the effect of our nanopattern on both OBs and MΦs, additional research towards the inflammatory response during co-culture on the nanopattern has to be done. Furthermore, multiple different patterns, including the ones that do not promote M1-M2 polarization should be investigated during co-culture.

The effect of the patterns on pro- and anti-inflammatory cytokines could not be investigated using the ELISA kits, since the size of the pattern is negligible to the effects of cell behavior inside a 6 well plate. The osteoimmunological response could therefore only be measured locally using staining. CCR7/CD206 (M1/M2) fluorescent staining was used in a preliminary study to look upon MΦ polarization in co-cultures (appendix E.6). No significant differences between M0, M1 and M2s were found. Therefore, we decided

that the investigation of the cytokines and resulting osteogenic behavior of the static co-cultures could be connected to the outcomes of solely Runx2 staining and BF imaging of the patterns. The usage of our proposed lab-on-a-chip device, could enable ELISA measurements of the cells on the nanopatterns, because the nanopattern will cover more of the cell growth surface within the channel. There even exists the ability to cover only the nanopattern with a microchannel, by changing the inlet and outlet positions or the size of the channels.

4.3 Shear stress induces osteogenic activity

For all conditions, the highest amount of cells were present near the inlets, especially for the static cultures. Most likely because here fresh medium was present. For the dynamic culture an continuous source of nutrients is available for the cells throughout the entire channel, for the static cultures however, only near the inlets, enough fresh medium is available for the cells. Another possibility is that air bubbles were trapped within the system, this is a common problem for these systems, and it is difficult to prevent bubbles from entering the system [57]. The induced shear stress inside the channels had a positive effect on osteogenic activity of MC3T3-E1 monoculture inside the channel. Runx2 expression was significantly higher and PGE2 significantly lower than the static cultures and both co-cultures. This clearly shows the relationship between low PGE2 rates and enhanced osteogenic activity [51]. This enhanced Runx2 expression by the addition of a shear stress on MC3T3-E1s was expected [58, 59, 60]. The relative Runx2 expression of sCO12 was higher than for the CO12 co-culture study. One major difference between these two studies, was the total amount of cells measured within the channels, which was limited, compared to the cells present in a 24 well plate. It is not possible to compare these to studies, since the cell numbers were not similar. The measurement of more channels and thus more cells could give a better insight in the relative Runx2 expression.

After 7 days of culture, the midsections of the dCO12 channels were almost empty, and cells grew mainly at the side of the channels. After washing the dynamic channels and imaging them prior to fixation. It was seen that MΦs, present in the inlets, detached and floated through the channels at a high speed. They bumped against cells present in the channel, which resulted in some cells detaching as well. It is possible that these loose MΦs are responsible for "cleaning" the whole channel, which resulted in a little amount of cells in the middle of the channels. It is also possible that the shear stress on MΦs was to high and lead to loosening of the cells. Not many studies have focused their attention on the effect of shear stress on MΦ adhesion and thus this remains unclear. Most osteoimmunological studies involving shear stress are focused on OB differentiation. Unstimulated and stimulated human THP-1 monocytes have been found to secrete more pro-inflammatory cytokines under influence of mechanical loading after 3 days [61]. This could result in an extended inflammatory response and have a negative effect on tissue regeneration, since less MΦs will polarize towards a M2 phenotype. However, the exact effect of shear stress on M1-M2 polarisation remains unclear. Additional research towards the effect of shear stress on pre-stimulated MΦs and the resulting polarization state after more than three days, will give us more insight.

In this study, I looked at the effect of shear stress on OBs and M1s inside a rectangular channel. There are several advantages of such a system. The flow can be controlled very precisely, and since the flow pattern inside the channel will be laminar, most of the cells inside the channel will be exposed to a similar shear stress [60]. A disadvantage would be that within perfusion pump models, the pressure inside the system can become extremely high around the inlets, which leads to leakage [57]. In addition, not only are the cells exposed to wall shear stress, but also to this pressure build-up inside the system. One possible solution, would be to develop a closed loop system, in which the medium will be pushed and pulled through the channel simultaneously. Such a closed loop system decreases the pressure build-up within the system [62]. Another advantage of such a system, in which a central culture medium reservoir is needed, is that medium can be easily refreshed [40]. On top of that, medium will be circulating through the system and thus cytokines that are secreted by the cells will also circulate through the system, which allows for better paracrine signalling interaction [21]. In our current model, the crosstalk caused by cytokines is minimal, since there is a continuous flow of fresh medium present inside the channel. The need of a specialised microfluidic chip is one major disadvantage of fluidic pump systems, another solution for the introduction of shear stress within culture models, is by rocking a multiple well plate during culture. Multiple conditions could be tested simultaneously, and there is no need for a special developed microfluidic system [60]. However, not every cell will be exposed by the same shear stress within the wells, this depends on the place in the well and can range from 0.01 to 2.5 dyne$/$cm^2 [60].

4.4 Design and fabrication of microfluidic chip

In this thesis I have introduced a microfluidic chip design that could introduce a shear stress on top of a nanopattern. With the use of this dynamic co-culture system the effect of the pattern on cellular behavior could increase the osteogenic response. On the one hand, because the pattern will cover most of the area within the microchannel, and thus all cells present will have a direct interaction with the surface. On the other hand, the *in-vivo* microenvironment could be simulated in more detail, by the addition of microflows. The proposed design consists out of one channel with a cell surface of approximately 1 cm^2, this way the dynamic cultures could be compared to static co-cultures inside 48 well plate. One of the major difficulties at the beginning was the lack in polymerization of PDMS inside the moulds made out of grey resin [63, 64]. By optimization of the post-processing of the mould, i.e. an additional overnight baking period and clean IPA wash, the clearness of the PDMS layer was improved. However, there is still room for improvement. The use of different resin for the SLA printer, or different moulding techniques, such as laser etching, could improve the clearity of the PDMS layer. In the end, three out of five chips developed cracks, immediately after the clamp was introduced to the system. It was believed that the lasercutter was used at a too high power output, which caused the cracks around the inlets, and eventually the breaking of the top PMMA layer. Only the first two fabricated chips remained intact, and after culturing OBs inside these channels, I found that OBs only grew inside the channel, and remained viable after 2 days of culture. This proofs that the concept of my chip has the potential to work. However different approaches towards the fabrication of the top layer has to be found, such as soft lithography or more precise CO_2 laser etching, before this lab-on-a-chip is suitable for experiments [44, 65]. This lab-on-a-chip will have the potential to investigate a biomaterial under physiological relevant conditions.

4.5 Recommendations for future research

Manual stimulation of the M1-M2 polarization after three days by the administering of IL-4 in the wells, could enhance the *in-vitro* co-culture inflammatory response profile. This way, we can better compare the patterns polarization abilities results to a IL-4 induced polarization.

More research should be done towards in-direct co-culture models. An in-direct co-culture system has the advantage that the cells can be stained and quantified separately from each other. One disadvantage would be the lack of direct cell-cell contact and the choice of culturing only one cell type on the biomaterial surface.

Instead of using MC3T3-E1 pre-osteoblasts, MSC-MΦ co-culture is also interesting to look upon. MSCs are mainly the first cells present after bone injury, and could give a better insight on what really happens in the bone-niche after implant surgery. In addition, MSCs have better regenerative abilities, and improve the polarization of MΦs towards an M2 phenotype.

Different medium combinations could be tested in more detail. For instance, only DMEM, RPMI 1640 or a combination of those, this could improve the osteogenic activity as well.

More emphasis should be put on Macrophage specific biomarkers, and especially on the quantification of those markers. Instead of solely using fluorescent imaging, flow cytometry could give better results with respect to the quantification of M1/M2 phenotypes.

The use of a perfusion pump instead of a syringe pump with a small container of medium that will be pumped through the system could have the potential to improve our dynamic culture system. A central reservoir of culture medium not only provides for a system that is less sensitive to contamination. But also could enable ELISA measurements at different time points, whenever the central reservoir has to be refreshed. Such a system will also reduce the amount of pressure near the inlets of the system.

Additional Runx2 staining of mono- and co-cultures inside the microfluidic chip on more days, could give more insight in the proliferation and differentiation of the cells inside the channels over time.

The effect of shear stress on J774A.1 Macrophages should be evaluated in more detail. There is not much research towards the effect of different flow rates on MΦ behavior. One possibility to quickly assess this effect is by using a rocking plate, to introduce shear stress on MΦs inside a multiple well plate.

Sandwiching a chip with a diameter of $31mm$ and not introducing cracks or leaks in the system proved to be very hard. Other designs using CO_2 laser engraved microchannels for instance, or PDMS-glass layers can enhance the chip. Additional different mould materials, such as PMMA, or other resins could improve the PDMS polymerization.

CHAPTER 5

Conclusion

Co-culture models within a microfluidic system possess the ability to improve *in vitro* research of biomaterials. The presence of microflows and multiple cell types are able to mimic *in vivo* behavior more closely. In this study, we have established to not only do research on the influence of nanopatterns and shear stress on M1 stimulated J774A.1 MΦ and MC3T3-E1 co-cultures, but also to provide a proof of concept of a lab-on-a-chip model suitable for the evaluation and validation of a biomaterial during and after the initial inflammatory response.

We established to determine the inflammatory and osteogenic behaviour inside a static co-culture system with respect to mono-cultures. Unfortunately, our co-culture model did not have an increased osteogenic response (Runx2, ALP, ARS). On top of that, the pro-inflammatory response of the co-culture was increasing from days 1 to 7 (TNF-α, PGE2). This indicates that the macrophages did not polarize towards a M2 anti-inflammatory phenotype after three days and that the presence of M1s inhibited OB activity. The presence of a nanopattern showed a trend in osteogenic activity (Runx2) for the co-culture only. This supports our believe that this prototype nanopattern induces M1-M2 polarization, and therefore provides for a more favourable environment for OBs. To our knowledge, this is the first time that the influence of shear stress on this direct co-culture model has been investigated. Low shear stresses ($\pm$ 0.5 dyn/cm^2) applied on OB mono-culture for 5 days resulted in an significant increase in Runx2 and decrease in PGE2 expression. Dynamic co-culture resulted in no significant differences during 7 days of culturing. The combination between a co-culture model that had a negative influence on osteogenic behavior with the shear stress resulted in morbidity inside the channels. Research towards the effect of shear stress on M1 or MΦs in general could give more insight about the source of this behavior. The research towards bone-on-a-chip devices that imitate the inflammatory response is limited. We have provided a prototype lab-on-a-chip model suitable for the evaluation and validation of a biomaterial during the FBR. Our findings in the design and fabrication of the chip can help in future research towards the osteoimmunodulatory properties of biomaterials.

There is still much unknown in osteoimmunological research, the intercellular interactions between different cell types, and their response to their environment is difficult to predict. However, in knowing and steering the response of cells lies the key to the development of better implants. The introduction of shear stress to a co-culture model is only the first step in this process and in time we will reach the pinnacle of osteoimmunological models.

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APPENDIX **A**

Protocols cell pre-culturing

A.1 J774A.1 Cell culture media

1. Requirements

1.1 Equipment

- 50ml tubes
- 5ml plastic pipettes
- 25ml plastic pipettes
- Suction system
- Water bath at 37 ^oC

1.2 Reagents

- DMEM (Thermo Fisher #11594446)
- Penicillin-Streptomycin (Thermo Fisher #15140122)
- Fetal Bovine Serum one shot (Thermo Fisher # A3160802)

2. Procedure

2.1 Protocol

1. Thaw 5ml Pen-strep and 50ml FBS
2. Withdraw and dispose 55 ml DMEM
3. Add 5 ml Pen-strep to the DMEM bottle(445ml) using a 5ml plastic pipette
4. Add 50 ml FBS to the DMEM bottle using a 25ml plastic pipette
5. Write your name, date and content on the medium bottle. Store at 4 ^oC

A.2 J774A.1 pre-culture

1. Requirements

1.1 Equipment

- 0.5 ml tubes
- 50ml tubes
- P20 pipette + filter tips

- P1000 pipette + filter tips
- 2ml plastic pipettes
- 5ml plastic pipettes
- 10ml plastic pipettes
- Suction system
- Water bath at 37 ^oC
- 50ml tube centrifuge (sigma centrifuge)

1.2 Reagents

- Complete culture medium: DMEM

1.3 Cells

- 1 vial with frozen J774A.1 cells

2. Procedure

2.1 Protocol

1. Fill 1 50ml tube with 10ml of culture medium.
2. Take 1 vial with cells from the -80.
3. Directly put the vial in the water bath, watch the vial closely and remove as soon as the last sliver of ice melts. **Hold the vial during this step, do not put it completely under water.**
4. Wipe the vial with ethanol before placing it in the biosafety cabinet.
5. Pipet the cells with the P1000 pipette into the 50 ml tube. Rinse the vial 2 times with 1 ml medium and add to the same 50 ml tube.
6. Adjust the volume of medium to 25ml.
7. Spin down for 5 minutes at 200 x g.
8. Carefully remove the supernatant by using the suction system.
9. Resuspend the cells in 1ml medium by pipetting up and down until no clumps of cells are visible anymore.
10. Transfer 2 samples of $10\mu l$ each into a 0.5ml tube to count the amount of cells
11. Seed 7×10^5 viable cells per 75 cm2 flask in 10 ml medium.
12. Distribute cells evenly by moving the flask quickly according to a cross-like pattern.
13. Transfer the flask into a CO2 incubator (37 ^oC, 5% CO2).
14. Replace the medium when needed:
    Remove 10ml medium from flask carefully by using the suction system.
    Add 10ml pre warmed medium.
15. Keep the cells in pre-culture for 3-4 days until confluence is reached.

A.3 J774A.1 Stimulation

1. Requirements

1.1 Equipment

- 50ml tubes

- P20 pipette + filter tips
- P200 pipette + filter tips
- 10ml plastic pipettes
- Suction system
- Water bath at 37 ^oC
- 50ml tube centrifuge (sigma centrifuge)

1.2 Reagents
- Complete culture medium: DMEM
- IL- 4 (10 ug/ml)
- LPS (100 ug/ml)
- INF-γ (10 ug/ml)

1.3 Cells
- (3x) 75 cm2 flasks with confluence J774A.1 cells

2. Procedure

2.1 Protocol

1. Take IL-4, LPS and INF-γ from the fridge and place the vials in the waterbath
2. Fill 3 50ml tubes with 10ml of pre-warmed culture medium.
3. Mark the tubes M0, M1 and M2
4. Wipe the thawed vials with ethanol before placing it in the biosafety cabinet.
5. Prepare M1 stimulation medium by adding 10 μl INF-γ (10 ug/ml) and 10 μl LPS (100 ug/ml) to the M1 50 ml tube (dilution 1:1000)
6. Prepare M2 stimulation medium by adding 10 μl IL-4 (10 ug/ml) to the M2 50 ml tube (dilution 1:1000)
7. Retrieve cells (75cm2 flasks) from the incubator and carefully remove the supernatant by using the suction system.
8. Replace the medium of the flasks by 10ml M0, M1 and M2 prepared stimulation media
9. Write the stimulation factors on the flask and transfer the flasks into a CO2 incubator (37 ^oC, 5% CO2).
10. Transfer the flask into a CO2 incubator (37 ^oC, 5% CO2).
11. Keep the cells in stimulation medium for 3 days.

A.4 MC3T3-E1 Cell culture media

1. Requirements

1.1 Equipment
- 50ml tubes
- 5ml plastic pipettes
- 25ml plastic pipettes
- Suction system

- Water bath at 37 ^oC

1.2 Reagents
- αMEM without ascorbic acid (Thermo Fisher #A1049001)
- Penicillin-Streptomycin (Thermo Fisher #15140122)
- Fetal Bovine Serum one shot (Thermo Fisher # A3160802)

2. Procedure
2.1 Protocol

1. Thaw 5ml Pen-strep and 50ml FBS
2. Withdraw and dispose 55 ml αMEM
3. Add 5 ml Pen-strep to the αMEM bottle(445ml) using a 5ml plastic pipette
4. Add 50 ml FBS to the αMEM bottle using a 25ml plastic pipette
5. Write your name, date and content on the medium bottle. Store at $4\ ^oC$.

A.5 MC3T3-E1 pre-culture

1. Requirements
1.1 Equipment
- 0.5 ml tubes
- 50ml tubes
- P20 pipette + filter tips
- P1000 pipette + filter tips
- 2ml plastic pipettes
- 5ml plastic pipettes
- 10ml plastic pipettes
- Suction system
- Water bath at 37 ^oC
- 50ml tube centrifuge (sigma centrifuge)

1.2 Reagents
- Complete culture medium:αMEM without ascorbic acid

1.3 Cells
- 1 vial with frozen MC3T3-E1 cells

2. Procedure
2.1 Protocol

1. Fill 1 50ml tube with 10ml of culture medium.
2. Take 1 vial with cells from the -80.
3. Directly put the vial in the water bath, watch the vial closely and remove as soon as the last sliver of ice melts. **Hold the vial during this step, do not put it completely under water.**

4. Wipe the vial with ethanol before placing it in the biosafety cabinet.
5. Pipet the cells with the P1000 pipette into the 50 ml tube. Rinse the vial 2 times with 1 ml medium and add to the same 50 ml tube.
6. Adjust the volume of medium to 25ml.
7. Spin down for 5 minutes at 200 x g.
8. Carefully remove the supernatant by using the suction system.
9. Resuspend the cells in 1ml medium by pipetting up and down until no clumps of cells are visible anymore.
10. Transfer 2 samples of $10\mu l$ each into a 0.5ml tube to count the amount of cells
11. Seed 5×10^5 viable cells per 75 cm2 flask in 10 ml medium.
12. Distribute cells evenly by moving the flask quickly according to a cross-like pattern.
13. Transfer the flask into a CO2 incubator (37 ^oC, 5% CO2).
14. Replace the medium every 2/3 days:
    Remove 10ml medium from flask carefully by using the suction system.
    Add 10ml pre warmed medium.
15. Keep the cells in pre-culture for 7 days until confluence is reached.

A.6 Mixed medium

1. Requirements

1.1 Equipment

- 50ml tubes
- 25ml plastic pipettes
- Water bath at 37 ^oC

1.2 Reagents

- 50 ml tubes with complete culture medium: αMEM without ascorbic acid & DMEM

2. Procedure

2.1 Protocol

1. Resuspend 25 ml αMEM in a fresh 50 ml tube.
2. Add 25 ml DMEM to the two tubes containing 25 ml αMEM
3. Write your name, date and content on the medium bottle. Store at $4\ ^oC$

A.7 Osteogenic Mixed medium

1. Requirements

1.1 Equipment

- 50ml tubes
- 25ml plastic pipettes
- Water bath at 37 ^oC

1.2 Reagents

- 50 ml tubes with mixed medium: αMEM without ascorbic acid: DMEM
- $50\ \mu g/ml$ A.A. (Sigma-Aldrich; # A4403-100MG)
- $4\ mM\ \beta$-GL (Sigma-Aldrich; # G9422-50G)

2. Procedure

2.1 Protocol

1. Add $50\ \mu g/ml$ A.A. (Sigma-Aldrich, Germany) and $4\ mM\ \beta$-GL (Sigma-Aldrich, Germany) to the mixed medium.
2. Write your name, date and content on the medium bottle. Store at $4\ ^oC$

APPENDIX **B**

Protocols cell experiments

B.1 Static Co-culture experiment

Summary
This protocol describes the harvesting and seeding of mono and co-cultures of J774A.1 macrophages (M0, M1, and M2 stimulated) and MC3T3-E1 preosteoblasts (OB).

1. Requirements

1.1 Equipment

- 1.5 ml tubes
- 15ml tubes
- 50ml tubes
- P20 pipette + filter tips
- P1000 pipette + filter tips
- 2ml plastic pipettes
- 10ml plastic pipettes
- Suction system
- Water bath at 37 ^oC
- 50ml tube centrifuge (sigma centrifuge)
- Cell scraper

1.2 Reagents

- Preheated Complete culture medium: αMEM without ascorbic acid
- Preheated Complete culture medium: DMEM
- Preheated Mixed Medium
- Preheated 1x Trypsin-EDTA (Thermo fisher # 10779413)
- 1x DPBS (Thermo Fisher #14200083)
- 50 $\mu g/ml$ A.A. (Sigma-Aldrich; # A4403-100MG)
- $4\ mM$ β-GL (Sigma-Aldrich; # G9422-50G)

1.3 Cells

- Pre cultured T75 flask with M0, M1, & M2 J774A.1
- Pre cultured T75 flask with MC3T3-E1 cells

2. Procedure

2.1 Protocol

1. Insert coverslips, using tweezers, into 48 well plate, 4x4
2. Sterilize the coverslips by adding 200 ul 70% ethanol, followed by a 1x DPBS wash twice.
3. Harvest M0, M1 and M2s
    a) Retrieve T75 flask containing M0s from the incubator
    b) Remove 10 ml of medium from the flask by using the suction system.
    c) Use a cell scraper to detach the cells from the bottom of the flask
    d) Pipet the cell suspension into a 50ml tube
    e) Add 10 ml preheated medium to the flask, move the flask gently and the medium to the 50ml Tube.
    f) Adjust the volume to 25ml with medium.
    g) Centrifuge for 5 minutes at 200 x g.
    h) Carefully remove the supernatant by using the suction system.
    i) Resuspend the cell pellet in 1ml preheated mixed medium by pipetting up and down until no clumps of cells are visible anymore.
    j) Transfer 2 samples of 10µl each into a 1.5ml tube to count the amount of cells.
    k) Add an appropriate amount of mixed medium for a 1×10^6cells/ml solution
    l) Repeat this procedure from step b, for M1s and M2s.
4. Harvest MC3T3-E1 cells
    a) Retrieve T75 flask containing MC3T3-E1 from the incubator
    b) Remove the medium from the flask carefully by using the suction system.
    c) Wash the cells twice with 10 ml PBS.
    Make sure to not add the solution directly onto the cell layer.
    d) Remove the PBS from the flask by using the suction system.
    e) Add 1ml preheated trypsin dropwise on top of the cell layer. Move the flask to distribute the drops over the complete cell layer. Put the flask into the incubator.
    f) Check after 5 minutes under the microscope whether the cells have already detached from the bottom of the flask (the cells should be round shaped by this time).
    g) Add 10 ml preheated medium to the flask, move the flask gently and transfer the cell suspension to a clean 50ml tube. Repeat this with another 10 ml preheated medium and add to the same tube.
    h) Adjust the volume to 25ml with medium.
    i) Centrifuge for 5 minutes at 200 x g.
    j) Carefully remove the supernatant by using the suction system.
    k) Resuspend the cell pellet in 1ml preheated medium by pipetting up and down until no clumps of cells are visible anymore.
    l) Transfer 2 samples of 10µl each into a 0.5ml tube to count the amount of cells.
    m) Add an appropriate amount of mixed medium for a 1×10^6cells/ml solution
5. Mark 6 tubes with M0, M1, M2, OB, CO11, and CO12

Table B.1: Exprimental conditions and amount of cells seeded per well for the static co-culture experiment

EC	# cells /well	1×10^6 cells/ml	mixed medium	final conc. cells/well
M0	5.000	N x 5 ul	N x 195 μl	5.000/well (200ul)
M1	5.000	N x 5 ul	N x 195 μl	5.000/well (200ul)
M2	5.000	N x 5 ul	N x 195 μl	5.000/well (200ul)
OB	5.000	N x 5 ul	N x 195 μl	5.000/well (200ul)
CO12	5.000 + 5.000	N x 5 ul OB + N x 5 ul M1	N x 190 μl	10.000/well (200ul)
CO12	5.000 + 10.000	N x 5 ul OB + N x 10 ul M1	N x 185 μl	15 000/well (200 ul)

6. Pipet the following amounts of mixed medium and 1×10^6cells/ml solutions into the appropriate 15 ml tubes.
   N is equal to the amounts of wells needed for your experiment, e.g. 4 wells with M0 –> 20 ul M0 cell solution + 780 ul mixed medium
7. Seed 200 ul of the cells of every Experimental condition in the appropriate wells
8. Distribute the cells evenly by moving the plate quickly according to a cross-like pattern.
9. Store the plate into a CO2 incubator (37 ^oC, 5% CO2).
10. Replace the mixed medium after two days with osteogenic mixed medium, i.e. supplement mixed medium with 50 $\mu g/ml$ A.A. (Sigma-Aldrich, Germany) and 4 mM β-GL (Sigma-Aldrich, Germany).
11. Replace the medium every 2/3 days with fresh osteogenic mixed medium
12. store and freeze supernatant from days 1, 3, 7, 11, 14 and 21 at -80 ^oC

B.2 pattern co-culture experiment

Summary

This protocol describes the harvesting and seeding of MC3T3-E1 (OB) mono-cultures and OB:J774A.1 (MΦ) co-cultures on top of a p2 nanotopographical pattern inside a 6 well plate.

1. Requirements & Procedure

This protocol used exactly the same steps and cell concentrations as the static direct co-culture protocol. Additional requirements were: wavers with and without a p2 pattern. Furthermore only OB and CO12 co-culture were investigated in this experiment so only these cell solutions were prepared.

Note: Carefully administer liquids on the side of the wells to avoid damaging the nanopillars

B.3 Dynamic co-culture experiment

Summary

This protocol describes the harvesting and seeding of MC3T3-E1 (OB) mono-cultures and OB:J774A.1

(MΦ) co-cultures inside a microfluidic chip.

1. Requirements

1.1 Equipment

- 1.5 ml tubes
- 15ml tubes
- 50ml tubes
- P20 pipette + filter tips
- P1000 pipette + filter tips
- 2ml plastic pipettes
- 10ml plastic pipettes
- Suction system
- Water bath at 37 ^oC
- 50ml tube centrifuge (sigma centrifuge)
- Cell scraper
- Syringe pump 270 (KD scientific, USA)
- Sterile PTFE tubing (Darwin Microfluidics, UK)
- Sterile luer connectors
- Sterile Flow channels chip (MB-P1E-1x-010-PDMS'; Darwin Microfluidics)
- Sterile 10 ml luer lock syringe (BD systems, US)
- Nalgene syringe filter, 0.2 um, 25 mm, sterile (723-2520; Thermo Fisher Scientific)

1.2 Reagents

- Preheated Complete culture medium: αMEM without ascorbic acid
- Preheated Complete culture medium: DMEM
- Preheated Mixed Medium - Preheated osteogenic Mixed Medium
- Preheated 1x Trypsin-EDTA (Thermo fisher # 10779413)
- 1x DPBS (Thermo Fisher #14200083)
- Fibronectin bovine plasma ($50\ \mu g/ml$) (F4759-1MG; Sigma Aldrich)

1.3 Cells

- Pre cultured 6 well plate with M1 stimulated J774A.1
- Pre cultured 6 well plate with MC3T3-E1 cells

2. Procedure

1. Place all the reagents in the waterbath
2. Flush the channels of the microfluidic chip with mixed medium twice by pipetting 70 ul and 50 ul in opposite inlets. Wait 5 minutes between each washing step.
3. Place in the incubator for 30 minutes.
4. Retrieve chip, aspirate the mixed medium and coat the channels with FN (50 ug/ml), approximately 25 ul, use a luer connector. Incubate for 30-45 minutes
5. Harvest 6 well plate with MC3T3-E1
    a) Wash the well twice with 2 ml/well PBS
    b) Trypsinize for 3-5 minutes with 1ml/well pre-warmed trypsin

c) Add 2 ml/well pre-warmed Amem to stop the reaction
d) Add enough Medium to get a 10 ml solution
e) Centrifuge 200xg for 5 minutes
f) Aspirate the supernatant, add 0.5 ml medium and count the cells
g) Make a $1.5x10^6$cells/ml solution

.

6. Harvest M1 Macrophages (stimulated for 3 days with 100 ng/ml LPS and 100ng/ml IFN-gamma)
   a) Aspirate half of the medium, but .
   b) Use the scraper to crosswise detach the cell layer from the bottom
   c) Check under the microscope whether all cells have detached
   d) Pipet the cells in a 50 ml tube and add enough medium to get 25 ml solution in the tube
   e) Centrifuge 200xg for 5 minutes
   f) Aspirate the supernatant, add 0.5 ml and count the cells
   g) Make a $1.5x10^6$ cells/ml solution
7. Prepare OB (160 ul) and O12(160:320) dynamic experiment solutions
8. Retrieve the chip with coated channels from the incubator after 30-45 minutes
9. flush the channels once with amem-dmem (70-50 ul)
10. Aspirate the medium and add 50 ul of medium in the inlet that will be seeded first and 30 ul in the opposite inlet.
11. Add 20 ul cell solution in one inlet, up to 70 ul, wait 2 minutes and fill the other inlet with 20 ul, up to 50 ul, cell solution. The 20 ul height difference between inlets creates a flow through the channel that will help the cells to go in the channel.
12. Check under the microscope for cell adhesion
13. Repeat step 6 if necessary with a higher concentration of cells
14. Place in incubator
15. refresh the medium every day by carefully aspirating the medium from the inlets, and adding 70 and 50 ul in opposite inlets.
16. Switch to osteogenic mixed medium on day 2, and connect luer connectors and PTFE tubing to start a dynamic flow through the chip.
17. Fill two syringes and tubing of the push positions on the perfusion pump with osteogenic medium.
18. Connect the syringe to the nalgene filter and the nalgene filter to the PTFE tubing, be carefull to limit the amount of bubbles present in the system.
19. Fill two syringes and tubing of the pull positions on the perfusion pump with 1x DPBS. Connect the syringe to the nalgene filter and the nalgene filter to the PTFE tubing, be carefull to limit the amount of bubbles present in the system.
20. Place the syringes on the perfusion pump and lead the PTFE tubing through the back of the incubator. Make sure the hole is closed with a plug, through which only the tubing can go.
21. Connect the outlets of the channels opposite from the inlets form the **push** syringes, to empty PTFE tubing that leads to an empty 15 ml conical tube.
22. Connect the outlets of the channels opposite from the inlets form the **pull** syringes, to PTFE tubing filled with osteogenic medium that leads to a filled 15 ml conical tube containing osteogenic medium.

23. Carefully place the chip in the incubator and connect the inlets of the syringe tubing at the right spots.
24. Turn on the dynamic flow, and leave this system for 5 days (until 7 days after seeding).
25. Refresh the static channels every day with osteogenic medium.

APPENDIX **C**

Protocols assays

C.1 Griess Assay

1. Requirements

1.1 Equipment

- 1.5 ml tubes
- 96 well plate
- P1000 pipette + filter tips
- P200 pipette + filter tips
- P20 pipette + filter tips
- centrifuge
- plate reader

1.2 Reagents

- Griess reagent kit (abcam, ab234044)

1.3 Cells

- Stimulated J774A.1 cells

2. Procedure

2.1 Preparation of reagents

1. Nitrate assay buffer: ready to use as supplied. (store at 4^oC)
2. Griess reagent 1: ready to use as supplied. (store at 4^oC)
3. Griess reagent 2: ready to use as supplied. (store at 4 4^oC)
4. Nitrite standards: Reconstitute with $100\mu l$ Nitrite Assay buffer to generate $100mM$ nitrite standard solution. Keep on ice during use. (reconstituted standard is stable for 4 months when stored at 4^oC)

2.2 Standard preparation

1. Dilute nitrite standard 100-fold by adding $5\mu l$ of $100mM$ nitrite standard to $495\mu l$ nitrite assay buffer to obtain $1mM$ nitrite standard solution.
2. Prepare a standard curve by using the $1mM$ nitrite standard solution:

Standard	$1mM$ nitrite standard (μl)	Assay buffer(μl)	Final volume standard in well (μl)	End amount nitrite standard in well (nmol/well)
1	0	200	100	0
2	4	196	100	2
3	8	192	100	4
4	12	188	100	6
5	16	184	100	8
6	20	180	100	10

Each dilution has enough standard to set up duplicate readings ($2 \times 100\mu l$)

2.3 Sample preparation

Medium:

1. Collect $200\mu l$ of the cell culture medium an pipet into a 1.5ml tube.
2. Centrifuge at 10.000 g for 5 minutes.
3. Transfer the supernatant to a fresh tube

2.4 Reaction wells set up:

1. Standard wells: $100\mu l$ standard solution.
2. Sample wells: $100\mu l$ sample.
3. Sample background control wells: $100\mu l$ sample

2.5 Reaction mix:

1. For each well, add the following reagents:

Table C.1: Reaction Mix Griess assay

Component	Reaction mix (µl)	Background reaction mix (µl)
Griess reagent 1	10	10
Griess reagent 2	10	-
Nitrite assay buffer	80	90

2.6 Measurement:

1. Incubate the plate for 10 minutes at room temperature.
2. Measure the absorbance at 540nm.

2.7 Data analysis:

1. Average the duplicate reading for each standard, control and sample.
2. Subtract the mean value of the blank (zero standard) from all standards, controls and sample readings. (this is the corrected absorbance)
5. Subtract the sample background control from the sample readings.
6. Plot the corrected values for each standard to generate a standard curve.
7. Apply the corrected sample reading to the standard curve to get the nitrite (B) amount in the sample well (nmol/well).

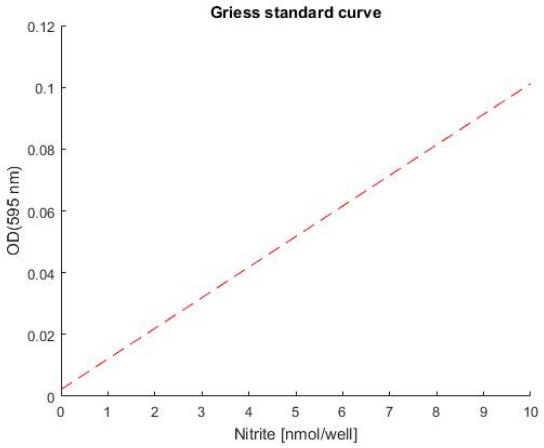

Figure C.1: Griess assay standard calibration curve.

8. Concentration of nitrite in mM is calculated as:

$$\text{Nitrite concentration} \; = \; B/V \times D \tag{C.1}$$

B = amount of Nitrite in the sample well calculated from standard curve in [nmol].
V = sample volume added in the sample wells [ml].
D = sample dilution factor if sample is diluted to fit within the standard curve range (prior to reaction well set up).

The standard curve obtained is shown in figure C.1

C.2 TNF-α

This protocol was provided together with the "Mouse TNF-α High Sensitivity ELISA kit" (Abcam, #BMS607HS). It has been altered for the use of cell culture supernatant and optimized for our experiment.

Summary:
1. An anti-mouse TNF-α coating antibody is adsorbed onto microwells.
2. Mouse TNF-α present in the sample or standard binds to antibodies adsorbed to the microwells. A biotin-conjugated anti-mouse TNF-α antibody is added and binds to mouse TNF-α captured by the first antibody.
3. Following incubation unbound biotin-conjugated anti-mouse TNF-α antibody is removed during a wash step. Streptavidin-HRP is added and binds to the biotin-conjugated anti-mouse TNF-α antibody.
4. Following incubation unbound Streptavidin-HRP is removed during a wash step, and amplification reagent I (Biotinyl-Tyramide) is added to the wells.
5. Following incubation unbound amplification reagent I is removed during a wash step and amplification reagent II (Streptavidin-HRP) is added.
6. Following incubation unbound amplification reagent II is removed during a wash step and substrate solution reactive with HRP is added.
7. A coloured product is formed in proportion to the amount of mouse TNF-α present in the sample or standard. The reaction is terminated by addition of acid and absorbance is measured at 450 nm. A standard curve is prepared from 7 mouse TNF-α standard dilutions and mouse TNF-α sample concentration determined.

1. Requirements

1.1 Equipment

- 1.5 ml tubes
- 96 well plate
- P1000 pipette + filter tips
- P200 pipette + filter tips
- P20 pipette + filter tips
- 5 ml pipetts
- 10 ml pipettes
- plate shaker
- centrifuge
- plate reader
- Deionized water

1.2 Reagents Provided

- 1 aluminium pouch with a Microwell Plate coated with monoclonal antibody to mouse TNF-α
- 1 vial (70 μl) Biotin-Conjugate anti-mouse TNF-α monoclonal antibody
- 1 vial (150 μl) Streptavidin-HRP
- 2 vials mouse TNF-α Standard lyophilized, 200pg/ml upon reconstitution
- 1 bottle (5 ml) Calibrator Diluent

- 1 bottle (12 ml) Sample Diluent
- 1 vial (5 ml) Assay Buffer Concentrate 20x (PBS with 1% Tween 20 and 10% BSA)
- 1 vial (7 ml) Amplification Diluent Concentrate (2x)
- 1 vial (75 μl) Amplification Reagent I
- 2 vials (15 μl) Amplification Reagent II
- 2 bottles (50 ml) Wash Buffer Concentrate 20x (PBS with 1 % Tween 20)
- 1 vial (15 ml) Substrate Solution (tetramethyl-benzidine)
- 1 vial (15 ml) Stop Solution (1M Phosphoric acid)
- 8 Adhesive Films

reagent contains ethyl alcohol

3. Procedure

3.1 Preparation of reagents

1. *Wash buffer 1x*:
   Pour $40ml$ Wash Buffer (20x) into a clean $1000ml$ graduated cylinder. Bring to final volume of 1000 ml with deionized water. Mix gently to avoid foaming. Transfer to a clean wash bottle. And leave at room Temperature
   Note: store at 2 degrees.
2. *Assay Buffer (1x):*
   Pour $5ml$ of Assay Buffer Concentrate (20x) into a clean $100ml$ graduated cylinder. Bring to final volume of $100ml$ with deionized water. Mix gently to avoid foaming and leave at room temperature
   Note: Store at 2 to 8 degrees Celsius.
3. *Amplification Diluent (1x):*

3.2 Standard preparation

1. Reconstitute mouse TNF-α standard by addition of $500\mu l$ Sample Diluent
   Swirl or mix gently to insure complete and homogeneous solubilisation (concentration of reconstituted standard $=$ $200\ pg/ml$). Allow the standard to reconstitute for 10-30 minutes. Mix well prior to making dilutions.
2. Label eight tubes, Standards 1– 8.
3. Add $150\mu l$ of sample Diluent NS into numbers 2-8.
4. Use the Stock Standard to prepare the following dilution series. Standard #8 contains no protein and is the Blank control:

Standard #	Volume to dilute (ul)	Volume Diluent (ul)	Final Conc. (pg/ml)
1	300 ul Stock	0	200
2	150 ul Std # 1	150	100
3	150 ul Std # 2	150	50
4	150 ul Std # 3	150	25
5	150 ul Std # 4	150	12.5
6	150 ul Std # 5	150	6.25
7	150 ul Std # 6	150	3.13
8	0	150	0

3.3 Sample Preparation

1. Centrifuge cell culture media at 2,000 x g for 10 minutes to remove debris.
2. Collect supernatants and assay.
3. Store un-diluted samples at -20^oC or below. Avoid repeated freeze-thaw cycles.

3.4 protocol

The amounts used in this protocol were sufficient for 96 wells.

1. Prepare Wash Buffer 1x, assay buffer 1x and Amplification solution.
2. Prepare TNF-standards
3. Prepare Biotin-Conjugate. Make a 1:100 dilution of the concentrated Make a 1:100 dilution of the concentrated Biotin-Conjugate solution with Assay Buffer (1x) in a 15 ml tube. Dilute $0.06ml$ Biotin Conjugate with $5.94ml$ Assay Buffer (1x).
   Note: This solution should be used within 30 minutes after dilution
4. Wash the microwell strips twice with $400\mu l$/well Wash Buffer. Allow the Wash Buffer to sit in the wells for about 10 – 15 seconds before aspiration.
   After the last wash step, empty wells and tap microwell strips on absorbent pad or paper towel to remove excess Wash Buffer. Use the microwell strips immediately after washing. Do not allow wells to dry.
5. Add $50\mu l$ of Sample Diluent in duplicate to all wells.
6. Add $50\mu l$ of Standards 1-8 to the corresponding standard well
7. Add $50\mu l$ of each Sample to the sample wells.
8. Add $50\mu l$ of Biotin Conjugate to all wells
9. Cover with an adhesive film and incubate at room temperature for 2 hours on a microplate shaker set at 200 rpm.
10. Prepare Streptavidin-HRP. Make a 1:200 dilution of the concentrated Streptavidin-HRP and Assay Buffer (1x). Substitute $0.96ml$ Streptavidin-HRP with $11.94ml$ Assay Buffer (1x) in a 15 ml tube.
11. Remove adhesive film and empty wells.
12. Wash microwell strips 6 times with $400\mu l/$well Wash Buffer (1x) according to step 6
13. Add $100\mu l$ of diluted Streptavidin-HRP to all wells.
14. Cover with an adhesive film and incubate at room temperature (18^oC to 25^oC) for 1 hour on a microplate shaker set at 200 rpm.
15. Prepare Amplification Solution I diluted in Amplification Diluent (1x) (1:200) immediately prior to use. Substitute $0.06ml$ Amplication Reagent I with 11.94 ml Amplification Diluent
16. Wash microwell strips 6 times with 400 ul/well Wash Buffer (1x) according to step 6
17. Add 100 μl of Amplification Solution I to all wells.
18. Cover with an adhesive film and incubate at room temperature (18^oC to 25^oC) for exactly 15 minutes.
19. Prepare Amplification Solution II diluted in Assay Buffer Concentrate(1X) (1:500) immediately prior to use. Substitute $0.024ml$ Amplification Reagent II in 11.976 ml Assay Buffer (1x)
20. Remove adhesive film and empty wells. Wash microwell strips 6 times with $400\mu l/$well Wash Buffer (1x) according to step 6
21. Add 100 μl of Amplification Solution II to all wells.

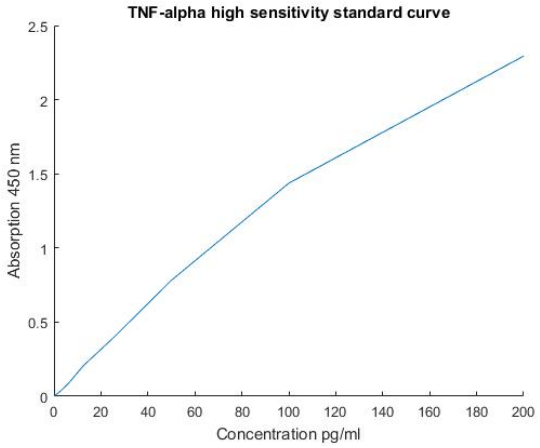

Figure C.2: TNF-α assay standard calibration curve.

22. Cover with an adhesive film and incubate at room temperature (18^oC to 25^oC) for exactly 30 minutes on a microplate shaker set at 200 rpm.
23. Remove adhesive film and empty wells. Remove adhesive film and empty wells. Wash microwell strips 6 times with 400 ul/well Wash Buffer (1x) according to step 6
24. Pipette 100 μl of TMB Substrate Solution to all wells.
25. Incubate the microwell strips at room temperature (18^oC to 25^oC) for about 10-20 min. Avoid direct exposure to intense light
    Note: It is recommended to add the stop solution when the highest standard has developed a dark blue colour. Alternatively the colour development can be monitored by the ELISA reader at $620nm$. *The substrate reaction should be stopped as soon as Standard 1 has reached an OD of 0.9 – 0.95.*
26. Stop the enzyme reaction by quickly pipetting 100 μl of Stop Solution into each well. It is important that the Stop Solution is spread quickly and uniformly throughout the microwells to completely inactivate the enzyme. Results must be read immediately after the Stop Solution is added or within one hour if the microwell strips are stored at $2 - 8^oC$ in the dark.
27. Read absorbance of each microwell on a spectro-photometer using 450 nm as the primary wave length (optionally 620 nm as the reference wave length; $610nm$ to $650nm$ is acceptable).

3.7 Data analysis:

1. Average the duplicate reading for each standard, control and sample.
2. Subtract the mean value of the blank (zero standard) from all standards, controls and sample readings. (this is the corrected absorbance)
5. Subtract the sample background control from the sample readings.
6. Plot the corrected values for each standard to generate a standard curve.
7. Apply the corrected sample reading to the standard curve to get the TNF-α amount in the sample

The standard calibration curve is shown in figure C.2

C.3 IL-10

This protocol was obtained from the supplier (Abcam, UK) together with the Mouse IL-10 SimpleStep ELISA kit (Abcam, #255729). It has been altered for the use of cell culture supernatant and optimized for our experiment.

1. Summary

The SimpleStep ELISA (ab255729) employs an affinity tag labeled capture antibody and a reporter conjugated detector antibody which immunocapture the sample analyte in solution. This entire complex (capture antibody/analyte/detector antibody) is in turn immobilized via immunoaffinity of an anti-tag antibody coating the well. To perform the assay, samples or standards are added to the wells, followed by the antibody mix. After incubation, the wells are washed to remove unbound material. TMB Development Solution is added and during incubation is catalyzed by HRP, generating blue coloration. This reaction is then stopped by addition of Stop Solution completing any color change from blue to yellow. Signal is generated proportionally to the amount of bound analyte and the intensity is measured at 450 nm. Optionally, instead of the endpoint reading, development of TMB can be recorded kinetically at $600nm$.

1. Prepare all reagents, samples and standards as instructed.
2. Add $50\mu l$ standard or sample to appropriate wells
3. $450\mu l$ antibody cocktail to all wells
4. incubate at room temperature for 1 hour
5. Aspirate and wash each well three times with $340\mu l$ 1x wash buffer PT
6. add $100\mu l$ TMB development solution to each well well (this step already takes 5 minutes) and incubate for 10 minutes.
7. Materials Supplied (store at 4 degrees Celsius)

2. Requirements

2.1 Materials Required

- Microplate reader capable of measuring absorbance at 450 or 600 nm.
- Deionized water.
- Multi- and single-channel pipettes.
- Tubes for standard dilution.
- Plate shaker for all incubation steps.

2.2 Reagents Provided

- Mouse IL-10 Capture Antibody 10X, 600 μl
- Mouse IL-10 Detector Antibody 10X, 600 μl
- Mouse IL-10 Lyophilized Recombinant Protein, 2 Vials
- Antibody Diluent CPR, 6 mL
- Cell Extraction Enhancer Solution 50X, 1 mL
- Sample Diluent 75BS, 20 mL
- Sample Diluent NS, 12 mL
- Wash Buffer PT 10X, 20 mL
- TMB Development Solution, 12 mL
- Stop Solution, 12 mL

- SimpleStep Pre-Coated 96-Well Microplate, 96 Wells
- Plate Seal, 1

Sample Diluent NS is provided but not necessary for this product.

3. Procedure

3.1 Reagent Preparation

- Equilibrate all reagents to room temperature (18-25°C) prior to use. The kit contains enough reagents for 96 wells. The sample volumes below are sufficient for 48 wells (6 x 8-well strips); adjust volumes as needed for the number of strips in your experiment.
- Sample Diluent 75BS may contain precipitate, this is normal. If precipitate is not dissolved by gentle mixing, the precipitate may be dissolved by gentle warming and mixing at 37°C for 10 minutes. If precipitate remains, gently spin down and avoid visible precipitates when pipetting.
- Prepare only as much reagent as is needed on the day of the experiment. Capture and Detector Antibodies have only been tested for stability in the provided 10X formulations.

1. *Sample Diluent 75BS + Enhancer:*
   Prepare Sample Diluent 75BS + Enhancer by adding 50X Cell Extraction Enhancer Solution to Sample Diluent 75BS. To make 5 mL Sample Diluent 75BS + Enhancer combine 4.9 mL Sample Diluent 75BS and 100 μl Cell Extraction Enhancer Solution 50X. Mix thoroughly and gently. If required protease inhibitors can be added.
2. *1X Wash Buffer PT:*
   Prepare 1X Wash Buffer PT by diluting Wash Buffer PT 10X with deionized water. To make 50 mL 1X Wash Buffer PT combine 5 mL Wash Buffer PT 10X with 45 mL deionized water. Mix thoroughly and gently. For whole plate, prepare 100 mL.
3. *Antibody Cocktail:*
   Prepare Antibody Cocktail by diluting the capture and detector antibodies in Antibody Diluent CPR. To make 3 mL of the Antibody Cocktail combine 300 μl 10X Capture Antibody and 300 μl 10X Detector Antibody with 2.4 mL Antibody Diluent CPR. Mix thoroughly and gently. For whole plate, make 6 mL.

3.2 Standard Preparation

1. Reconstitute the IL-10 standard sample(Mouse IL-10 Lyophilized Recombinant Protein?) by adding 500 μl of Sample Diluent 75BS + Enhancer. Mix thoroughly and gently. Hold at room temperature for 10 minutes and mix gently. This is the 24.13 ng/mL Stock Standard Solution.
2. Label eight tubes, Standards 1– 8.
3. Add 376 μL Sample Diluent 75BS + Enhancer into tube number 1 and 150 μL of Sample Diluent 75BS + Enhancer into numbers 2-8.
4. Use the Stock Standard to prepare the following dilution series. Standard #8 contains no protein and is the Blank control:

Standard #	Volume to dilute (ul)	Volume Diluent (ul)	Final Conc. (pg/ml)
1	34 ul Stock	376	2,000
2	150 ul Std # 1	150	1,000
3	150 ul Std # 2	150	500
4	150 ul Std # 3	150	250
5	150 ul Std # 4	150	125
6	150 ul Std # 5	150	62.50
7	150 ul Std # 6	150	31.25
8	0	150	0

3.3 Sample Preparation

1. Centrifuge cell culture media at 2,000 x g for 10 minutes to remove debris.
2. Collect supernatants and add 50X Enhancer to supernatant to a final concentration of 1X and assay. (2ul 50x in 90 ul supernatant)
3. Any serial dilutions should be made in Sample Diluent 75BS + ab255729 Mouse IL-10 SimpleStep ELISA Kit 10 Enhancer. Store un-diluted samples at -20°C or below. Avoid repeated freeze-thaw cycles.

3.4 Protocol

- Equilibrate all materials and prepared reagents to room temperature prior to use.
- We recommend that you assay all standards, controls and samples in duplicate.

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, reseal and return to 4ºC storage.
3. Add 50 μl of all sample or standard to appropriate wells.
4. Add 50 μl of the Antibody Cocktail to each well.
5. Seal the plate and incubate for 1 hour at room temperature on a plate shaker set to 400 rpm.
6. Wash each well with 3 x 340 μl 1X Wash Buffer PT. Wash by aspirating or decanting from wells then dispensing 340 μl 1X Wash Buffer PT into each well. Complete removal of liquid at each step is essential for good performance. After the last wash invert the plate and blot it against clean paper towels to remove excess liquid.
7. Add 100 μl of TMB Development Solution to each well (this step already takes 5 minutes) and incubate for 10 minutes in the dark on a plate shaker set to 400 rpm. Given variability in laboratory environmental conditions, optimal incubation time may vary between 5 and 20 minutes.
   Note: The addition of Stop Solution will change the color from blue to yellow and enhance the signal intensity about 3X. To avoid signal saturation, proceed to the next step before the high concentration of the standard reaches a blue color of O.D.600 equal to 1.0.
8. Add 100 μl of Stop Solution to each well. Shake plate on a plate shaker for 1 minute to mix. Record the OD at 450 nm. This is an endpoint reading.
9. Analyze the data

3.5 Data analyis:

1. Average the duplicate reading for each standard, control and sample.
2. Subtract the mean value of the blank (zero standard) from all standards, controls and sample readings.

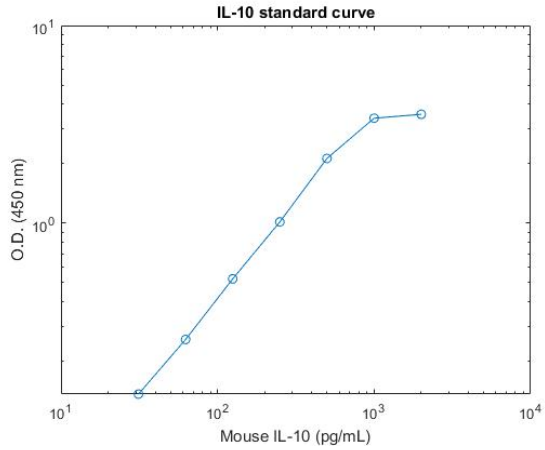

Figure C.3: IL-10 standard calibration curve.

(this is the corrected absorbance)

5. Subtract the sample background control from the sample readings.
6. Plot the corrected values for each standard to generate a standard curve.
7. Apply the corrected sample reading to the standard curve to get the IL-10 amount in the sample

The standard curve obtained is shown in figure C.3

C.4 PGE2

This product and protocol was obtained from abcam (Abcam's Prostaglandin E2 (PGE2) in vitro competitive ELISA (Enzyme- Linked Immunosorbent Assay) kit # ab133021; Abcam, UK). It has been altered for the use of cell culture supernatant and optimized for our experiment.

1. Summary Abcam's Prostaglandin E2 (PGE2) in vitro competitive ELISA (Enzyme- Linked Immunosorbent Assay) kit is designed for the accurate quantitative measurement of Prostaglandin E2 in serum, saliva, urine and tissue culture media and other biological fluids. A mouse IgG antibody has been precoated onto 96-well plates. Standards or test samples are added to the wells, along with an alkaline phosphatase (AP) conjugated-Prostaglandin E2 antibody. After incubation, the excess reagents are washed away and pNpp substrate is added and is catalyzed by AP to produce a yellow color. The intensity of the yellow coloration is inversely proportional to the amount of Prostaglandin E2 captured in the plate. Prostaglandin E2 is formed in a variety of cells from PGH2, which itself is synthesized from arachidonic acid by the enzyme prostaglandin synthetase. Prostaglandin E2 has been shown to have a number of biological actions, including vasodilation, both anti- and proinflammatory action, modulation of sleep/wake cycles, and facilitation of the replication of human immunodeficiency virus. It elevates cAMP levels, stimulates bone resorption, and has thermoregulatory effects. It has been shown to be a regulator of sodium excretion and renal hemodynamics.

1. Prepare all reagents, samples, and standards as instructed
2. Add standards and samples to appropriate wells.
3. Add prepared labeled AP-conjugate to appropriate wells.
4. Add PGE2 antibody to appropriate wells. Incubate at room temperature.
5. Add pNpp substrate to each well. Incubate at room temperature. Add Stop Solution to each well. Read immediately.

2. Requirements

2.1 Materials Required

- Microplate reader capable of measuring absorbance at 450 or 600 nm.
- Deionized water.
- Multi- and single-channel pipettes.
- Tubes for standard dilution.
- Plate shaker for all incubation steps.

2.2 Reagents Provided

3. Procedure

3.1 Reagent Preparation

- Equilibrate all reagents to room temperature ($18 - 25\ ^oC$) prior to use.
- The kit contains enough reagents for 96 wells

Prepare only as much reagent as is needed on the day of the experiment.

1. *PGE2 Alkaline Phosphatase Conjugate:*

Allow the Prostaglandin E2 Alkaline Phosphatase Conjugate to equilibrate to room temperature. Any unused conjugate should be aliquoted and stored at $-20\ ^oC$.

2. *1X Wash Buffer:*
   Prepare the 1X Wash Buffer by diluting 5 mL of the 20X Wash Buffer Concentrate in 95 mL of deionized water. Mix thoroughly and gently. 1X Wash buffer can be stored at room temperature for three months, or until the expiration date of the kit, whichever is earlier.

3.2 Standard Preparation

- Always prepare a fresh set of standards for every use.
- Prepare serially diluted standards immediately prior to use.
- Diluted standards should be used within 60 minutes of preparation.

1. Allow the $50,000\ pg/mL$ PGE2 Stock Standard solution to warm to room temperature. Any unused Stock Standard solution should be stored at $-20\ ^oC$.
2. Label eight tubes #1 - #8.
3. Add $950\ \mu l$ mixed medium into tube # 1 and $500\ \mu l$ mixed medium into tubes #2 – #7.
4. Prepare a $2,500\ pg/ml$ Standard 1 by adding $50\ \mu l$ of the $50,000\ pg/ml$ Stock Standard to $950\ \mu l$ mixed medium into tube #1. Mix thoroughly and gently.
5. Use Standard 1 to prepare the following dilution series

Standard #	Volume to dilute (ul)	Volume Diluent (ul)	Final Conc. (pg/ml)
1	50 ul Stock	950	2,500
2	500 ul Std # 1	500	1,250
3	500 ul Std # 2	500	625
4	500 ul Std # 3	500	313
5	500 ul Std # 4	500	156
6	500 ul Std # 5	500	78.1
7	500 ul Std # 6	500	39.1

3.3 Sample Preparation

1. Centrifuge cell culture media at 2,000 x g for 10 minutes to remove debris.
2. Collect supernatants and assay
3. Any serial dilutions should be made in Appropriate diluent (mixed medium). Store un-diluted samples at $-20\ ^oC$ or below. Avoid repeated freeze-thaw cycles.

3.4 Plate Layout

Table C.2: Plate layout PGE2. Bs = Blank; contains substrate only.TA = Total Activity; contains conjugate ($5\mu L$) and substrate. NSB = Non-specific binding; contains standard diluent, assay buffer,conjugate and substrate. B0 = $0pg/mL$ standard; contains assay buffer, conjugate, antibody and substrate.

	Standard			**Day 0**		**Day 1**		**Day 3**		**Day 7**		**Medium**
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	B_s	std #1	std #5	OB-D0 #1	M1-D0 #1	OB-D1 #1	CO11-D1 #1	OB-D3 #1	CO11-D3 #1	OB-D7 #1	CO11-D7 #1	aMEM
B	B_s	std #1	std #5	OB-D0 #2	M1-D0 #2	OB-D1 #2	CO11-D1 #2	OB-D3 #2	CO11-D3 #2	OB-D7 #2	CO11-D7 #2	aMEM
C	TA	std #2	std #6	OB-D0 #3	M1-D0 #3	OB-D1 #3	CO11-D1 #3	OB-D3 #3	CO11-D3 #3	OB-D7 #3	CO11-D7 #3	DMEM
D	TA	std #2	std #6	OB-D0 #4	M1-D0 #4	OB-D1 #4	CO11-D1 #4	OB-D3 #4	CO11-D3 #4	OB-D7 #4	CO11-D7 #4	DMEM
E	NSB	std #3	std #7	M0-D0 #1	M2-D0 #1	M1-D1 #1	CO12-D1 #1	M1-D3 #1	CO12-D3 #1	M1-D7 #1	CO12-D7 #1	Mixed -
F	NSB	std #3	std #7	M0-D0 #2	M2-D0 #2	M1-D1 #2	CO12-D1 #2	M1-D3 #2	CO12-D3 #2	M1-D7 #2	CO12-D7 #2	Mixed -
G	B_0	std #4		M0-D0 #3	M2-D0 #3	M1-D1 #3	CO12-D1 #3	M1-D3 #3	CO12-D3 #3	M1-D7 #3	CO12-D7 #3	Mixed +
H	B_0	std #4		M0-D0 #4	M2-D0 #4	M1-D1 #4	CO12-D1 #4	M1-D3 #4	CO12-D3 #4	M1-D7 #4	CO12-D7 #4	Mixed +

3.4 Protocol

- Equilibrate all materials and prepared reagents to room temperature prior to use.
- We recommend that you assay all standards, controls and samples in duplicate.

1. Add $100\ \mu L$ mixed medium into the NSB & B0 wells.
2. Add $100\mu L$ of the prepared standards (#1-#7) into appropriate wells
3. Add $100\mu L$ supernatant to appropriate wells.
4. Add $50\mu L$ of Assay Buffer into the NSB wells only.
5. Add $50\mu L$ of PGE2 Alkaline Phosphatase Conjugate (blue) into NSB, B0, standard and sample wells, i.e. not the Total Activity (TA) and Bs wells.
6. Add $50\mu L$ of PGE2 Antibody (yellow) into B0, standard and sample wells, i.e. not Bs, TA and NSB wells.
7. Note: Every well used should be green except the NSB wells which should be blue. Bs and TA wells are empty at this point and have no color. (see table below)
8. Incubate the plate at room temperature on a plate shaker for 2 hours at 500 rpm. The plate may be covered with the plate sealer provided.
9. Empty the contents of the wells, by tapping them on a sufficient amount of lint free cloth and wash by adding $400\mu L$ of 1X Wash Buffer to every well. Repeat the wash 2 more times for a total of 3 washes. After the final wash, empty or aspirate the wells, and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.

Table C.3: PGE2 protocol timeline. Add the reagents to the wells from the left to the right.

	Sample Diluent (Mixed Medium)	Std	Sample	Assay Buffer	PGE2 Alkaline Phosphatase Conjugate (Blue)	PGE2 Antibody (yellow)
B_s						
TA						
NSB	100 μl			50 μl	50 μl	
B_0	100 μl				50 μl	50 μl
Std		100 μl			50 μl	50 μl
Sample (supernatant)			100 μl		50 μl	50 μl

10. Add $5\mu L$ of the PGE2 Alkaline Phosphatase Conjugate to the TA wells only.
11. Add $200\mu L$ of the pNpp Substrate solution to all wells. Incubate at room temperature for 45 minutes without shaking.
12. Add $50\mu L$ Stop Solution to all wells. The plate should be read immediately.
13. After blanking the plate reader against the Bs (blank) wells, read optical density at 405 nm, preferably with correction between 570 and 590 nm. If the plate reader is not able to be blanked against the Bs wells, manually subtract the mean optical density of the blank wells from all readings.

C.5 Fluorescent Staining (Runx2 & phenotype staining)

1. Requirements

1.1 Equipment

- 50ml tubes
- P20 pipette + filter tips
- P1000 pipette + filter tips
- Disposable pipettes
- tweezer
- object glass

1.2 Reagents

- DPBS (Thermo Fisher #14200083)
- Demi water
- Formaldehyde solution (Sigma-Aldrich # 252549)
- BSA (Sigma-Aldrich # A2058-1G)
- Triton (Sigma-Aldrich # T8787-250ml)
- Tween (Sigma-Aldrich # P1379-100ml)
- Prolong gold with dapi (Thermo Fisher # 11549306)

Phenotype staining

Primary antibodies:

- Anti-Mannose Receptor antibody - CD206 (# ab8918, Abcam) 1:100
- Recombinant Anti-CCR7 antibody (# ab32527, Abcam) 1:100

Secondary antibodies:

- Alexa-Fluor 488 donkey anti-mouse (#10544773,ThermoFisher Scientific, US) 1:50
- Alexa-Fluor 594 donkey anti-rabbit (#10798994, ThermoFisher Scientific, US) 1:150

Runx2 staining

Primary antibody:

- RunX2 (Abcam, #ab192256) 1:250

Secondary antibody:

- Alexa 488 donkey anti rabbit (# A-21206, Thermo Fisher Scientific) 1:200

1.3 Cells

- Pre cultured wells plate

2. Procedure

2.1 Preparation of solutions

- Dilute the PBS 10 times by adding 450ml demi water to 50ml 10x PBS
- Dilute the Formaldehyde solution to 4% by adding 5.4 ml Formaldehyde to 44.6 ml 1x PBS solution. this step should be performed in the chemical hood)
- Dilute 1G of BSA in 100ml 1xPBS (final concentration of 1% BSA or 10mg/ml). Aliquot per 2 ml and store at $-20\ ^oC$.
- Add 2.5ml of Triton to 500ml 1xPBS (0.5% Triton/PBS)
- Add 2.5ml Tween 20 to 500ml 1xPBS (0.5% Tween/PBS)

2.2 Protocol

1. Wash the cells 2 times with PBS.
2. Transfer the cells to the chemical hood.
3. Add an appropriate volume of 4% formaldehyde solution to the cells.
4. Incubate for 15 minutes at room temperature.
5. Discard the formaldehyde solution into the correct chemical waste bin.
6. Wash the cells twice with 1x PBS.
7. Permeabilize the cells with 0.5% Triton/PBS for 5 minutes at 4^oC.
8. Discard the Triton solution.
9. Add 1% BSA/PBS for 5 minutes at 37^oC.
10. Dilute primary antibody in 1% BSA/PBS and incubate for 1hour at 37^oC.
11. Wash cells with 0.5% Tween/PBS 3 times 5 minutes at room temperature.
12. Dilute secondary antibody in 1% BSA/PBS and incubate for 1hour at room temperature.
13. Wash cells with 0.5% Tween/PBS 3 times 5 minutes at room temperature.
14. Wash cells with 1x PBS for 5 minutes at room temperature.
15. Mount glass slide with prolong gold (containing dapi) to object glass.

C.6 Live/dead staining

Equipment

- P2 pipette + filter tips
- P20 pipette + filter tips
- P1000 pipette + filter tips
- 1.5 ml tubes
- Fluorescent microscope

Reagents

- LIVE/DEAD Viability/Cytotoxicity Kit, for Mammalian Cells (#L3224 ThermoFisher Scientific, US)
- Calcein stock is 4mM
- Ethd stock is 2mM
- DPBS (Thermo Fisher #14200083)

Cells

- Pre cultured well plate

Procedure

1. Wash the cells with 10x PBS (12 wells, 2.4 ml)
2. Wash the cells with 1x PBS (12 wells, 2.4 ml)
3. Prepare Live/dead solution
    a) Ostoeblasts: 1ml 1xPBS, 0.5 uL Calcein AM stock (2 uM/ml) + 1.5 ul EthD stock (3uM/ml). (need 1.2ml, 0.6 ul Calcein & 1.8 ul EthD)
    b) Macrophages: 1ml 1xPBS, 0.2µl Calcein AM stock (0.8 uM/ml) + 1.5µl EthD-1 stock (3 uM/ml) (need 1.2ml, 0.24 ul Calcein & 1.8 ul EthD)
4. Incubate sample for 30 minutes.
5. Discard solution and replace with 1x PBS.
6. Image by fluorescent microscope. Live cells: bright green signal Dead cells: red + green signal

C.7 ALP

This protocol was attached to the ALP Assay Fluoremetric kit (Abcam, #ab83371). It has been altered for the ALP measurements of supernatants.

1. Requirements

1.1 Equipment

- 1.5 ml tubes
- 96 well plate
- P1000 pipette + filter tips
- P200 pipette + filter tips
- P20 pipette + filter tips
- centrifuge
- plate reader

1.2 Reagents

- ALP Assay Fluoremetric kit (Abcam, #ab83371)

1.3 Cells

- Stimulated MC3T3-E1 cells

2. Procedure

2.1 Preparation of reagents

1. ALP Assay Buffer: Ready to use as supplied. Equilibrate at room temperature before use. Store at -20^oC
2. MUP Substrate (MUP Standard): Dissulve MUP substrate in 1.2 ml Assay Buffer to generate 5 mM MUP substrate solution. Store at -20^oC and avoid multiple thaw, freeze cycles. (use within 2 months)
3. ALP Enzyme Solution: Reconstitute ALP Enzyme with 1 mL Assay Buffer. The reconstituted enzyme is stable for up to 2 months at 4^oC. Do not freeze! Keep on ice whilst in use.
4. Stop Solution: Ready to use as supplied. Equilibrate to room temp before use. Store at -20^oC

2.2 Standard Preparation

1. Prepare a 50 um MUP standard by diluting 5 ul 5 mM MUP substrate with 495 ul Assay Buffer
2. Using 50 um standard, prepare standard curve dilution as described in the table in a microplate or microcentrifuge tubes.

Standard #	Volume of Standard (ul)	Assay Buffer (ul)	Final volume standard in well (ul)	End [MUP] in well (nmol/well)
1	0	360	120	0
2	6	354	120	0.1
3	12	348	120	0.2
4	18	342	120	0.3
5	24	336	120	0.4
6	30	330	120	0.5
7	36	324	120	0.6
8	42	318	120	0.7
9	48	312	120	0.8
10	54	306	120	0.9

2.2 Protocol

- Equilibrate all materials and prepared reagents to room temperature prior to use.
- It is recommended to assay all standards, controls and samples in duplicate.

1. Set up reaction wells:
    a) Standard wells = 120 ul/well
    b) Sample wells = 1-110 ul/well (adjust volume to 110ul/well with assay buffer). I used 100 ul supernatant/well.
2. MUP Reaction Mix.
    a) Prepare reaction mix for each well. 2 ul 5mM MUP substrate + 18 ul Assay buffer
    b) Add 20 ul reaction mix to sample wells and background control (assay buffer only)
    c) Add 20 ul stop solution to sample control (mixed medium)
    d) Add 10 ul ALP enzyme to standard curve wells
    e) Incubate for 30 minutes at 25 degrees protected from light.
    f) Add 20 ul stop solution to all wells, except for sample background wells.
    g) Gently shake the plate.
    h) Measure output. 360/440 nm

2.3 Calculations

- Samples producing signals greater than that of the highest standard should be further diluted in appropriate buffer and reanalyzed, then multiplying the concentration found by the appropriate dilution factor.
- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates).

1. Average the duplicate reading for each standard and sample.
2. Subtract the appropriate background from all standard and sample readings if applicable.
3. Subtract the mean absorbance value of the blank (Standard #1) from all standard and sample readings. This is the corrected absorbance.
4. Plot the corrected absorbance values for each standard as a function of the final concentration of 4-MU.

5. Draw the best smooth curve with 4LP function in Matlab through these points to construct the standard curve.
6. Apply sample readings to the standard curve to get the amount of 4-MU generated by ALP sample
7. Activity (mU/mL) of alkaline phosphatase in the test samples is calculated as:

$$\text{ALP activity} = B/V/T \tag{C.2}$$

Where:
B = amount of 4-MU generated by samples (in nmol).
V = volume of sample added in the assay well (in mL), $100\mu l$.
T = reaction time (in minutes), 25 minutes.

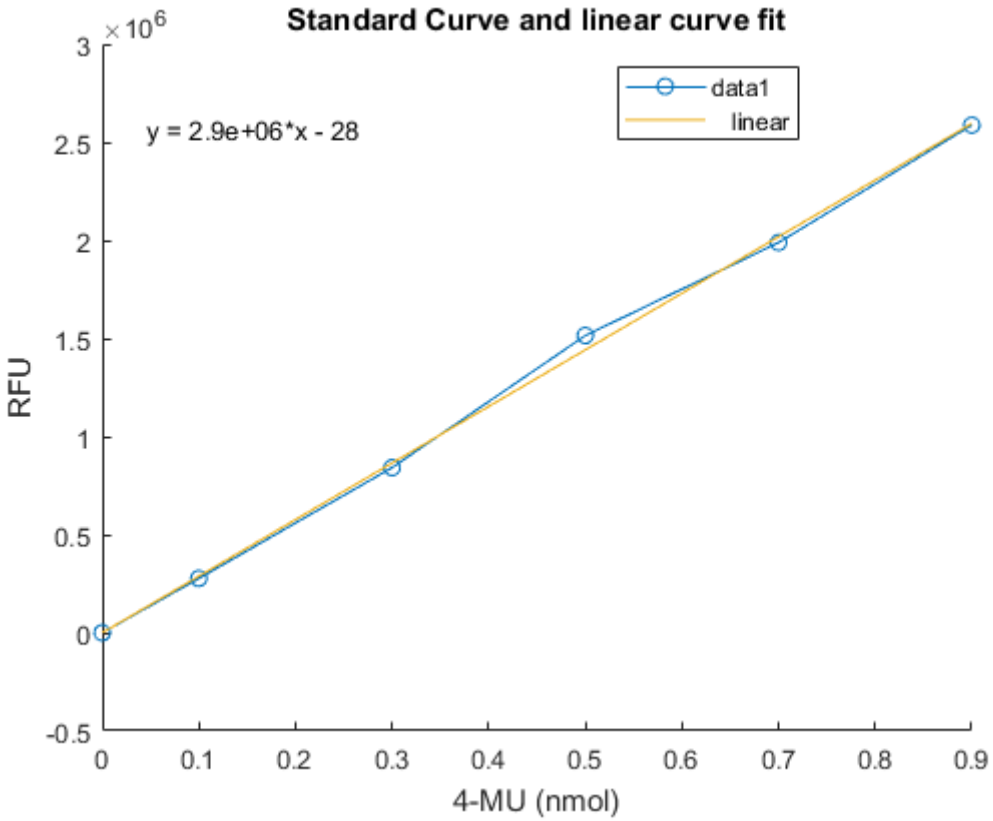

Figure C.4: ALP standard curve

C.8 Alizarin Red

1. Requirements

Equipment

- 50ml tubes
- P1000 pipette + filter tips
- P200 pipette + filter tips
- Disposable pipettes
- 0.22 μM filter

1.2 Reagents

- DPBS (Thermo Fisher #14200083)
- Demi water
- Formaldehyde solution (Sigma-Aldrich 252549)
- Alizarin red S (Sigma-Aldrich A5533-25G)

1.3 Cells

- Pre cultured cells in 24-well plates

Procedure

2.1 Preparation of solutions

1. Dilute the PBS 10 times by adding 450ml demi water to 50ml 10x PBS
2. Dilute the Formaldehyde solution 10 times by adding 9ml of 1x PBS to 1ml Formaldehyde solution (this step should be performed in the chemical hood). The concentration will be around 4%.
3. Dilute 1g Alizarin red s in 50ml distilled water to make a solution of 2% and adjust the pH to 4.1-4.3. Filter the solution before using trough a $0.22\mu M$ filter. The correct pH is critical!

2.2 Protocol

1. Wash the cells 2 times with PBS.
2. Transfer the cells to the chemical hood.
3. Add $100\mu l$ of 4 % formaldehyde solution to the cells.
4. Incubate for 15 minutes at room temperature.
5. Discard the formaldehyde solution into the correct chemical waste bin.
6. Wash the cells twice with $200\mu l$ distilled water.
7. Add $100\mu l$ of 2% alizarin red s solution and incubate at room temperature on shaker (300 rpm) for 20-30minutes (in the dark).
8. Discard the Alizarin red S and wash the cells 5 times with distilled water for 5 minutes on a shaker.
9. After removal of the final wash, leave the plates at an angel for 2 minutes, aspirate remaining fluid and dry.
10. Image the samples with a Brightfield microscope and define mineralization as % red in the wells

APPENDIX **D**

Results Optimization

D.1 Culture Medium

Table D.1: Overview of Direct Co-Culture media. The first column shows the origin and seeding ratio of MΦs and OBs. Cells originated from Mice or Humans are indicated with (M) or (H) respectively.

Cells	Medium			FBS	Pen-strep	L-glutamine	A.A	B-GL	Dexa	Other	Reference
MΦ : OB (ratio)	**DMEM**	**α-MEM**	**RPMI 1640**								
Raw 264.7 (M) : MG-63 (H) (2:1)	1	0	0	10%	1%	-	-	-	-	-	[23]
Donor (H) : Donor (H) (1:1)	1	0	0	5%	1%	$2mM$	-	$10mM$	$10^{-8}M$	-	[66]
Donor (H) : Donor (H) (1:1)	1	0	0	5%	$25\mu g/mL$	$2mM$	$50\mu g/mL$	-	-	-	[51]
C57BL/6J (M) :C57BL/6J (M) (1:1) & (5:1)	1	0	1	10%	1%	-	$100mg/mL$	$10mM$	$10 \times 10^{-8}M$	$10ng/mL$ M-CSF 30% LCM	[43]
U937 (H) : Donor MSCs (H) (2:1)	0	0	1	-	-	-	-	-	-	-	[67]
C57BL/6 (M) : C57BL/6 (M) (2:1)	0	0	1	10%	1%	-	-	-	-	-	[68]
C57BL/6Jc (M) : MC3T3 (M) (1:1)	0	1	1	10%	1%	-	$50\mu g/mL$	$10mM$	$10^{-8}M$	1% Glutamax 5% LCM	[22]
Raw 264.7 (M) : ADMSC (H) (1:1)	0	1	0	-	1%	$2mM$	$0.2mM$	$10mM$	$10^{-8}M$	5% platelet lysate $10U/mL$ heparin	[24]
C57BL/6J (M) : MC3T3 (M) (1:1)	0	1	1	10%	1%	-	$50\mu g/mL$	$10mM$	$10^{-8}M$	1% Glutamax 5% LCM	[69]
THP-1 (H) : Donor (H) (2:1, 1:1 & 0.5:1)	0	1	1	10%	-	-	$100\mu M$	$10mM$	$10^{-8}M$	-	[70]

D.2 Live/Dead

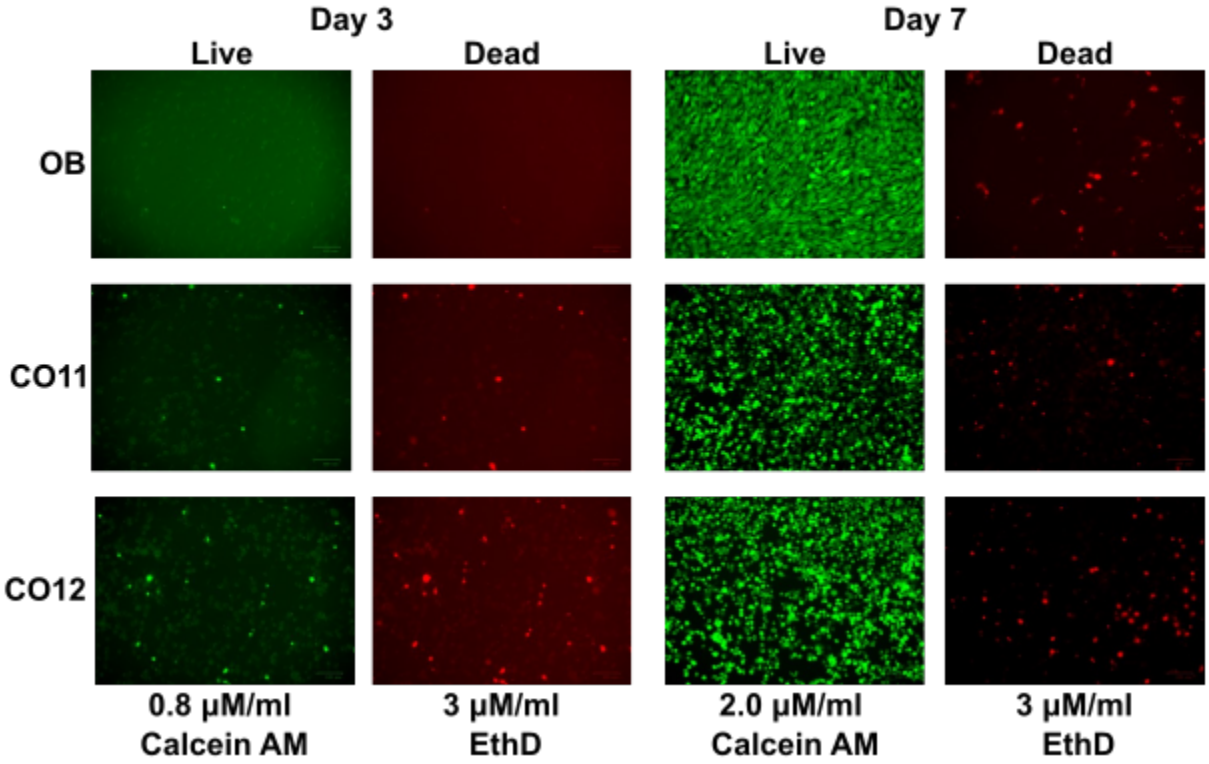

Figure D.1: Live/Dead (green/red) staining fluorescent images for OB, CO11 and CO12 on day 3 and day 7 under different concentrations of Calcein AM staining.

D.3 Dynamic cell seeding concentrations

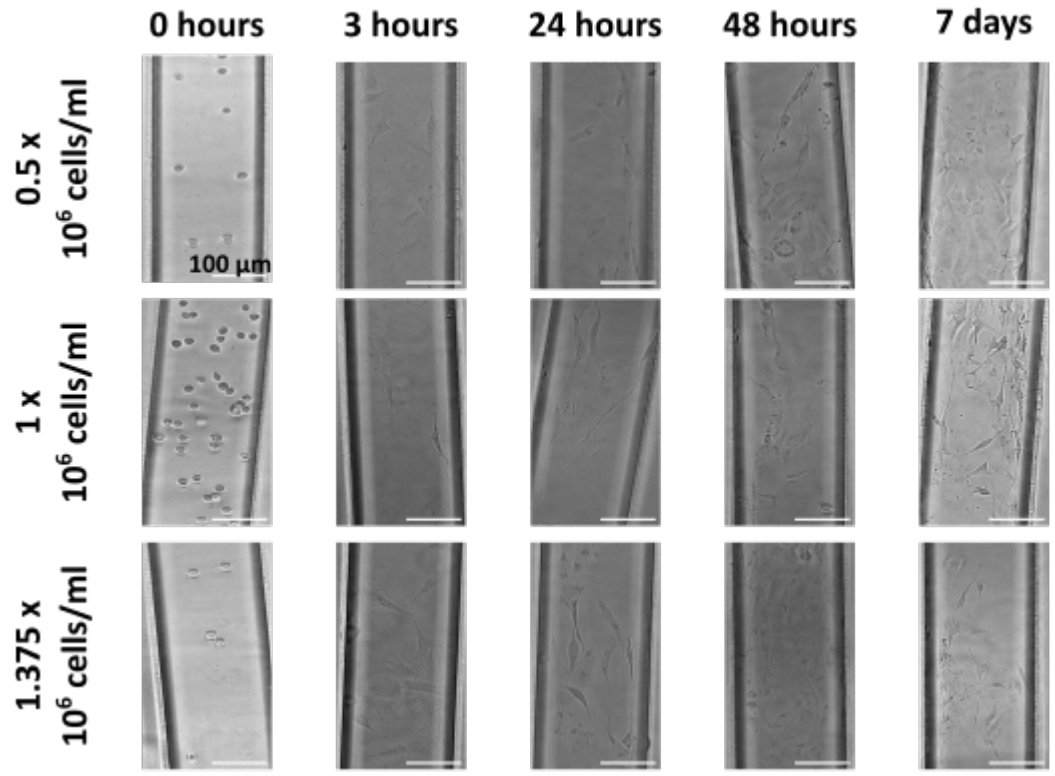

Figure D.2: Brightfield images of three different OB seeding concentrations, 0.5-, 1- and 1.375-$\times 10^6$ cells$/ml$

Appendix E

Additional Results

E.1 ARS results static direct co-culture

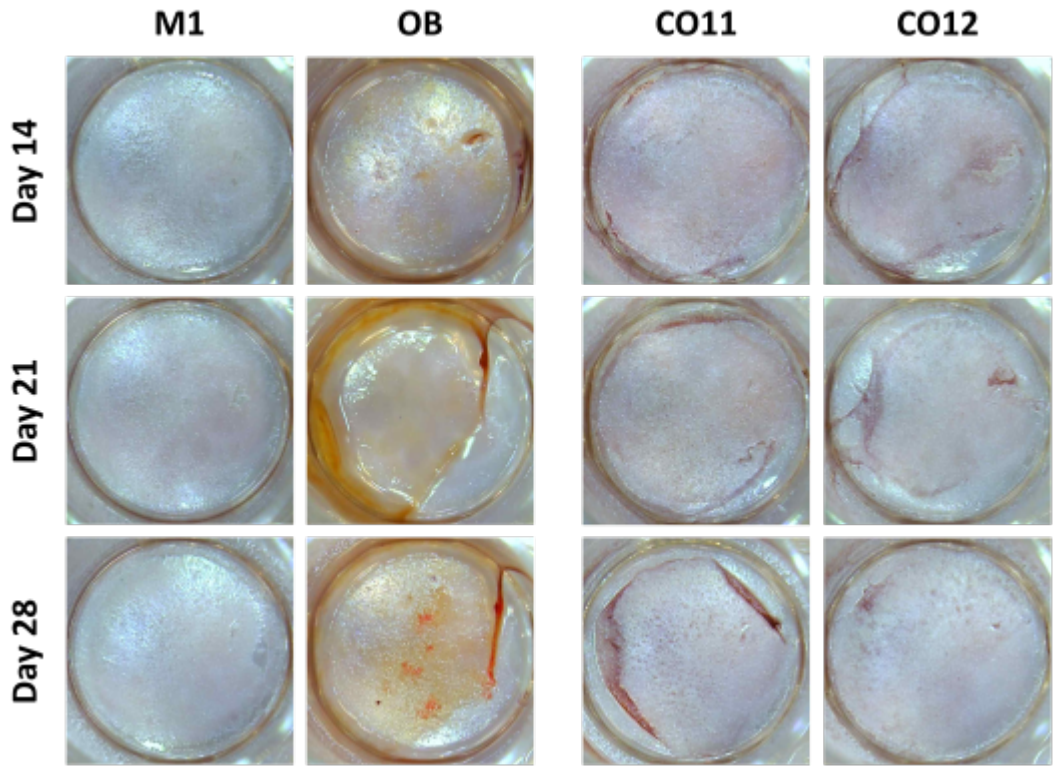

Figure E.1: Brightfield images of Alizarin Red Staining of M1, OB, CO11 and CO12 on days, 14, 21 and 28 after seeding.

E.2 Pattern Bright Field Images

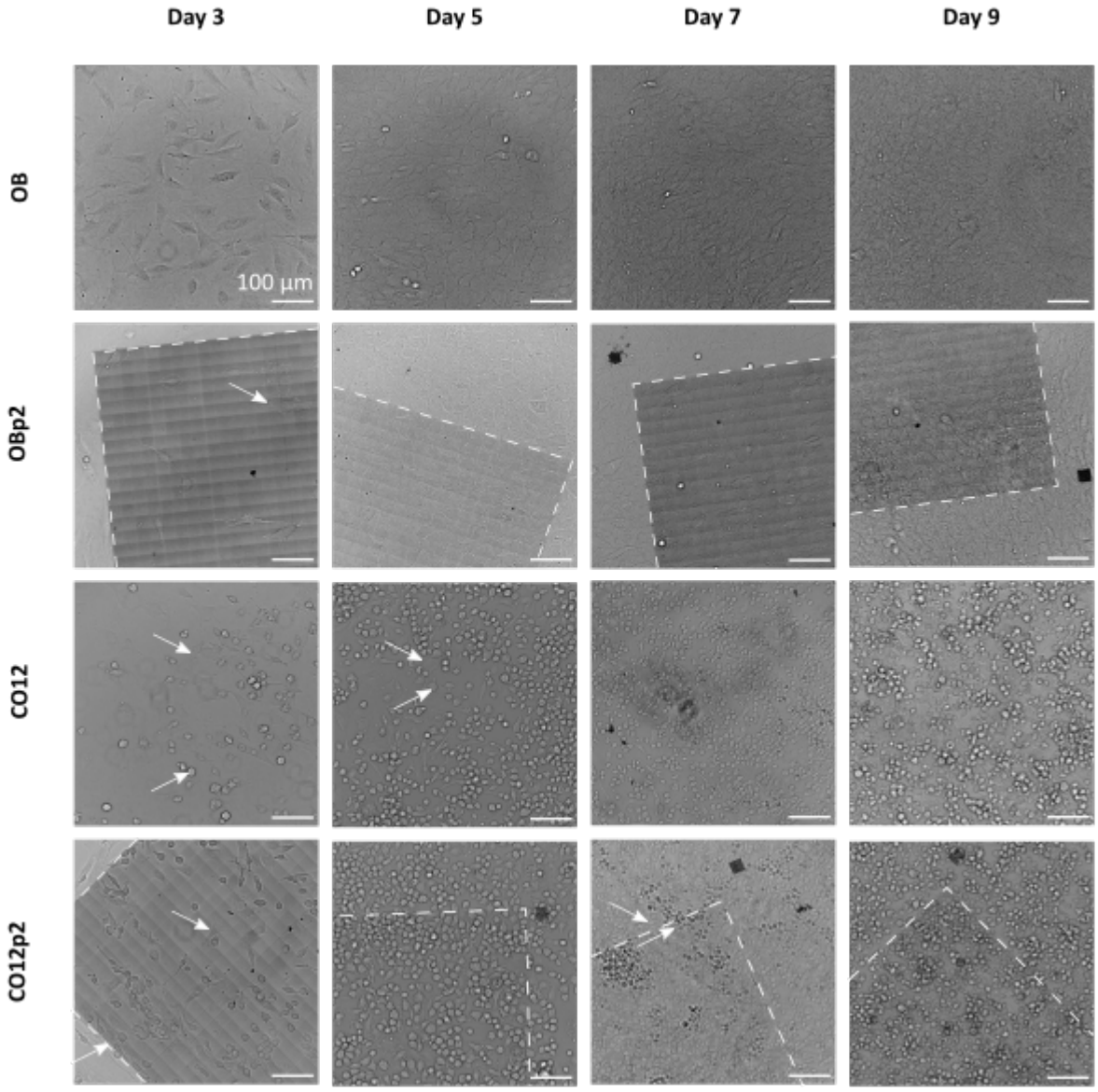

Figure E.2: Brightfield images of OB, OBp2, CO12 and CO12p2 on days, 3, 7 and 9 after seeding. White and Black arrows indicate M1 and OB cells respectively. The pattern borders have been indicated with a white line. Image contrast have been enhanced using FIJI [37]

E.3 Pattern ARS results D11

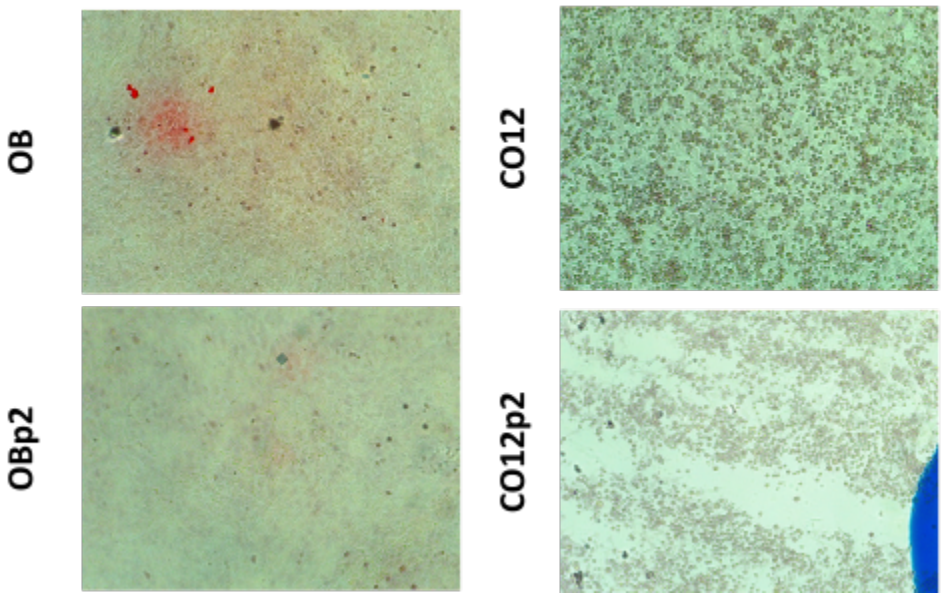

Figure E.3: Alizarin Red staining on pattern with OB and CO12

E.4 Dynamic direct co-culture Brightfield images

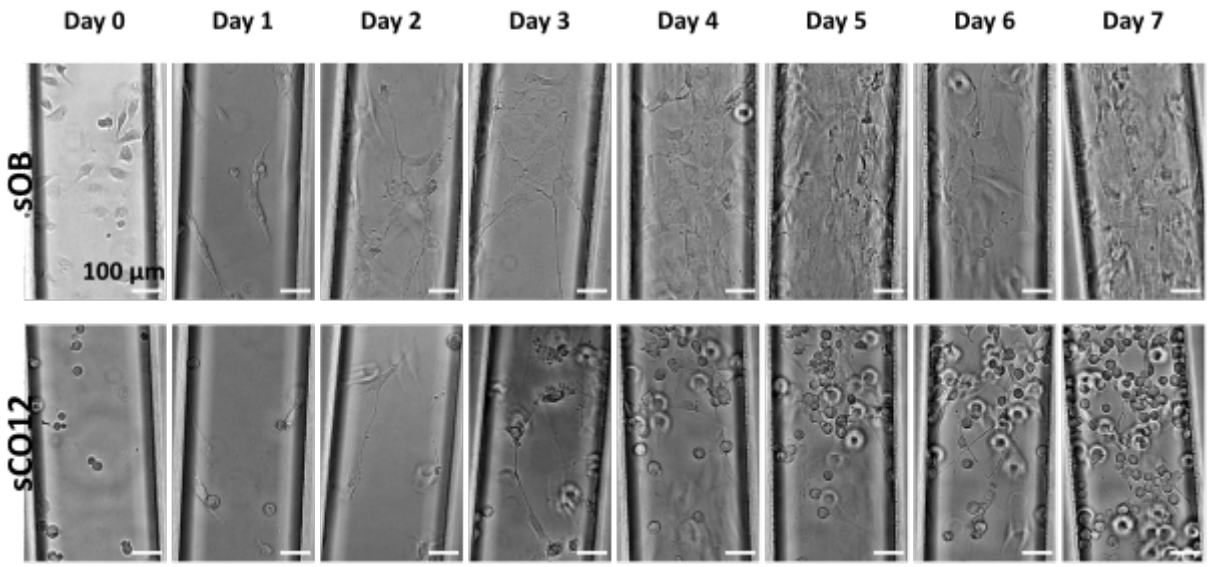

Figure E.4: sOB & sCO12 brightfield images on days 1-7

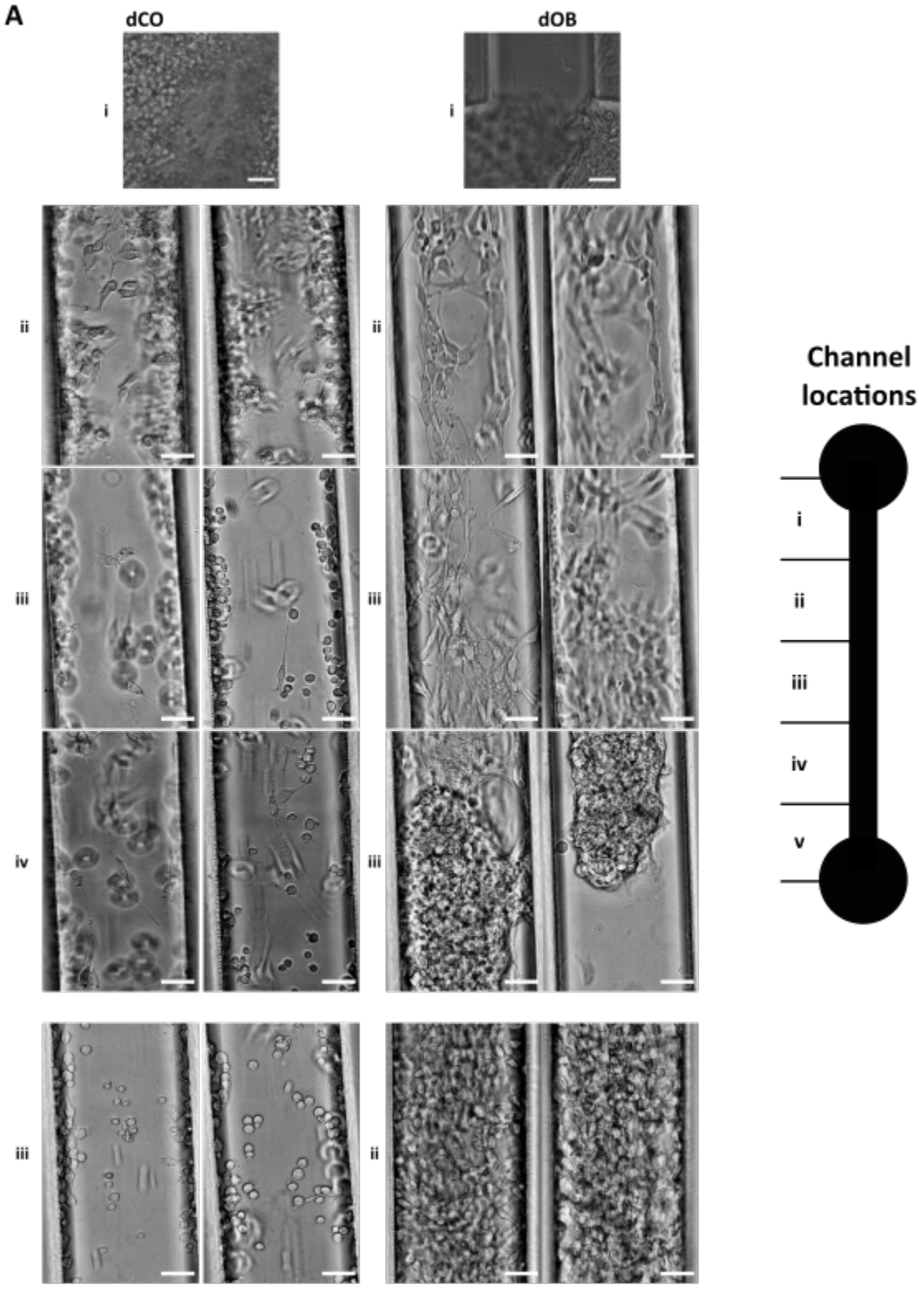

Figure E.5: sOB & sCO12 brightfield images on day 7 at different locations inside the channel

E.5 Dynamic co-culture microfluidic channel shear stresses

Table E.1: Resulting Wall shear stress τ_w and Reynolds number using different heights (h_0), widths (w_o) and flow rates (Q) of the channel. The bold and underlined rows are within the $0.03 - 0.7$ dyn/cm^2 range.

h_0 $[\mu m]$	w_0 $[mm]$	Q $[m^3/s]$	Q $[\mu l/min]$	Reynolds	τ_w $[dyn/cm^2]$
50	***1***	***1.67E-11***	***1***	***1.02E-10***	***0.00258***
100	1	1.67E-11	1	9.69E-11	6.45E-04
500	1	1.67E-11	1	7.11E-11	2.58E-05
1000	1	1.67E-11	1	5.33E-11	6.45E-06
50	3	1.67E-11	1	3.49E-11	8.60E-04
100	3	1.67E-11	1	3.44E-11	2.15E-04
500	3	1.67E-11	1	3.05E-11	8.60E-06
1000	3	1.67E-11	1	2.66E-11	2.15E-06
50	***1***	***1.67E-10***	***10***	***1.02E-09***	***0.0258***
100	***1***	***1.67E-10***	***10***	***9.69E-10***	***0.00645***
500	1	1.67E-10	10	7.11E-10	2.58E-04
1000	1	1.67E-10	10	5.33E-10	6.45E-05
50	***3***	***1.67E-10***	***10***	***3.49E-10***	***0.0086***
100	***3***	***1.67E-10***	***10***	***3.44E-10***	***0.00215***
500	3	1.67E-10	10	3.05E-10	8.60E-05
1000	3	1.67E-10	10	2.66E-10	2.15E-05
50	***1***	***1.67E-09***	***100***	***1.02E-08***	***0.258***
100	***1***	***1.67E-09***	***100***	***9.69E-09***	***0.0645***
500	***1***	***1.67E-09***	***100***	***7.11E-09***	***0.00258***
1000	1	1.67E-09	100	5.33E-09	6.45E-04
50	***3***	***1.67E-09***	***100***	***3.49E-09***	***0.086***
100	***3***	***1.67E-09***	***100***	***3.44E-09***	***0.0215***
500	3	1.67E-09	100	3.05E-09	8.60E-04
1000	3	1.67E-09	100	2.66E-09	2.15E-04
50	***1***	***1.67E-08***	***1000***	***1.02E-07***	***2.58***
100	***1***	***1.67E-08***	***1000***	***9.69E-08***	***0.645***
500	***1***	***1.67E-08***	***1000***	***7.11E-08***	***0.0258***
1000	***1***	***1.67E-08***	***1000***	***5.33E-08***	***0.00645***
50	***3***	***1.67E-08***	***1000***	***3.49E-08***	***0.86***
100	***3***	***1.67E-08***	***1000***	***3.44E-08***	***0.215***
500	***3***	***1.67E-08***	***1000***	***3.05E-08***	***0.0086***
1000	***3***	***1.67E-08***	***1000***	***2.66E-08***	***0.00215***

E.6 CCR7/CD206

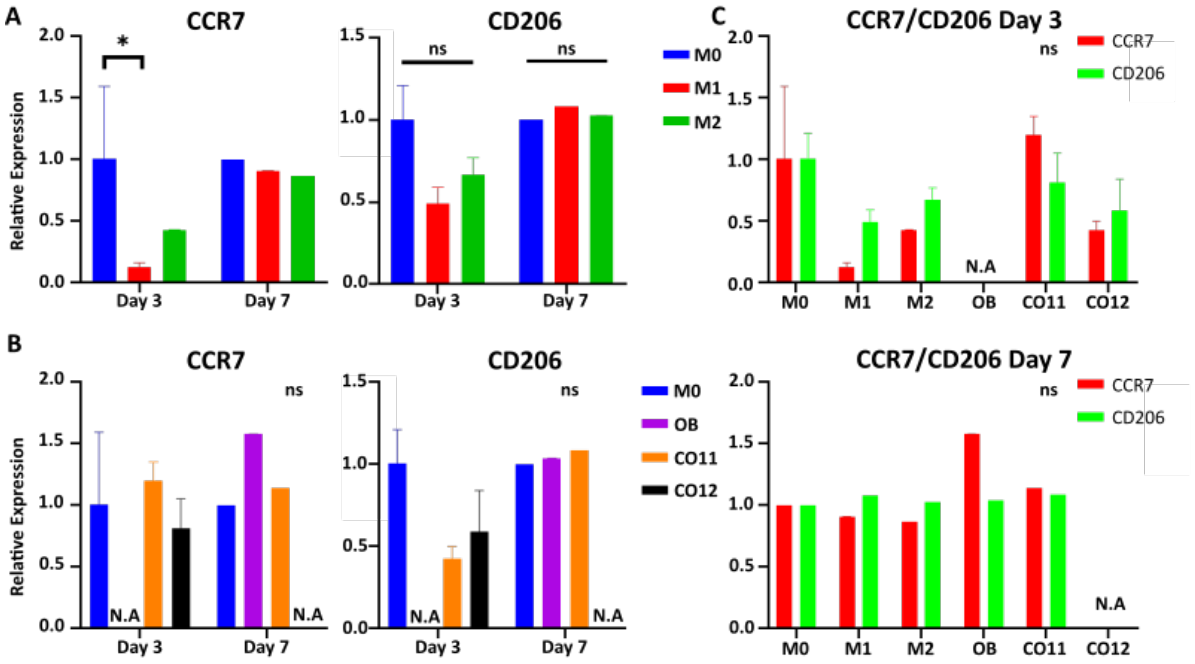

Figure E.6: **A)** Relative CCR7 and CD206 Expression (mean ± SD) of J774A.1, M0, M1 and M2 on days 4 and 7. **B)** Relative CCR7 and CD206 Expression (mean ± SD) of MC3T3-E1 (OB), and MC3T3-E1:J774A.1 co-cultures with ratios 1:1 and 1:2 (CO11 & CO12). **C)** CCR7/CD206 differences for all experimental conditions on Day 3 and Day 7. N.A. = images were not available

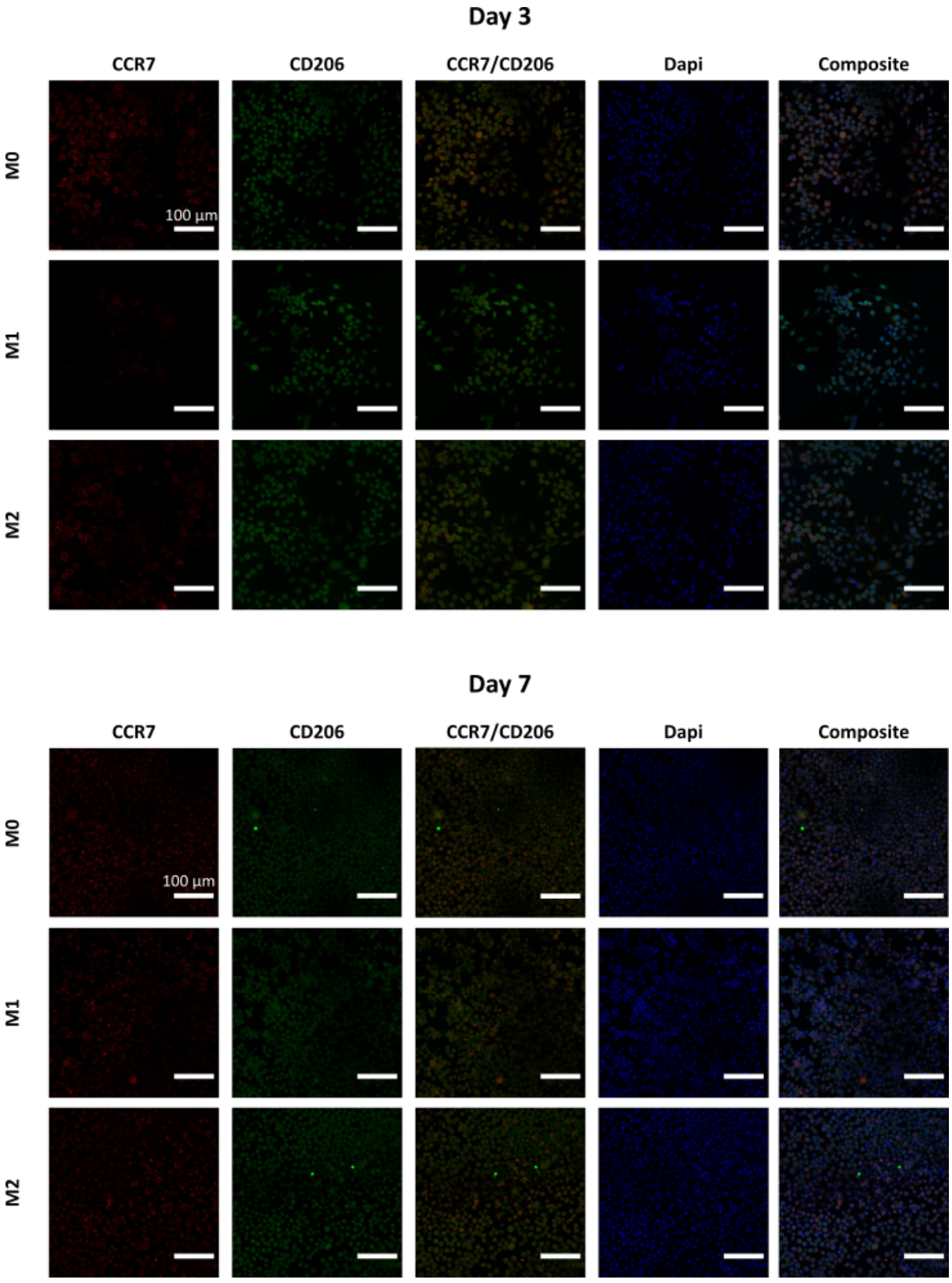

Figure E.7: Fluorescent images CCR7/CD206 of M0, M1 & M2 on day 3 and 7. N.A. = images were not available

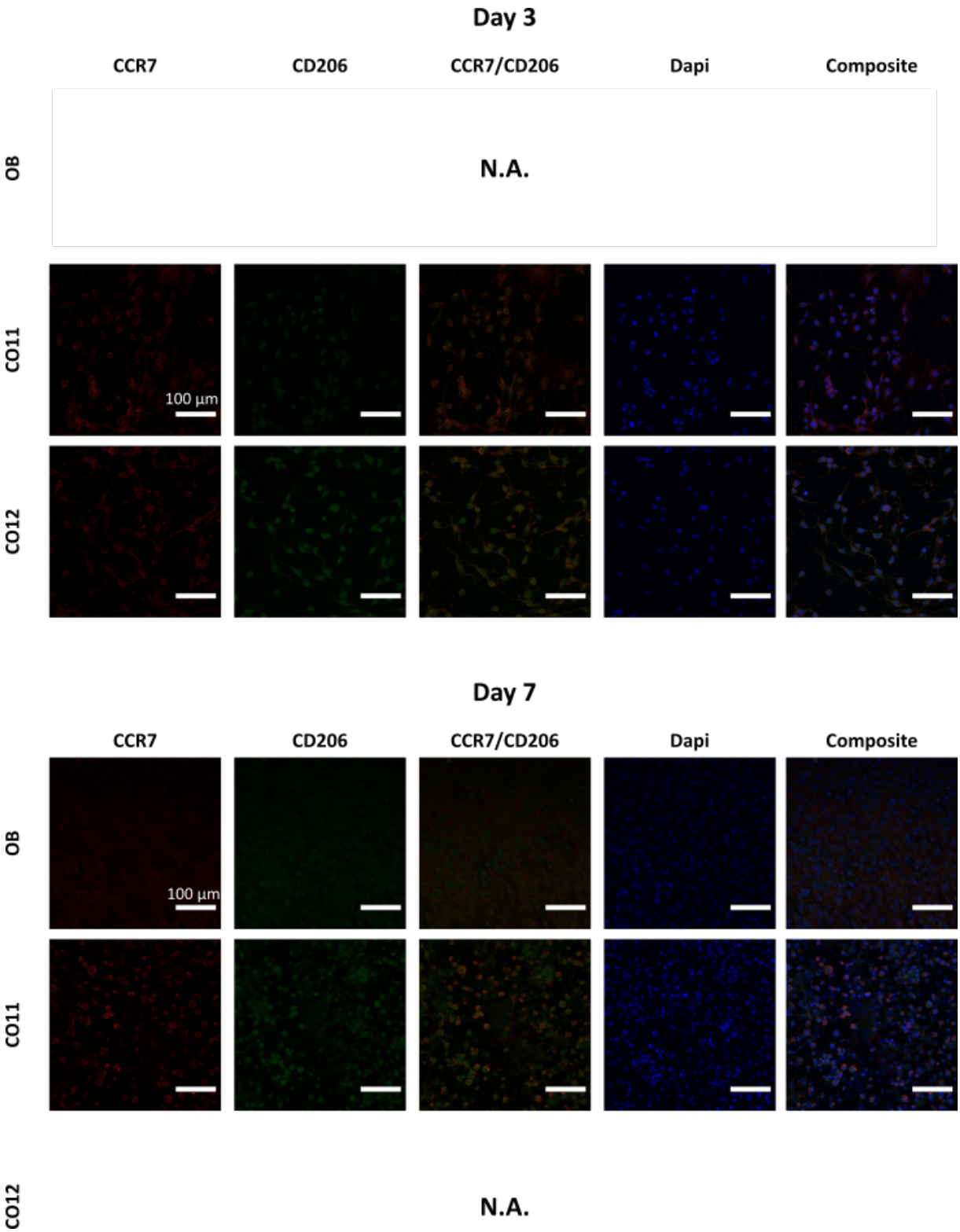

Figure E.8: Fluorescent images CCR7/CD206 of oB, CO11 & CO12 on day 3 and 7. N.A. = images were not available