Incorporation of Resident Macrophages in Engineered Tissues: Multiple Cell Type Response to Microenvironment Controlled Macrophage-laden Gelatin Hydrogels

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Abstract

The success of tissue engineering strategy is strongly related to the inflammatory response, mainly through the activity of macrophages that are key cells in initial immune response to implants. For engineered tissues, the presence of resident macrophages can be beneficial for maintenance of homeostasis and healing. Thus, incorporation of macrophages in engineered tissues can facilitate the integration upon implantation. In this study, we developed an *in-vitro*

model of interaction between encapsulated naive monocytes, macrophages induced with M1/M2 stimulation and incoming cells for immune assisted tissue engineering applications. To mimic the wound healing cascade, Naive THP-1 monocytes, endothelial cells, and fibroblasts were seeded on the gels as incoming cells. The interaction was first monitored in the absence of the gels. In order to mimic resident macrophages, THP-1 cells were encapsulated in the presence or absence of IL-4 to control their phenotype and then these hydrogels were seeded with incoming cells. Without encapsulation, activated macrophages induce apoptosis in endothelial cells. Once encapsulated no adverse effects were seen. Macrophage-laden hydrogels attracted more endothelial cells and fibroblasts compared to monocytes-laden hydrogels. The induction (M2 stimulation) of encapsulated macrophages did not change the overall number of attracted cells; but significantly affected their morphology. M1 stimulation by a defined media resulted in secretion of both pro and anti-inflammatory cytokines compared to M2 stimulation. We demonstrated that there is a distinct effect of encapsulated macrophages on the behavior of the incoming cells; this effect can be harnessed to establish a microenvironment more prone to regeneration upon implantation.

Keywords: Macrophage, Hydrogel, Cell/Cell interactions, Foreign Body Response, Gelatin, Microenvironment

1. Introduction

Once a material is implanted into the body, adverse immune reactions can be triggered. Cells receive a diverse range of signals from their surrounding microenvironment and adjust their responses accordingly.(Kzhyshkowska, Gudima et al. 2015) The immune system recognizes the material as foreign, initiating a macrophage-mediated acute inflammatory phase.(Sridharan, Cameron et al. 2015) Inflammatory cells initially are recruited to the site of inflammation due to the endothelial response to injury Monocyte responds to monocyte chemoattractant protein-1 (MCP-1), which is secreted by cytokine-stimulated endothelial and smooth muscle cells in the intima. In response to cytokines secreted by endothelial cells, fibroblasts, and other cells, the incoming monocytes at the site of injury differentiates into macrophages.(Takahara, Kashiwagi et al. 1996) This chain of events makes macrophages an important factor in the acceptance or rejection of implanted biomaterials.

Another important aspect of the fate of engineered tissues is their integration with the host vasculature; as angiogenesis is involved in wound healing by promoting the outgrowth of new blood vessels from preexisting vasculature. Inflammation also plays a major role in physiological angiogenesis. Inflammatory cells such as macrophages secrete a multitude of inflammatory mediators of which may influence angiogenesis.(Coussens and Werb 2002) Thus it is important to establish a strong control over the cross-talk between endothelial cells and macrophages in the vicinity of engineered tissues.

The success of the tissue engineering strategy is strongly related to the inflammatory response, mainly through the activity of macrophages. They can be polarized either into M1 or M2 phenotype, depending on the activation signals. (Kzhyshkowska, Gudima et al. 2015). M1 macrophages are considered to be pro-inflammatory, while M2 macrophages promote tissue regeneration. However, the exploitation of these cells to control engineered tissue integration

has not been widely studied.(Vrana 2016) Resident macrophages are cells observed in most of tissues with a heterogenous phenotype.(Davies, Jenkins et al. 2013) They are involved in wound healing and resolution of inflammation; thus encapsulation of monocytes or pre-differentiated macrophages in hydrogels can mimic this function in tissue engineering applications. Some examples of resident macrophages are microglia, Kupffer cells, alveolar macrophages and osteoclasts.(van de Laar, Saelens et al. 2016)

One of the main means for macrophages to control the microenvironment around the implanted biomaterials is the cytokine production, M1 macrophages produce larger amounts of the pro-inflammatory cytokines: TNF-α (tumor necrosis factor alpha), IL-12, and IL-1β. TNF-α is a master pro-inflammatory cytokine involved in chronic inflammation.(Munker, Gasson et al. 1986) IL-12 promotes T_H1 cell-mediated responses, which have been implicated in the pathogenesis of a number of inflammatory and autoimmune diseases.(Teng, Bowman et al. 2015) IL-1β is an important mediator of the inflammatory response.(Dinarello 2009) M2 macrophages produce anti-inflammatory cytokines such as IL-1RA, CCL18. IL-1RA is a natural inhibitor of the pro-inflammatory effect of IL-1β. IL-1RA is used to treat autoinflammatory diseases such as rheumatoid arthritis and juvenile idiopathic arthritis. (Dinarello 2009) CCL18 is a chemokine, *i.e.* a cytokine with chemotactic activity.(Struyf, Schutyser et al. 2003) CCL18 is produced by myeloid cells and induces chemotaxis of lymphocytes and immature DCs, as well as collagen deposition by fibroblasts.(Schutyser, Richmond et al. 2005) Although, it is not one of the main components of macrophage secretome; Interleukin 4 (IL-4) is one of the main factors in macrophage polarization. It drives the differentiation of naive T helper (T_H) cells to T_H2 effector cells and monocytes and macrophages to an M2 (or alternatively) activated phenotype.(Martinez and Gordon 2014) As can be seen from the aforementioned activities of these cytokines, the cytokine

microenvironment around an engineered tissue would significantly affect its remodeling and integration and needs to be controlled.(Knopf-Marques, Singh et al. 2016)

Wound healing cascade can be divided in three steps. The initial inflammatory step is characterized first by the formation of blood clot and the mobilization of neutrophils and then macrophages to the site of injury.(Sinno and Prakash 2013) Then there is a "proliferative phase" with the formation of a granulation tissue. At this stage, endothelial cells are crucial because neovascularization will enable the procurement of the nutrients to the wound and this will lead to the formation of a new stroma. The last stage is the "maturation phase" which is characterized by the transition from granulation tissue to scar formation. (Levenson, Geever et al. 1965, Jun, Kim et al. 2015) In this step, fibroblasts are of interest because they will secrete collagen and they will be the key players to remodel extracellular matrix. In all these three stages and especially in the last two, macrophages will continuously secrete growth factors that will stimulate angiogenesis and collagen deposition and that is why these three cell types are the main actors for tissue remodeling after injury.

One way to understand and control these complex interactions is to develop simplified in vitro models.. However, currently there are no models that directly deal with the interaction of immune cells with connective tissue cells in tissue engineering context. A recent example of monitoring of cell-cell interactions is an intestine organ-on-chip study by Kim and colleagues (2016)(Kim, Li et al. 2016). The cells are exposed to physiological peristalsis-like motions and fluid flow. The systems involves a co-culture human intestinal endothelial and immune cells, in the presence of bacteria in order to elucidate how they contribute to host tolerance of infection and disease inflammation.(Kim, Li et al. 2016). However, in order to assess regeneration in a multi-cellular environment a model should integrate extracellular matrix component. One way to achieve this is to encapsulate cells in ECM like hydrogels as the three-dimensional (3D) cell

culture in vitro environment is a crucial key for acquiring phenotypes and for responding to stimuli analogous to *in vivo* biological systems.(Szot, Buchanan et al. 2011)

The main objective of this study is to develop an *in-vitro* model of interaction between incoming cells and encapsulated macrophages as a model of inclusion of resident macrophages in engineered tissues. Fibroblasts and endothelial cells have been selected in co-culture condition with macrophages due to their key role in wound healing process. Thus, in a tissue engineering setting it is important to control the incoming cells to have a precise control over the remodeling of the implanted engineered tissue. For this end, we observed the attachment, morphology and cytokine profile in different settings pertaining to wound healing cascade in the presence of macrophages in M1/M2 phenotype inducing microenvironments. Through this model, we aim to define the optimal inclusion conditions for macrophages in engineered tissues.

2. Materials and Methods

2.1. Materials

Gelatin Type A from porcine skin and PMA were purchased from Sigma-Aldrich (St Quentin Fallavier, France). M1 and M2 macrophage generating media, Interleukin-4, HUVEC cells, Endothelial cell growth medium, Apoptotic/Necrotic/Healthy Cell kit, Fluorimetric cell

viability kit I and DAPI were purchased from Promocell (Heidelberg, Germany). THP-1 (human) and NIH-3T3 (murine) cells were purchased from ATCC in frozen form (Manassas, US). RPMI-1640 and DMEM media, Dulbecco's Phosphate buffered saline, Fetal bovine serum, 0.05% trypsin/0.02% EDTA, TripLE™ Express (1x), β-mercaptoethanol, Cell tracker™ and Vybrant® Cell Adhesion Assay Kit were obtained from Life Technologies (Carlsbad, USA). Transwell plates were purchased from Millipore. Bacterial transglutaminase was kindly provided by Ajinomoto Inc (Tokyo, Japan).

2.2. Methods

The cell culture was done with different cell types: naïve THP-1, 3T3 fibroblasts, HUVEC, THP-1 cells in M1 and M2 macrophage polarization media. The experiments were done under two different conditions: **(A)** non-contact cell co-culture, it means with a transwell (Figure 1a) and **(B)** contact cell co-culture, with cells encapsulated in a gel, as shown in Figure 2a.

The THP-1 (a human monocytic cell line) were cultured in RPMI 1640 GlutaMAX supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 0.2% fungizone and 0.05 mM 2-mercaptoethanol. To differentiate the monocytes to macrophages, cells were treated with 50 ng/ml of phorbol myristate acetate (PMA) which is dissolved in media (RPMI 1640 without 2-mercaptoethanol) for 24 h at 37 °C, 5% CO_2. Unattached cells were removed after washing with DPBS. PMA activated THP-1 cells were detached using TripLE ™ Express (1x), centrifuged and re-suspended in media (without 2-mercaptoethanol).

NIH-3T3 cells were cultured in RPMI 1640 (Gibco Life Technologies, USA) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, 0.2% fungizone at 37 °C in a 5% CO2 atmosphere. Prior to seeding, cells were harvested using 0.05% trypsin/0.02% EDTA, centrifuged and re-suspended in media.

Human Umbilical Vein Endothelial Cells (HUVEC) were used at passages between 4 and 8. The culture media used were endothelial cell growth medium supplemented with Supplement Mix C-39215 (mainly composed of heparin, hydrocortisone, fetal calf serum, basic fibroblast growth factor, epithelial growth factor and endothelial cell growth supplement) and 1% penicillin/streptomycin. Prior to seeding, cells were harvested using 0.05% trypsin/0.02% EDTA, centrifuged and re-suspended in media.

M1 and M2 phenotypes are generally characterized by specific cytokine inductions of a variety of phenotype marker expression, such as cytokines, surface markers, bioactive molecules such as ROS, NO and chemokines. The commercially available M1 and M2 generation media from PromoCell (Heidelberg, Germany) contains the necessary cytokines to induce M1 or M2 differentiation. In this configuration, they were used to induce a specific stimulation microenvironment. The cells were pre-treated with the differentiation medium prior to the seeding of incoming cells .(Gordon 2003)

(A) Non-contact cell co-culture

Transwell® permeable supports (3-µm porous polyethylene terephthalate (PET) inserts) (Millicell®, Millipore, France) were utilized for non-contact cell co-culture. THP-1 cells (1.10^6 cells.mL^{-1}) were added to mono-culture wells (except for the condition "no THP-1"), and 10 000 HUVEC cells were added in the Transwell® inserts (Figure 1A). THP-1 cells were either naive THP-1 or activated directly (presence of PMA in the culture medium: SET A) or pre-activated one day before the experiment (Activation in PMA containing medium before co-

culture experiments, to avoid the effects of PMA on the other cell type: SET B). SET C: THP-1 cells were either naive THP-1 or activated directly (presence of IL-4 in the culture medium) or pre-activated for six days before the experiment.

(B) Contact cell co-culture

For all cell experiments, the same protocol of seeding was used.

Gelatin type A solution was prepared in cell culture medium (RPMI 1640 GlutaMAX for THP-1) under sterile condition. All the solutions, 6% gelatin (w/v) and 20% (w/v) of TGA solution in PBS were filtered prior to use (0,22 µm). THP-1 cells were centrifuged in order to get cells pellet. Then gelatin type A solution was added in order to get a cell density of $6x10^6$ cells.mL^{-1} solution and kept in water bath at 37°C. 50 µL of gelatin solution with encapsulated cells (300 000 cells/hydrogel) were then deposited on top of 10 µL of TGA solution in order to get homogeneous crosslinking and then put in incubator at 37°C for at least 15 minutes prior to add cell culture medium in each well. The encapsulated cells were cultured for 3 days in different media (RPMI-1640 with or without 10 ng/mL of IL-4, M1 or M2 media). We have previously demonstrated that transglutaminase crosslinked gelatin structures can be loaded and can release growth factors and cytokines (Barthes, Vrana et al. 2015). On the 3rd day, the cell culture medium was removed and 50 000 or 100 000 incoming cells (HUVEC, THP-1 or 3T3) were added to all the samples. Cells were cultured for a further 3 days or 6 days. The metabolic activity of those cells was measured by Alamar Blue assay to assess cell proliferation, and cytokine (IL-1ß, IL-1RA, IL-4, IL-10, IL-12, CCL-18 and TNF-α) analysis of supernatants was done by ELISA.

2.2.1 Metabolic activity

To assess metabolic activity, samples were incubated with 10% v/v Resazurin (Fluorometric cell viability kit I, PromoKine, Germany) in cell culture medium for 2 h. The substrate will become fluorescent (red) when incubated with viable cells due to reduction. The amount of fluorescence was monitored with a SAFAS Xenius XML fluorescence reader (SAFAS, Monaco) at excitation 560 nm and emission wavelength 590 nm.

2.2.2 Apoptotic/Necrotic/Healthy Cell Assay

Apoptotic/Necrotic/Healthy cell assay allows to detect apoptotic (green), necrotic (red) and healthy (blue only) cells using fluorescent microscopy. Staining was made according to the manufacturer's procedure (PromoCell, Germany). Briefly, samples were washed twice with binding buffer. Incubated for 15 min at RT and protected from light in a solution containing FITC-Annexin V, Ethidium homodimer III and Hoechst 33342. For 100 µL of binding buffer 5 µL of each dye was added. Samples were washed again twice and kept in binding buffer for imaging. Fluorescence images were performed using Nikon Eclipse Ti-S with a 10x PL Fluor (0.30 NA) objective equipped with Nikon Digital Camera (with NIS-Elements software), and processed with ImageJ.

2.2.3 Cytokine detection by ELISA

Cell culture media was collected at days 3 and 6 and the cytokine amounts in the media was quantified by ELISA developer kits. As the concentration of specific cytokines are not known for M1 and M2 media, standard curves were done using the relevant sample media type. Absorbance measurements were done @450 nm. The cytokine amounts were calculated using the standard curves. OD cut-off is set as the OD value for standard concentration of 0 ng/mL. Following cytokines were quantified: IL-1ß, IL-1RA, IL-4, IL-10, IL-12, CCL-18 and TNF-α.

2.2.4 Cell Pre-Labelling

In order to differentiate the encapsulated cells from the incoming cells, a pre labelling was done with cell tracker or calcein-AM. Briefly, to stain 1 million cells, either 5µL calcein or 1µL cell tracker was added to 1 mL serum free medium. Cells were centrifuged in order to get cells pellet and resuspended in staining solution. Incubated for 30 minutes at 37°C in the dark and washed with PBS using centrifugation. Cells were resuspended in gelatin for encapsulation or in culture medium for the seeding on top of the gel. Fluorescence images were performed using Nikon Eclipse Ti-S with a 10x PL Fluor (0.30 NA) objective equipped with Nikon Digital Camera (with NIS-Elements software), and processed with ImageJ.

2.2.5 Scanning electron microscopy (SEM).

The samples were fixed with 4% glutaraldehyde. The specimen were washed with DPBS prior to following a dehydration protocol using an alcohol series of increasing concentration (70%, 95% and 2 × 100%), with incubation periods of 5 min for each. Subsequently, samples were incubated in 100% ethanol/hexamethyldisilazane (HMDS) (1:1) for 5 min, then only in HMDS for 2x 5 min and dried overnight. Samples were adhered onto titanium discs using a carbon tape and coated with gold/palladium in a sputter coater. The samples were sputtered at 7.5 mA for 3 min under argon atmosphere and images were acquired using a scanning electron microscope (Hitachi TM1000).

2.2.6 Statistical Analysis

The statistical significance of the obtained data was assessed using the t-test or Mann-Whitney test ($n \geq 3$). The error bars are representative of Standard Deviation (SD). Differences at $p \geq 0.05$ were considered statistically not significant.

3. Results and Discussion

Any implanted cell-containing material *in vivo* will be in contact with several incoming cell types including monocytes/macrophages, endothelial cells and connective tissue cells such as fibroblasts. Our working hypothesis is that addition of a macrophage component in such systems to mimic "resident macrophages" and their role in wound healing will help the regeneration process. As macrophage function is tightly linked to their physicochemical niche, encapsulation in hydrogels might provide the necessary signal for encapsulated macrophages to assume a more resident macrophage-like phenotype. In order to assess the effects of the macrophage presence, we have developed a system where pre-labelled cells of different types can be directly added onto macrophage-laden hydrogels in order to assess their interaction with the encapsulated macrophages. In order to determine the effect of encapsulation, the interaction was first monitored in the absence of the gels with a transwell system (paracrine interactions).

(A) Transwell system

In order to see the extent of the reaction of HUVECs to the presence of THP-1 cells without cell to cell contacts; naive and activated THP-1 cells were put into co-culture conditions with HUVECs with the help of a transwell inserts. In the absence of THP-1 cells, HUVECs formed a monolayer by 5 days with an increase in metabolic activity (Figure 1B); in the presence of THP-1 cells however, particularly with activated THP-1 cells; HUVECs had significantly lower metabolic activity (Figure 1B) with substantial number of cells going through apoptosis as evidenced by positive Annexin-V staining (Figure 1C, D). In order to see whether this effect has any direct relation with the macrophage phenotype; the macrophages were put in contact with IL-4 (M2 inducer) (Figure S1); aside from M2 inducing capacity of IL-4; it has also been shown that IL-4 dependent proliferation can be seen in resident macrophages.(Davies, Jenkins et al. 2013) Addition of IL-4 did not have a significant effect on metabolic activity of HUVECs;

however the number of alive attached cells were significantly higher in the presence of IL-4 (Figure S1 B and C). The IL-4 has been shown to induce V-CAM expression by vascular endothelial cells (Iademarco, Barks et al. 1995), but there are no reports on the effect of IL-4 on HUVEC proliferation; thus the differences in the metabolic activity can be attributed to the secretions of monocytes that changes with the presence of IL-4.

(B) Contact cell co-culture

In order to see the effect of encapsulated THP-1 cells on different cell types implicated in wound healing, (endothelial cells, fibroblasts and incoming monocytes/macrophages), we encapsulated THP-1 cells in the presence or absence of IL-4 to control their phenotype. Then we seeded these hydrogels with naive monocytes, endothelial cells and fibroblasts (Figure 2A). The presence of IL-4 does not have an effect on the metabolic activity of encapsulated THP-1 cells (Figure 2B). After 6 days, the number of attached incoming fibroblasts were significantly higher compared to monocytes and endothelial cells (Figure 2C and D). Even though there was no significant difference between the number of attached cells with respect to the IL-4 presence; in the case of fibroblasts, the cells on the gel containing IL-4 were more spread compared to no IL-4 conditions (Figure 2D). As IL-4 is known to induce synthesis of fibronectin (Postlethwaite, Holness et al. 1992) by fibroblasts; the increase in spreading can be attributed to the presence of more self-secreted ECM proteins on the gelatin substrate induced by either directly via IL-4 presence or the secretions of macrophages activated with IL-4. When the cross-sections of the hydrogels are observed; THP1 cells could be seen as individual cells within the hydrogel structure and the incoming cells on the surface of the structure. When the gel surfaces were observed with SEM; macrophages which escaped from the gel were visible together with individual endothelial cells (which had a more defined shape in the case of no IL-4, Figure 3A) and well spread, spindle shaped fibroblasts with significant granular material around them; which could be new ECM secretion. The organization of fibroblasts were more apparent in the

case of IL-4 treatment (Figure 3B). In both cases no significant in-growth of the incoming cells were observed in the given timeframe. On day 6 the IL-4 treated group had significantly more release of two important anti-inflammatory cytokines IL-1RA and CCL-18 (Figure 3C). The release profile of these cytokines were even more profoundly affected when the incoming cells are monocytes, due to the additional secretion by the incoming cells after paracrine interaction with the encapsulated macrophages (Figure S2B).

In tissue, following the initial immune reaction, resident macrophages contribute to the resolution of inflammation by IL-10 and TGF-beta dependent pathways; thus the presence of macrophages within an implanted tissue can facilitate the resolution process by having an indirect effect on the incoming cells. In order to see the distinct effect of M1/M2 inducing microenvironments, we have used specific media that has M1 and M2 macrophage polarization inducing properties (Figure 4A). The medium composition in these conditions has a significant effect on encapsulated macrophage metabolic activity within M1 induction conditions the metabolic activity was higher (Figure 4B). M1/M2 induction has opposing effects on fibroblast and endothelial cell attachment; where in M1 induction case more fibroblasts were seen to spread on the surface of the hydrogels; particularly at the borders (Figure 4C,D), in M2 induction the effect was more apparent on endothelial cells where in M2 conditions a nearly confluent layer of endothelium was formed on the gel surface (Figure 4 C). M1/M2 media induced the organization of the attached cells more apparently compared to no induction or IL-4 induction conditions. However, direct M1/M2 induction does not have a significant effect on cytokine secretions, as all tested cytokines except CCL18 were below detection limits (data not shown).

The observations here are in line with previous simulation work where Pettet et al (1995)(Pettet, Byrne et al. 1996) developed a mathematical model describing angiogenesis in soft tissue wound healing. This model is based on the evolution of capillary-tip endothelial cells and the

formation of new blood vessels in the direction of the wound site. Through a system of partial differential equations, they hypothesize that the migration of endothelial cells and the blood vessel density is deeply correlated to the local concentration of chemoattractant secreted by macrophages present in the wound site. Endothelial cells migrate in the direction of increasing macrophage derived chemoattractant which lead to the increase of blood vessel density. They also took into account a self-regulatory mechanism that will decrease the secretion of chemoattractant once blood vessel density reach a critical value. This example show the cross talking between endothelial cells and macrophage which is crucial for angiogenesis. With our system based on the encapsulation of macrophages, we aim to exert more control over the secretions of the chemoattractant by inducing a specific microenvironment for incoming macrophages. This should accelerate angiogenesis which is necessary to supply nutrient in the wound site. In the current model developed we demonstrated that the nature of induction (single stimulant or specific polarization inducing media) had a direct effect on incoming cell attachment and can be used to regulate the chemokine profile secreted by macrophages. With the aim of observing if M1/M2 stimulated macrophages have a pronounced effect on the cytokine profiles of incoming monocytes, the model was also done by incoming naive THP-1 cells; which induced moderate attachment of incoming cells. The M1/M2 induction resulted in similar amount of THP-1 cell attachment on gel surface (Figure S2); however there was a significant difference in the total amount of secreted cytokines (Figure 5). For both pro- and anti-inflammatory cytokines M1 induction medium was more potent in induction of secretion; for the case of pro-inflammatory cytokines there is an initial high secretion which then decreased (TNF-alpha and IL1beta); for anti-inflammatory IL1RA a similar trend was observed whereas for IL-10 and CCL-18 there is a time dependent manner increase . For both IL1RA and CCL-18 the secreted amount was higher than IL-4 induction cases; which demonstrates the specific advantage of using more defined induction cocktails. It should be pointed out that, due

to the presence of a surrounding 3D protein structure, the polarization effect of M1/M2 induction media did not create specific macrophage phenotypes. Thus, the effects observed were more linked to the cytokine microenvironment created than the specific phenotype of encapsulated macrophages (which can be expected to have a crosslinked gelatin microenvironment specific signature).

Finally, for modelling the movement of the cells from surrounding tissues; macrophage containing hydrogels are put in contact with endothelial cell laden hydrogels (Figure 6A). Over the course of two weeks, endothelial cells moved more towards the macrophage laden hydrogels in M2 media microenvironment (Figure 6B). Just as in direct contact conditions, M1 microenvironment was more potent in inducing both pro- and anti-inflammatory cytokine secretion (Figure 6C). For both conditions, after an initial high levels of pro-inflammatory TNF-alpha and IL-1beta release, the microenvironment has become more pro-regeneration with the presence of IL-1RA, CCL-18 and IL-4. It has been previously shown that the presence of endothelial cells improve the viability of cardiomyocytes in 3D hydrogel configuration (Narmoneva, Vukmirovic et al. 2004); thus establishment of a microenvironment that can induce the in-growth of endothelial cells would be advantageous. Also, the migration in the presence of two gels demonstrate that the effect of the encapsulated macrophages is not limited to the cells in suspension.

In regenerative medicine, the final aim is the complete integration of the implanted artificial tissue over time. This implies that the artificial tissue should integrate with the host circulatory system, nervous system and also the immune system. The immune system integration can be triggered by addition of macrophages in the artificial tissue formulation, in the manner described in this study, which can facilitate the establishment of a resident macrophage population. This can also facilitate the healing process and the resolution of inflammation induced by implantation.

4. Conclusion

The design and maturation of an engineered tissue is important for its integration within the host. Here we demonstrated in an in vitro model that the encapsulated macrophages, mimicking resident macrophages, have an impact on the behavior of incoming cells in a cell-type specific manner. The presence of pro- or anti-inflammatory cytokines can be impacted by the conditioning of the encapsulated macrophages. In the future, The incorporation of phenotype controlled macrophages in engineered tissues has an important potential the initial immune reaction upon implantation. Controlled delivery of polarization inducing cytokines could achieve partially a similar effect; but incorporation of macrophages would provider an active source of secretion for a variety of cytokines and bioactive agents for a longer term. Such a model can be used to better understand possible immunoengineering strategies using macrophages and also studies of immune response to xenogenic and allogenic cells in a controlled manner. Our future studies will focus on the elucidation of the encapsulation conditions on macrophage polarization for potential regenerative medicine applications.

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Author Contributions:

Dollinger C, Ciftci S, Knopf-Marques, Guner Rabia and Barthès J performed experiments. Dollinger C, Knopf-Marques H, Barthès J and Vrana NE designed experiments. Ghaemmaghami AM, Vrana NE, Debry C supervised experiments. Ghaemmaghami AM,

Vrana NE, Debry C, Dollinger C, Barthès J and Knopf-Marques H analyzed and interpreted data. Dollinger C, Barthès J, Knopf-Marques H and Vrana NE wrote the manuscript. All authors revised and corrected the final manuscript.

Competing Financial Interests:

Authors declare that there are no competing financial interests.

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Figures :

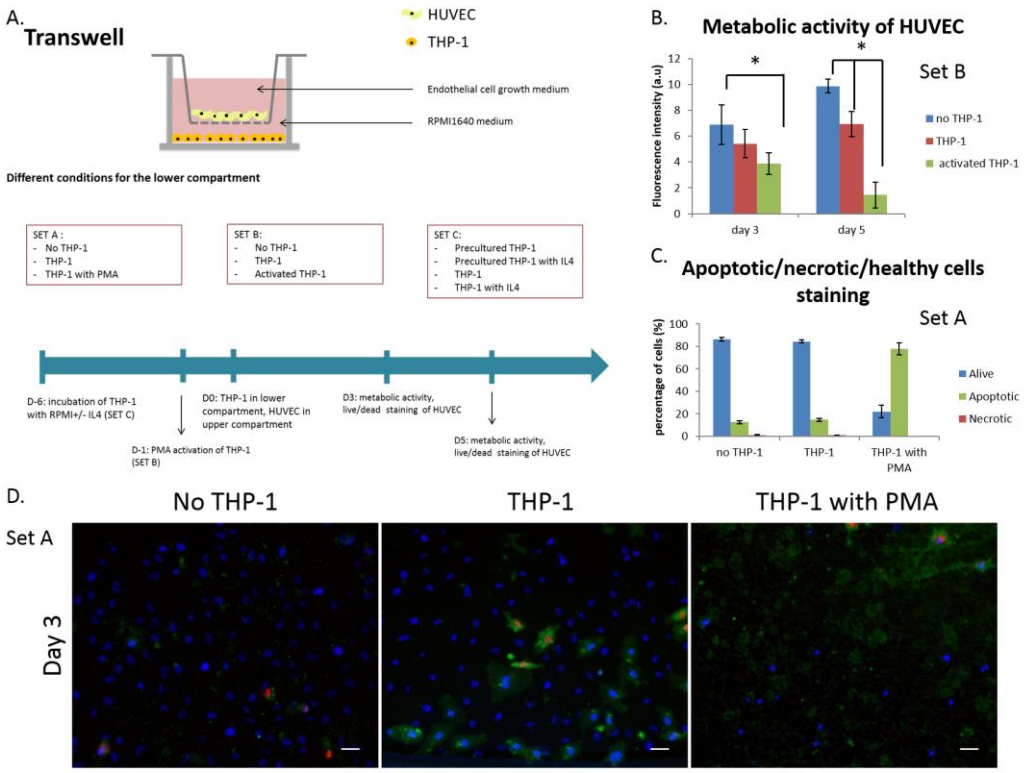


Figure 1. A) Experimental design of Transwell based co-culture of HUVECs with phenotype controlled THP-1 cells. B) Metabolic activity of HUVECs in the Transwell co-culture system (set B). C) Quantification of HUVECs viability after three days of culture using Apoptotic/Necrotic/Healthy cells kit (set A). D) Apoptotic(green)/Necrotic (red)/Live (blue) staining of HUVECs on Transwell after three days of culture in the presence of THP-1 cells (set A). Scale bar = 50 μm.

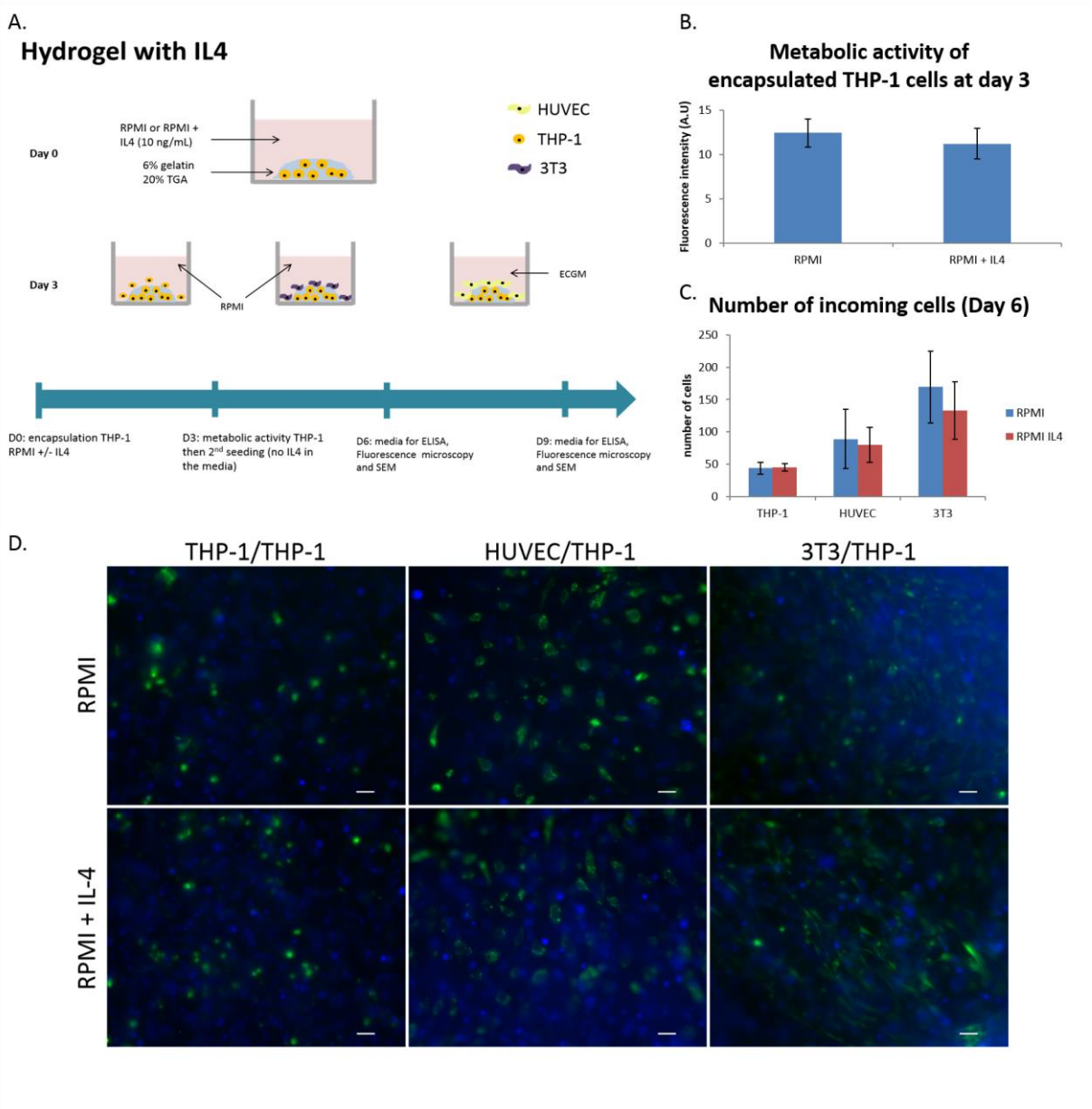


Figure 2. A) Experimental design of the hydrogel based co-culture system with pre culture of encapsulated THP-1 cells in RPMI medium with or without supplementation of IL-4. B) Metabolic activity after three days of culture of encapsulated THP-1 cells in the presence or absence of IL-4 in the medium. C) Average number of incoming cells. Incoming cells were pre-labelled with calcein-green and pictures were taken with epifluorescence microscope (10x) and cells per image were counted with four pictures. D) Epifluorescence pictures (10x) at day 6 showing the interaction between pre-labelled (calcein-green) THP-1, HUVECs or 3T3 cells with encapsulated THP-1 cells. Cells were cultured with or without IL-4 in the medium. Scale bar = 50µm

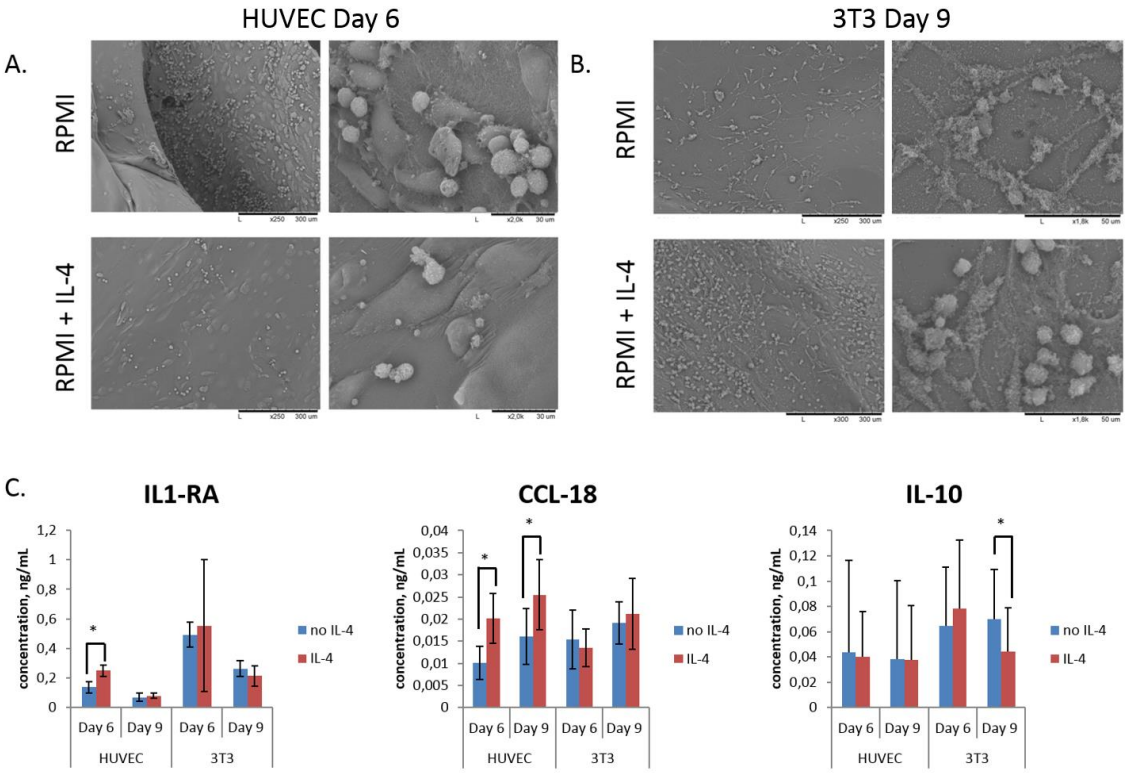


Figure 3. SEM pictures of incoming A) HUVECs (Day 6) or B) 3T3 cells (Day 9) on encapsulated THP-1 cells.THP-1 were pre cultured with or without IL-4 in the medium. C) ELISA quantification of anti-inflammatory cytokines in the supernatant at day 6 and day 9 of culture. Statistics were performed using t-test or Mann-Whitney ($p < 0,05$)

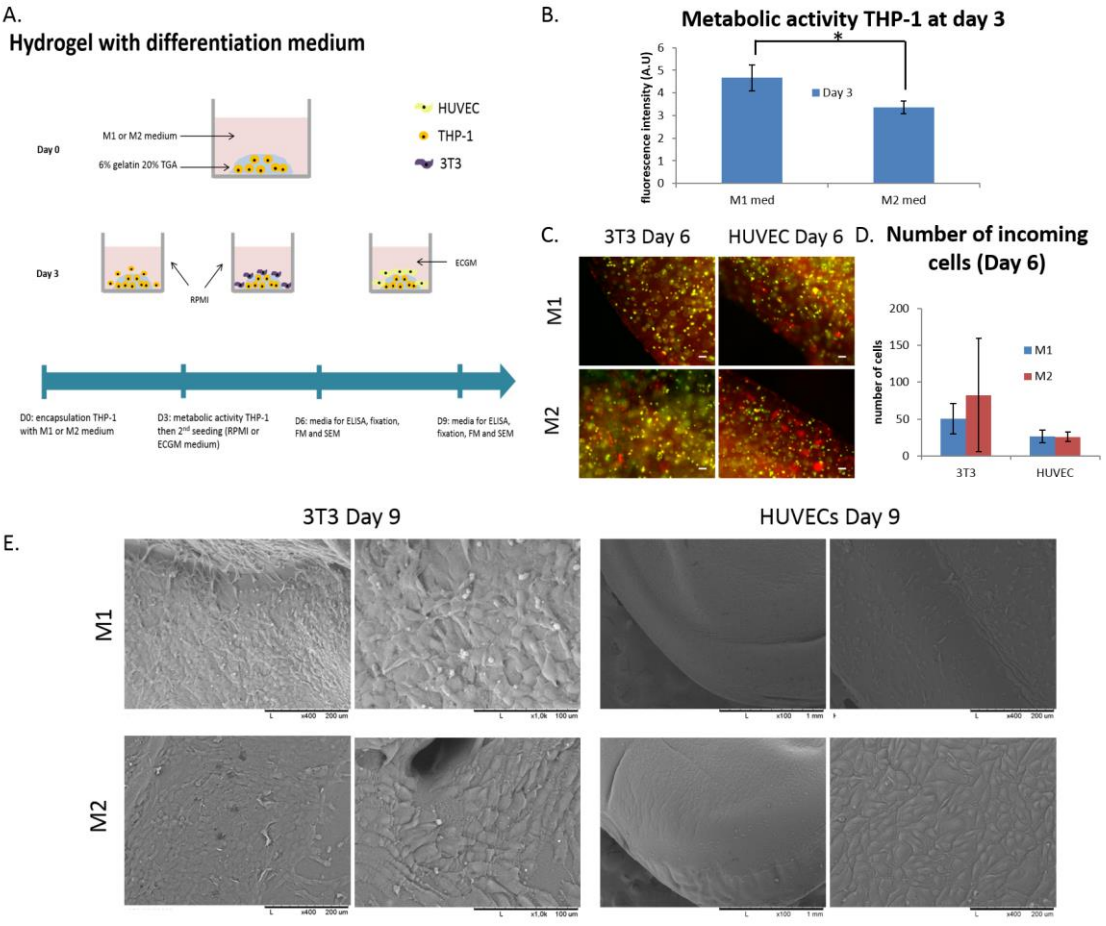


Figure 4. A) Experimental design of the hydrogel based co-culture system with pre culture of encapsulated THP-1 cells in M1 or M2 differentiation medium. B) Metabolic activity after three days of culture of encapsulated THP-1 cells in differentiation medium. C) Epifluorescence pictures (10x) at day 6 showing the interaction between incoming pre-labelled (red cell tracker) 3T3 cells or HUVECs in contact with pre-labelled (calcein green) encapsulated THP-1 cells. Scale bar = 50 μm. D) Average number of incoming cells. Incoming cells were pre-labelled with (red cell tracker) and pictures were taken with epifluorescence microscope (10x) and cells per image were counted with 5 pictures. E) SEM pictures of incoming 3T3 cells or HUVECs at day 9 in contact with encapsulated THP-1 cells. THP-1 were pre cultured with M1 or M2 differentiation medium.

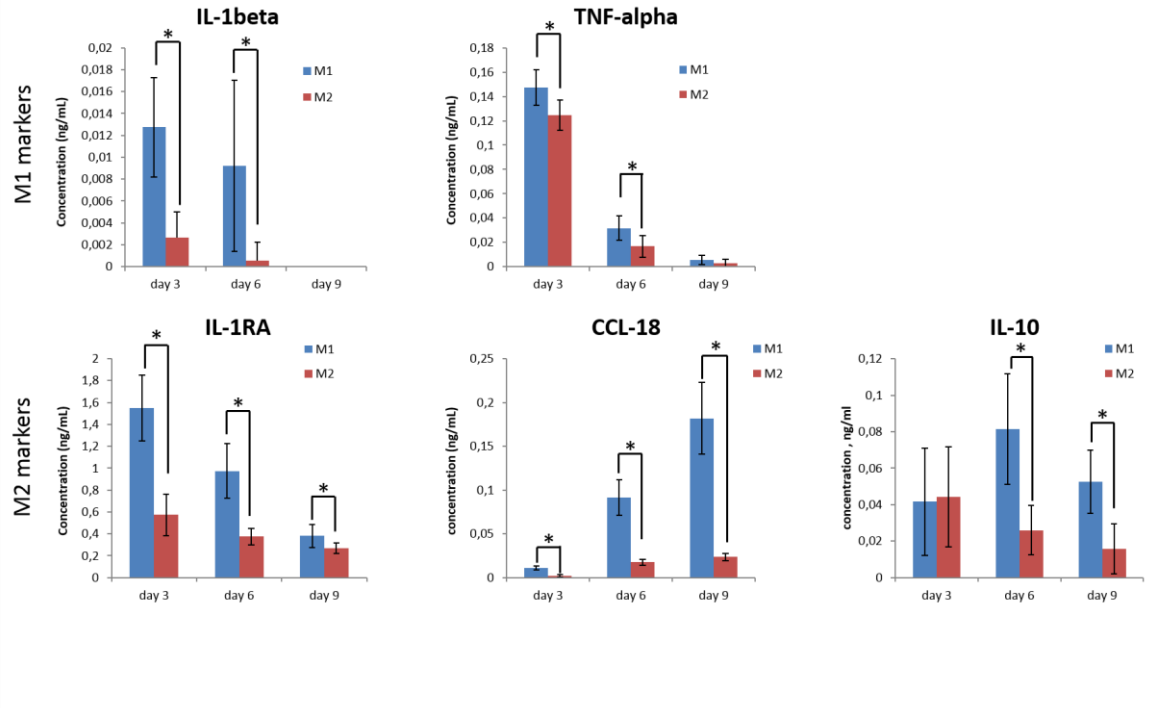


Figure 5. ELISA quantification of pro-inflammatory (M1) and anti-inflammatory (M2) cytokines in the supernatant at day 3, 6 and 9 of culture for hydrogel based co-culture system with incoming THP-1 cells in contact with encapsulated THP-1 cells pre cultured in M1 or M2 differentiation medium. Statistics were performed using t-test or Mann-Whitney ($p<0,05$).

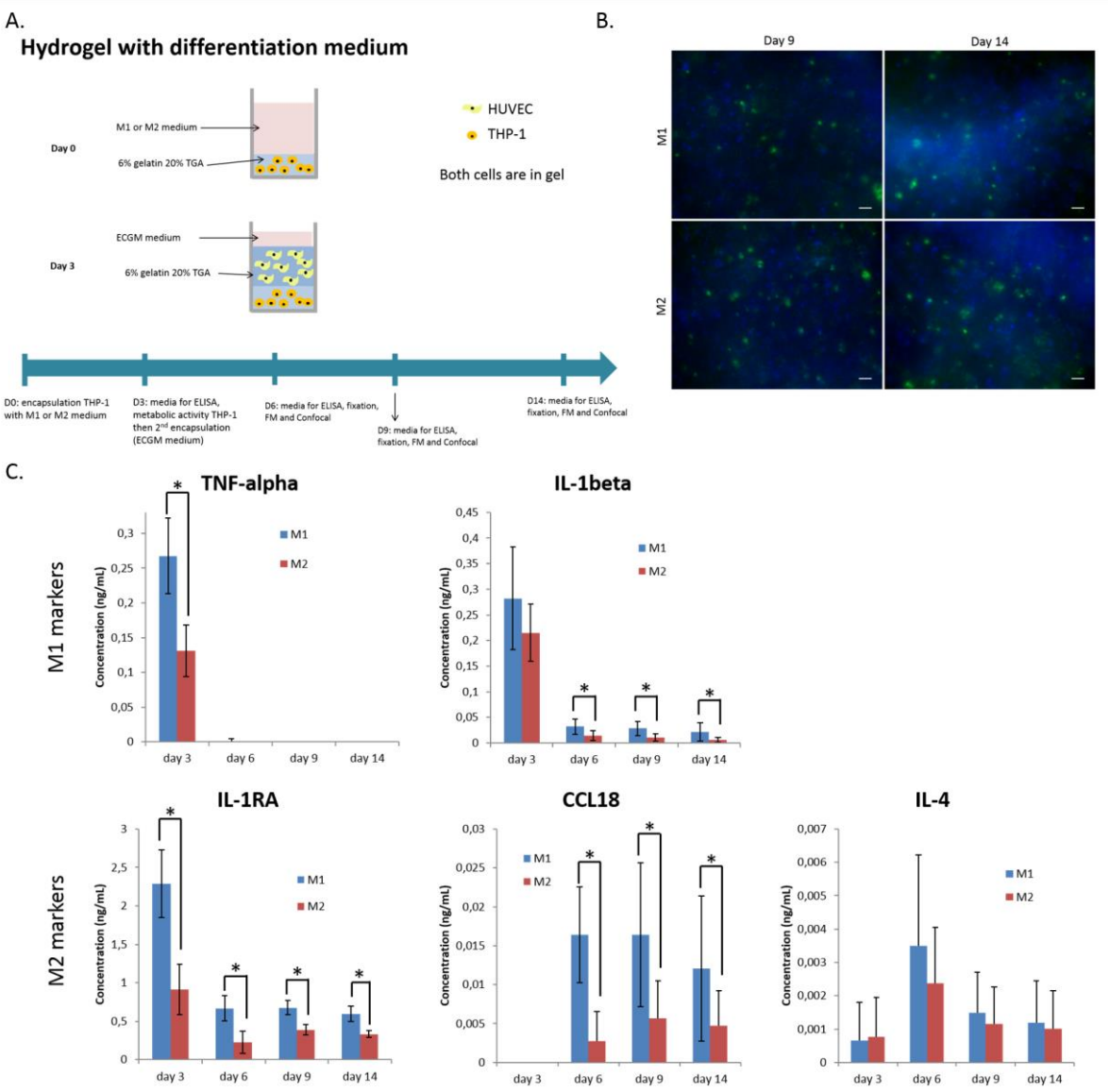
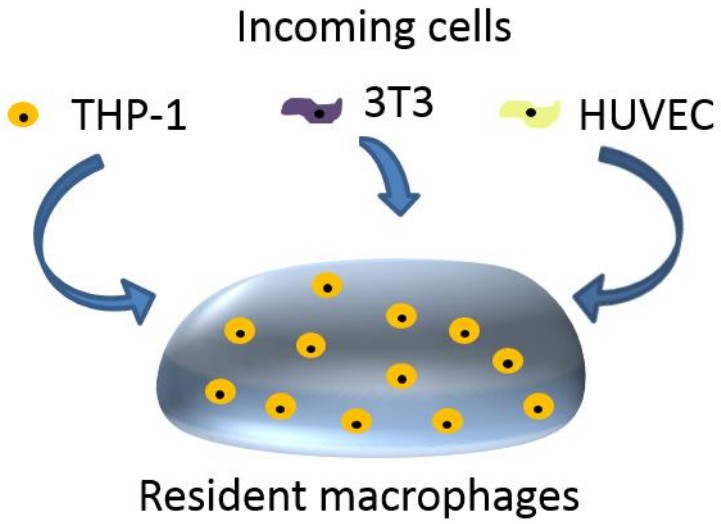


Figure 6. A) Experimental design of the hydrogel based co-culture system with pre culture of encapsulated THP-1 cells in M1 or M2 differentiation medium. Incoming HUVECs and encapsulated THP-1 cells are in a different gel. B) Epifluorescence pictures (10x) at day 9 and 14 showing the interaction between pre-labelled (calcein green) encapsulated HUVECs with pre-labelled (blue cell tracker) encapsulated THP-1 cells precultured in M1 or M2 differentiation medium. Scale bar = 50 µm. C) ELISA quantification of proinflammatory (M1) and anti-inflammatory (M2) cytokines in the supernatant at day 3, 6, 9 and 14. Statisticswere performed using t-test or Mann-Whitney ($p<0,05$).



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