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Human macrophage polarisation and regulation of angiogenesis and osteogenesis is dependent on culture extracellular matrix and dimensionality

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A B S T R A C T

The immune system plays a crucial role in tissue repair and regeneration. Macrophages have been identified as master regulators of the early immune response and healing outcome, by orchestrating the temporal nature of the initial inflammation phase and coordinating the fate of stem/progenitor cells involved in regeneration. However, traditional in-vitro models for the study of macrophages often fail to fully replicate the complexity of the in-vivo microenvironment, therefore generating models which do not fully capture the extensive spectrum of macrophage behaviour seen in native tissues. To this end, we used a hematoma-mimetic 3D fibrin matrix characteristic of early injured tissues to generate a 3D in-vitro model mirroring the local macrophage microenvironment. Leveraging this framework, we demonstrated significant effects of extracellular matrix and dimensionality on macrophage basal signalling and polarisation, achieving more pronounced regenerative phenotypes upon stimulation with the M2a polarisation factors compared to traditional 2D tissue culture conditions. Moreover, this enhanced physiological macrophage behaviour corresponded to increased coordination of angiogenesis and osteogenesis, better mirroring the healing processes seen in-vivo. Taken together, this study demonstrates the critical importance of integrating tissue composition and 3D architecture when investigating the macrophage behaviour in-vitro, establishing a powerful tool that overcomes known limitations associated with traditional 2D culture on plastic, and can be used to identify and validate novel immunomodulation strategies to enhance tissue regeneration.

1. Introduction

The immune system plays a crucial role in tissue repair and regeneration and thus modulation of the initial immune response following injury has grown as an attractive approach in regenerative medicine [1]. Specifically, regulating the response of macrophages, which are known to act as key mediators in the immune response, has been recognised to be crucial in eliciting a positive healing outcome [2]. In addition to controlling the temporal nature of the inflammatory response, macrophages establish a regenerative environment coordinating the fate of tissue-specific progenitor cells, which are integral to long-term regeneration [3]. In particular, macrophages infiltrate the injured area where they are exposed to inflammatory signals driving them into the classically termed 'pro-inflammatory' M1-like phenotype which elicits the release of inflammatory cytokines and growth factors promoting the recruitment of endothelial cells [4]. As the inflammation resolves, macrophages progressively shift into a pre-dominantly 'anti-inflammatory' M2-like phenotype which consolidates the onset of vascularisation and, in the context of bone healing, directs the osteogenic fate of progenitor mesenchymal stromal/stem cells (MSCs) [5]. However, in compromised wounds, resolution of the immune response can be delayed leading to poor healing. Therefore, given the critical role of the immune response in facilitating tissue repair, a thorough understanding of how the macrophage can be modulated to positively mediate regeneration is required.

Current gold standard modalities for assessing the role of macrophages in tissue repair remains in-vivo models. However these can be costly, time-consuming, and in the context of animal models fail to recapitulate the human condition. Therefore, in-vitro culture models remain the most commonly utilised approach, in which primary human blood-derived macrophages are cultured on 2D tissue culture plastic (TCP) [6]. However, these are subjected to significant donor variability, that can be age, gender and/or diet-dependent, therefore greatly impacting the reproducibility of experimental outcome [7]. In addition,

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their inability to proliferate extensively results in recurrent labour-intensive isolations. As a result, human macrophage cell lines represent an attractive alternative. Specifically, the THP-1 monocyte cell line offers the potential to be propagated indefinitely from a single donor therefore eliminating donor-variability and recurring ethical considerations, thus easing scalability [8]. However, while the THP-1 macrophage has been recognised to be highly responsive to inflammatory M1 signals, previous studies have pointed to their limited polarisation potency towards the more regenerative M2 phenotype [9,10]. In particular, THP-1 macrophages upon M2 treatment fail to enhance the secretion of IL-10, an anti-inflammatory cytokine that plays a multifaceted role in tissue repair [11]. Failure to reproduce macrophage behaviour with regenerative functionalities is a significant limitation of current in-vitro modalities.

Upon tissue injury, local blood vessel disruption leads to the formation of a fibrin rich hematoma which acts as a scaffold for immune cells such as macrophages. In addition to local soluble factors which can regulate immune responses, biophysical cues in the form of extracellular matrix (ECM), structure, and mechanics have also emerged as potent stimuli. Therefore, culturing macrophages on 2D TCP represents an alien environment when compared to the native hematoma. As fibrin constitutes the primary component of the provisional ECM that forms upon injury, studying the interaction between macrophages and fibrin may be more physiologically relevant in the context of healing. In addition to recapitulating the fibrin-rich ECM of the injured tissue, integrating the 3D dimensionality of the matrix needs to be equally considered [1]. Surprisingly, little is known about the effect of 3D culture conditions on macrophages. Bartneck et al. [12] cultured human macrophages on 2D and 3D hydrogels of similar material, and observed both decreased secretion of inflammatory cytokines, and increased release of angiogenic factors when cultured in 3D. However, there is still limited knowledge on the synergistic role that both fibrin and 3D architecture play in the modulation of the phenotypical behaviour of macrophages, let alone on the subsequent coordination of angiogenesis and osteogenesis [13].

Therefore, this study aimed to elucidate the combined effects of ECM and culture dimensionality on the functionality of macrophages embedded in 3D fibrin-rich hematoma mimetic hydrogels, establishing a novel physiologically relevant in-vitro macrophage cell culture platform.

2. Methods

2.1. Cell Culture

2.1.1. Human monocytes (hM_0)

Human monocyte reporter cell line THP-1 was cultured in RPMI-1640 medium (Sigma-Aldrich) supplemented with 10 % (v/v) fetal bovine serum (FBS), 1 % L-Glutamine, 1 % Penicillin Streptomycin (P/S) (Sigma-Aldrich) under standard conditions.

2.1.2. Human umbilical vein endothelial cells (HUVECs)

Green Fluorescent Protein-HUVECs were cultured in Endothelial Basal Medium (EBM-2) supplemented with EGM-2 BulletKit (Lonza) under standard conditions. For angiogenesis studies, serum free HUVEC media was used and consisted of growth media depleted in FBS and VEGF-A supplements.

2.1.3. Human Bone Marrow Stromal/Stem cells (hMSCs)

HMSCs (Lonza) were expanded in growth media consisting of high glucose Dulbecco's modified Eagle's medium (DMEM) GlutaMAX (Sigma-Aldrich) supplemented with 10 % (v/v) FBS (Gibco) and 1 % P/S (Sigma-Aldrich).

2.2. Macrophage differentiation and polarisation in 2D/3D culture conditions

Monocyte differentiation into macrophages (hMφ), in 2D culture conditions was achieved by first plating 1 million cells/mL THP-1 monocytes and treating with 100 nM Phorbol 12-Myristate 13-Acetate (PMA; Sigma-Aldrich) for 3 days, and then priming into the traditional M1-like phenotype with LPS (100 ng/mL; EnzoLife Sciences) and IFN-γ (20 ng/mL; Miltenyi Biotec) for 24 h, or into the M2a-like phenotype with IL-4 (20 ng/mL; Miltenyi Biotec) and IL-13 (20 ng/mL; Miltenyi Biotec) for 24 h, or leaving untreated for 24 h to obtain the M0-like phenotype. Serum free and supplemented macrophage CM were then collected, and cells were lysed for RT-PCR. Experimental design is illustrated in Fig. 1A.

Monocyte differentiation into macrophages in 3D culture conditions was achieved by first crosslinking fibrin hydrogels (Ø = 5 mm; h = 3 mm) under 37 °C for 45 min to obtain final fibrinogen, thrombin and cell concentrations of 50 mg/mL, 2.5 U/mL and 3 million cells/mL, respectively, and then treating with 100 nM PMA for 24 h. Following an additional 24 h rest period, 3D THP-1 macrophages were then primed into the traditional M1-like, M2a-like and M0-like phenotypes as described. Serum free and supplemented macrophage CM were then collected, and cells were lysed for RT-PCR.

2.3. Gene expression

To assess gene expression, total RNA extraction was performed by following the TRIzol reagent manufacturer's protocol. 500 ng of RNA was reverse transcribed to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). qPCR was performed using 7500 Fast System machine (Applied Biosystems) and SYBR Select Master Mix (Applied Biosystems) for custom designed primers (Sigma-Aldrich). The relative mRNA amounts of each sample were calculated using the $2^{-\Delta\Delta Ct}$ method.

2.4. Cytokine production

Cytokine concentrations of IL-8, VEGF-A (Biolegend), and IL-10, TNF-α (Invitrogen) were quantified in the collected supplemented supernatants by ELISA according to manufacturer's protocol.

2.5. Angiogenesis assay

Following hMφ supernatant collection, 48-well culture plates were plated with 100 μL/well of reduced growth factor basement membrane matrix Geltrex (ThermoFisher) and allowed to polymerise for 45 min at 37 °C and 5 % CO_2. GFP-HUVECs were then seeded at a cell density of 25,000 cells/cm^2 in 50 % serum free conditioned media and 50 % serum free HUVEC media and incubated for 10 h at 37 °C in humidified air with 5 % CO_2 to allow for tubule formation. Cells were then fluorescently imaged at 488 nm with an Olympus IX83 microscope. Tube formation was evaluated using the Angiogenic plugin on ImageJ and characterised with relative expression of segments, junctions and nodes.

2.6. Osteogenesis assay

Following hMφ supernatant collection, hMSCs were plated at a cell density of 6,500 cells/cm^2 for 4 days in supplemented high glucose DMEM + Glutamax. Cells were then incubated for 48 h in supplemented macrophage conditioned media except for negative control cultured in standard growth media and lysed for evaluation of osteogenic gene expression, detailed above.

2.7. Statistical Analysis

A one-way ANOVA test was used for the comparison of treatments

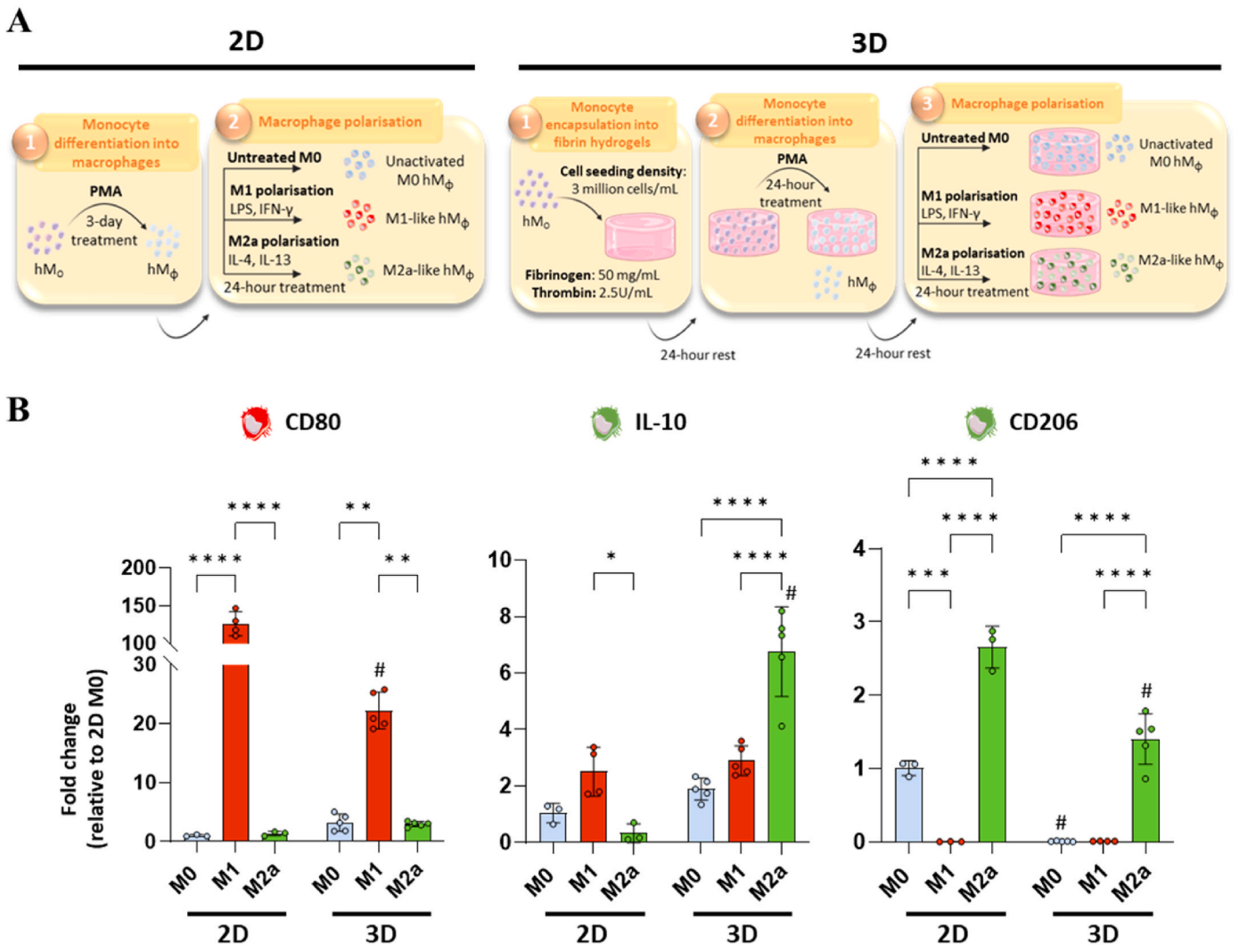

Fig. 1. Macrophage signalling upon standard polarisation treatments is dependent on culture conditions. (A) Study design schematics. **(B)** mRNA expression levels of M1-like and M2-like markers. (*P ≤ 0.05; #≤0.05 denotes significance with corresponding 2D control).

within the same culture dimension, with the Tukey's post-test. For differences in treatments in both 2D and 3D culture conditions, a two-way ANOVA was performed using GraphPad Prism 9.00.

3. Results

3.1. THP-1 macrophage polarisation is dependent on culture ECM and dimensionality

We first sought to investigate the impact of culturing human THP-1 macrophages in 3D hematoma mimetic fibrin hydrogels when compared to standard 2D culture, with an initial emphasis on M0 basal signalling, followed by a detailed characterisation on M1/M2a polarisation.

Interestingly, the basal expression of M1-associated CD80 and M2-associated IL-10 gene expression in M0 macrophages was unaffected by 3D fibrin culture, while M2-associated CD206 expression was significantly downregulated (Fig. 1B). However, despite minimal changes in basal signalling, the response of THP-1 macrophages to M1/M2a polarisation stimuli is dramatically affected by the culture conditions (interaction, $p < 0.0001$). M1 polarisation resulted in a significant 126-fold and 6.91-fold increase in CD80 gene expression in 2D and 3D respectively when compared to their corresponding M0 controls ($p < 0.0001$ and $p < 0.01$, respectively; Fig. 1B). However, M2a polarisation treatment resulted in a significant 2.65-fold and 189-fold increase in CD206 M2 marker gene expression in 2D and 3D culture respectively when compared to their corresponding M0 controls ($p < 0.0001$). It is worth noting that the large increase in 3D is primarily due to the very low expression in M0 macrophages cultured in 3D. Importantly, despite no differences in basal expression of IL-10 in 2D vs 3D M0 macrophages, the macrophage regenerative signalling outcome was largely dependent on culture dimensionality, as evident by the opposite trends in IL-10 expression following M2a polarisation with a non-significant 0.33-fold decrease in 2D and a significant 3.57-fold increase in 3D culture when compared to their corresponding M0 controls ($p > 0.05$ and $p < 0.0001$, respectively; Fig. 1B). Taken together, our findings clearly demonstrate that the degree of M1 and M2a polarisation is dependent on matrix and culture dimensionality of the macrophage, with 3D culture facilitating a more regenerative signalling profile.

3.2. The secretome of THP-1 macrophages is dependent on culture ECM and dimensionality

Given that human THP-1 macrophage basal signalling and polarisation potential were dependent on culture ECM and dimensionality, we next investigated whether these effects translated to the protein level by evaluating the secretome composition.

We first analysed the basal release of TNF-α and IL-8 inflammatory cytokines in M0 macrophages, which were significantly enhanced 102-

fold and 12.5-fold respectively in 3D when compared to 2D culture ($p < 0.0001$; Fig. 2A–B). Importantly, both 2D and 3D macrophages were capable of responding to M1 induction with enhanced TNF-α secretion (187-fold, $p < 0.0001$; 1.54-fold, $p < 0.01$, respectively) (Fig. 2A), reaching a similar level across both 2D and 3D cultured macrophages, therefore demonstrating successful M1 polarisation. This was further confirmed in 2D with the significant 5.86-fold ($p < 0.0001$) increase in IL-8 secretion upon M1 treatment (Fig. 2B), although not to a level seen in M0 macrophages cultured in 3D. Interestingly, IL-8 secretion in 3D was not further enhanced following LPS treatment. We next investigated the basal release of IL-10 anti-inflammatory cytokine, which appeared to be unaffected by 3D culture (Fig. 2C), therefore consistent with our gene expression data (Fig. 1B). Moreover, VEGF-A production was significantly enhanced 1.77-fold ($p < 0.001$) in 3D culture (Fig. 2D) when compared to 2D controls. Importantly, no noticeable increase in IL-10 cytokine release was observed upon M2a induction in 2D cultured macrophages, while a significant 2.53-fold increase ($p < 0.01$) in IL-10 secretion was identified in the 3D cultured macrophages, when compared to their corresponding M0 controls, which is in alignment with that seen at the gene level. Interestingly, M1 induction with LPS promoted a significant 2.21-fold ($p < 0.05$) increase in IL-10 release compared to M0 controls in 2D while no change was seen in 3D (Fig. 2C). Given the established nature of IL-10 as a marker of M2 polarisation and its role in tissue regeneration [11], the discovery that IL-10 secretion is particularly sensitive to culture ECM and dimensionality is an important consideration for the study of macrophage behaviour. Additionally, 2D cultured macrophages experienced a significant 0.48-fold decrease ($p < 0.05$) in the secretion of the angiogenic cytokine VEGF-A upon M2a

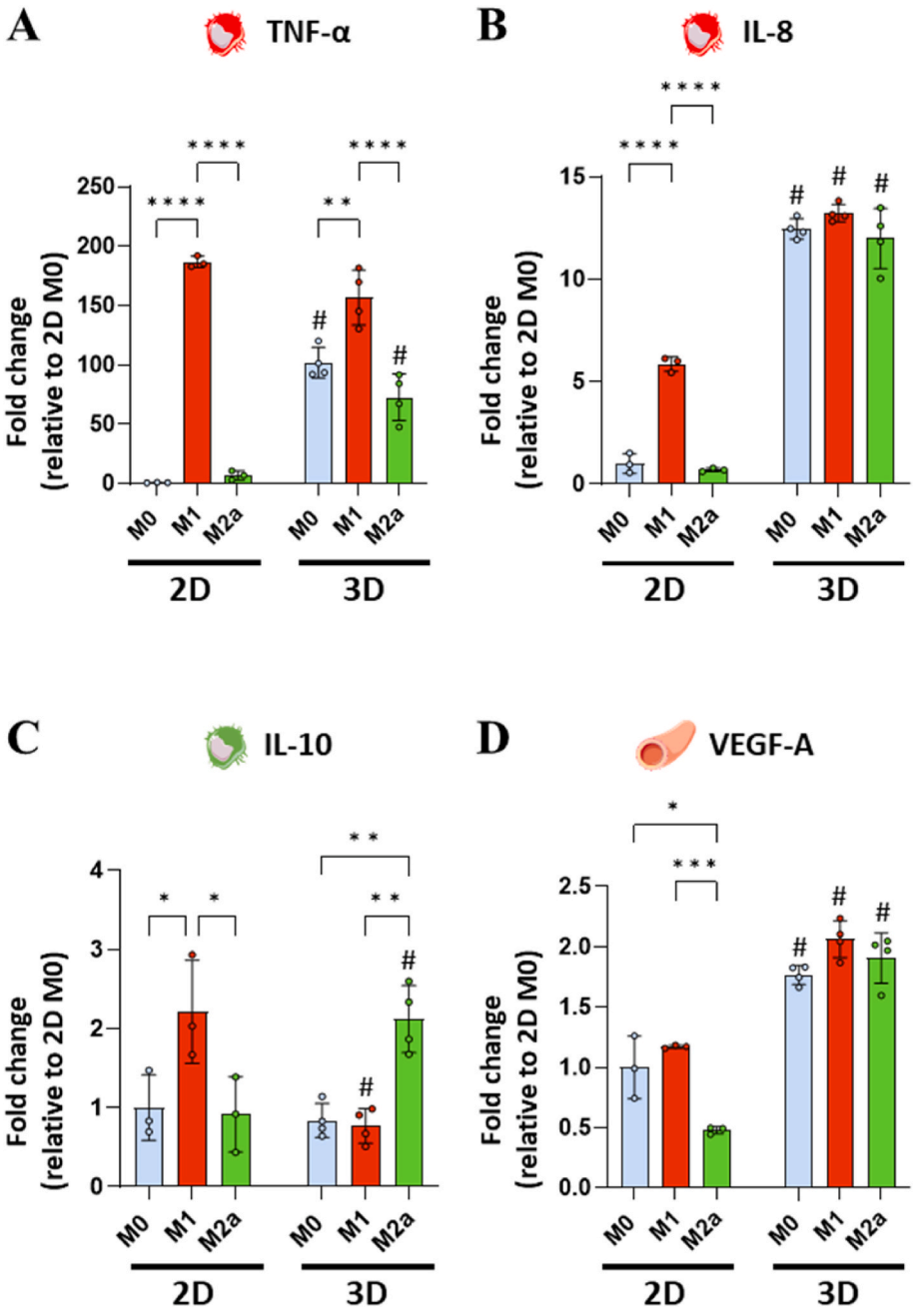

Fig. 2. Macrophage cytokine production upon standard polarisation treatments is dependent on culture conditions. ELISA to quantify the production of **(A)** TNF-α and **(B)** IL-8 pro-inflammatory cytokines, as well as **(C)** IL-10 pro-regenerative cytokine and **(D)** VEGF-A angiogenic factor. (*P ≤ 0.05; # ≤0.05 denotes significance with corresponding 2D control).

induction (Fig. 2D), while no changes were identified in 3D cultured macrophages, therefore further supporting the dependency of regenerative cytokine production on macrophage culture conditions.

Collectively, these results indicate that THP-1 macrophages are exquisitely sensitive to the culture ECM and dimensionality, whereby 3D hematoma mimetic fibrin hydrogel culture is conducive to the upregulation of phenotype-specific gene and cytokine release associated with higher regeneration.

3.3. The angiogenic properties of the polarised THP-1 macrophage secretome is dependent on culture ECM and dimensionality

Long-term healing of most tissues, such as bone, is highly dependent on the early vascularisation of the defect area [13]. Therefore, it was next of interest to assess the angiogenic potency of the THP-1 secretome collected from samples cultured and polarised in either 2D or in our 3D in-vitro model.

We first investigated the effect of macrophage conditioned media (CM) collected from differentially polarised THP-1 macrophages cultured in 2D on HUVECs capacity to form angiogenic tube-like structures. Interestingly, secreted factors from both M0 and M1 polarised macrophage did not alter vascularisation (Fig. 3A–B). However, secreted factors from M2a polarised macrophages cultured in 2D demonstrated potent angiogenic properties when compared to all other groups, with significant increases in the formation of segments (2.00-fold, $p < 0.0001$), junctions (1.71-fold, $p < 0.0001$) and nodes (1.71-fold, $p < 0.0001$).

We then investigated the effect of macrophage CM collected from THP-1 macrophages cultured in 3D. Similarly to the 2D M0 macrophage CM, no significant effect on HUVECs was found upon treatment with 3D M0 macrophage CM (Fig. 3C–D). However, secreted factors from M1 polarised macrophages cultured in 3D elicited a significant increase in segments (4.00-fold, $p < 0.0001$), junctions (2.41-fold, $p < 0.0001$) and nodes (2.25-fold, $p < 0.0001$) (Fig. 3C–D). It is worth noting that the 3D M1 macrophage CM promoted tube formation to a higher extent to that seen with the 2D M2a phenotype. Finally, secreted factors from M2a polarised macrophage CM induced the highest vascularisation outcome with significant increases in segments (5.72-fold, $p < 0.0001$), junctions (3.07-fold, $p < 0.0001$) and nodes (3.03-fold, $p < 0.0001$) (Fig. 3C–D), a response that was significantly improved when compared to that seen with secreted factors from either M1 or M0 macrophages.

Collectively, these data demonstrate that our 3D culture conditions further promote the angiogenic properties of THP-1 macrophages, and confirm that by identifying the right culture conditions, we can generate an improved THP-1 macrophage model better mimicking in-vivo macrophage behaviour.

3.4. The osteogenic properties of the polarised THP-1 macrophage secretome is dependent on culture ECM and dimensionality

Given that the crosstalk between the immune system and stem/stromal cell populations is a pivotal process in tissue regeneration, particularly in bone, we next sought to determine the osteogenic potency of the THP-1 secretome collected from samples cultured and polarised in either 2D or 3D fibrin environments.

Initially, we investigated the effect of macrophage CM on hMSC osteogenic gene expression. Interestingly, the expression of RUNX2 and ALP were significantly downregulated with either 2D cultured M0, M1, or M2a polarised macrophage CM, indicating that macrophages cultured in 2D have a tendency to inhibit osteogenic lineage commitment regardless of their polarisation state (Fig. 4A). As large bone defects can heal via endochondral ossification, we next sought to explore whether the chondrogenic master transcription factor SOX9 is influenced by macrophage secreted factors. SOX9 expression appeared unaffected following treatment with M0 or M1 polarised macrophage CM, although a slight but non-significant increase (1.76-fold, $p = 0.43$) was noticeable in the M0 treated group. However, upon M2a polarised macrophage CM treatment, a significant 2.32-fold increase ($p < 0.05$) in SOX9 expression was detected (Fig. 4A), indicating a potential preference towards the chondrogenic lineage. The preference for an osteogenic or chondrogenic lineage is best visualised via the RUNX2/SOX9 ratio [14], which appeared to be significantly downregulated upon M0, M1, or M2a polarised macrophage CM treatment, therefore further confirming that THP-1 macrophages cultured in 2D secrete factors that are detrimental to osteogenesis.

We then explored the effect of macrophage CM collected from differentially polarised THP-1 macrophages cultured in 3D. Similarly to 2D, expression of RUNX2 and ALP were significantly downregulated with either 3D M0, M1, or M2a polarised macrophage CM, although a trend of increase in RUNX2 was observed upon M2a CM treatment when compared to M0 treatment (Fig. 4B). We then explored the expression of SOX9 and while the 3D M0 or M1 polarised macrophage CM did not seem to impact its expression, 3D M2a macrophage CM treatment resulted in a significant 0.60-fold decrease ($p < 0.05$) in SOX9 expression (Fig. 4B), therefore suggesting a trend towards the osteogenic lineage. This is in opposition to our 2D M2a CM treatment which significantly enhanced SOX9 expression. Importantly, this finding was further confirmed when displaying the RUNX2/SOX9 ratio with an appreciable recovery of the ratio, which appeared even slightly enhanced (1.12-fold, $p > 0.05$) upon M2a CM treatment from macrophages cultured in 3D (Fig. 4B).

Taken together, these results indicate that THP-1 macrophages secrete factors that inhibit osteogenic lineage commitment regardless of polarisation state in 2D, but that this effect can be mitigated using our 3D culturing platform and by priming macrophages into the M2a-like phenotype.

4. Discussion

Following injury, macrophages have been identified as master regulators of the early immune response and healing outcome, by orchestrating the temporal nature of the inflammation phase and coordinating the fate of stem/progenitor cells [4]. However, traditional in-vitro models for the study of macrophages often fail to fully replicate the complexity of the in-vivo microenvironment, and thus do not fully capture the spectrum of macrophage behaviour seen in-vivo [9,10]. Therefore, in this study we incorporated a hematoma mimetic fibrin ECM composition and 3D spatial arrangement characteristic of early injured tissues to generate a 3D in-vitro model mirroring the local macrophage microenvironment. Leveraging this framework, we demonstrated significant effects of ECM composition and dimensionality on macrophage basal signalling and polarisation, achieving more pronounced regenerative phenotypes upon M2a treatments compared to traditional 2D tissue culture conditions, overcoming known limitations associated with traditional 2D culture on plastic. Moreover, this increased physiological macrophage behaviour corresponded to enhanced coordination of angiogenesis and osteogenesis, better mirroring the healing processes seen in-vivo. Taken together, the results of this study demonstrate the critical importance of tissue composition and 3D architecture when investigating the macrophage behaviour in-vitro, establishing a powerful tool to identify and validate novel immunomodulation strategies to enhance regeneration.

M0-like macrophages cultured in a 3D hematoma mimetic platform secreted higher levels of pro-inflammatory TNF-α and IL-8 cytokines when compared to standard 2D culture, suggesting a potential pro-inflammatory synergistic effect of fibrin ECM and 3D culture on macrophage signalling. Upon M1 polarisation, macrophages were capable of maintaining their pro-inflammatory response, although to a lesser extent compared to that seen in 2D. These milder inflammatory phenotypical changes in 3D may be more physiologically relevant. Consistent with our findings, Hsieh et al. [15] achieved a reduction in TNF-α secretion in macrophages cultured on 2D fibrin substrates when

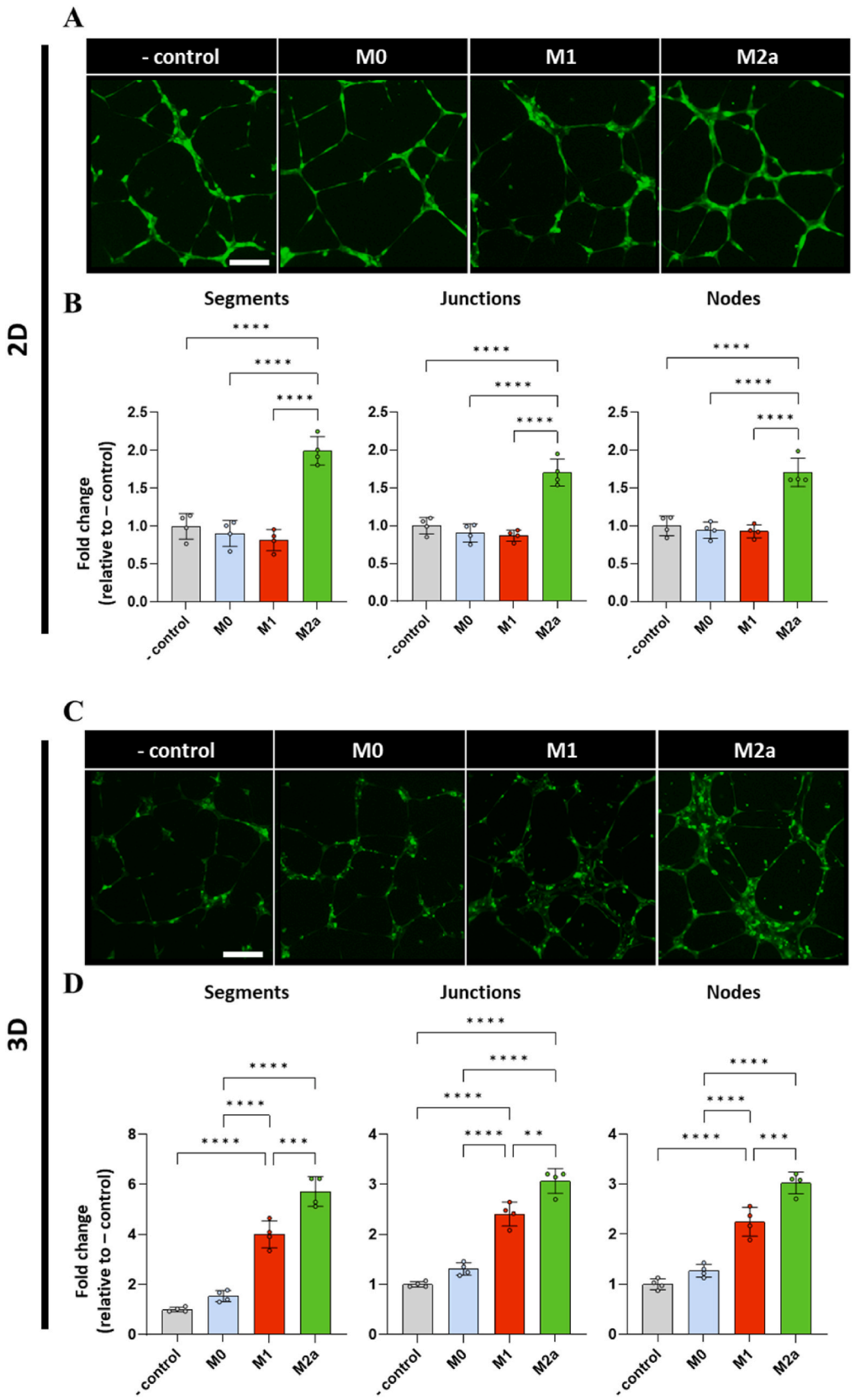

Fig. 3. Blood vessel formation via modulation of macrophage immune secretome is dependent on culture conditions. **(A)** Representative images of endothelial cell tubule (Scale bar: 200 μm). **(B)** Quantification of blood vessel formation. **(C)** Representative images of endothelial cell tubule (Scale bar: 200 μm). **(D)** Quantification of blood vessel formation. (*P ≤ 0.05).

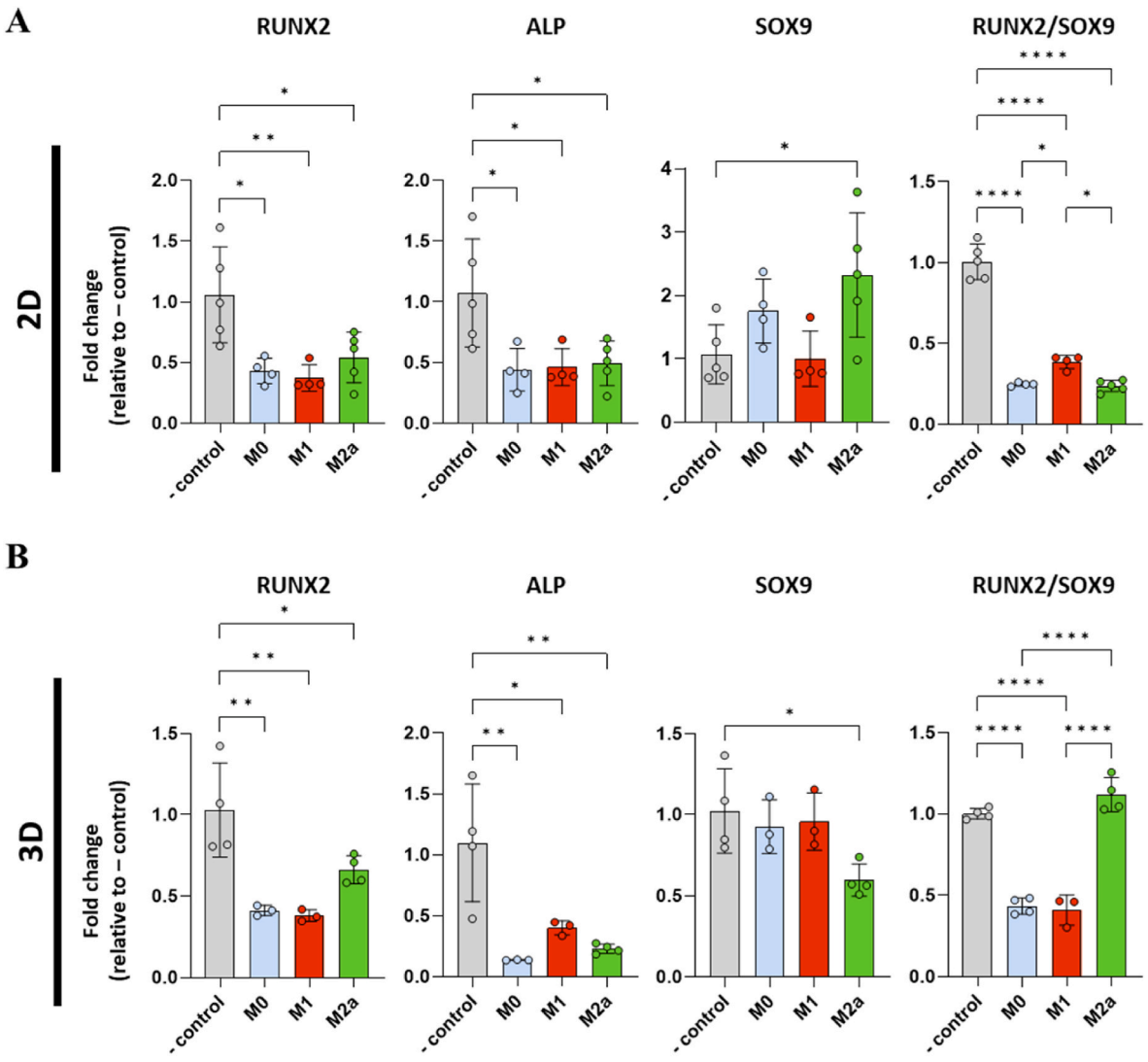

Fig. 4. The osteogenic commitment of human MSCs via the modulation of macrophage immune secretome is dependent on culture conditions. mRNA levels of osteogenic RUNX2 and ALP markers, as well as chondrogenic SOX9 marker and RUNX2/SOX9 ratio were analysed upon **(A)** 2D macrophage secretome and **(B)** 3D macrophage secretome treatment. (*P $\leq$ 0.05).

compared to TCP cultured controls. As fibrin matrix forms in injured tissues which are characterised by elevated inflammation, our findings point to the combined effects of fibrin and 3D architecture in dampening inflammatory levels in macrophages during early wound healing, therefore actively contributing to the pro-inflammatory to pro-regenerative transition necessary for normal healing. Interestingly, in terms of M2a induction into a pro-regenerative macrophage, traditional 2D models appear incapable of driving IL-10 expression in macrophages [9,10], yet our model was successful in significantly enhancing IL-10 gene expression and cytokine release upon IL-4/IL-13 treatment. It should be noted that previously mentioned 2D fibrin substrates failed to elicit increased IL-10 secretion following M2a activation [15]. Our findings therefore demonstrate that considering the fibrin ECM composition in combination with 3D culture dimensionality is essential to unlocking the regenerative potency of macrophages in-vitro.

3D hematoma mimetic culture of macrophages influences the secretion of regenerative factors mediating angiogenesis and osteogenesis. While only the CM from the 2D M2a phenotype elicited a significant increase in tube formation, when polarised in the 3D hematoma mimetic models, the M1 macrophage secretome was successful in driving enhanced tubular formation, notably to a higher extent than our 2D M2a control, and the 3D M2a phenotype elicited an even greater vascularisation outcome. Moreover, in terms of MSC differentiation, both 2D and 3D M0/M1-like phenotypes promoted early chondrogenesis. This is in agreement with the work of Miyamoto et al. [16] who reported that co-culture of cartilage with M1-like macrophages promoted cartilage specific protein production. It is worth noting that in large defect healing the initial inflammatory phase is followed by the endochondral bone growth. Therefore, we hypothesize that the induction of chondrogenesis in hMSCs from the initial pro-inflammatory M1 phenotype may be integral to a successful long-term bone healing process. Importantly, our generated 3D M2a macrophage secretome induced an early osteogenic commitment, therefore eliciting an opposite differentiation outcome compared to their 2D corresponding controls. However, more recent studies support an osteogenic role of the M2 macrophage notably through IL-10 [17], which may be indicative of a small stable fracture which would heal via intramembranous ossification.

5. Conclusion

In summary, we developed a 3D in-vitro hematoma mimetic fibrin hydrogel which incorporates two key cues that macrophages are exposed to during the early stages of healing, and which can be

employed to more closely mimic the in-vivo behaviour of macrophages compared to traditional 2D tissue culture plastic conditions.

CRediT authorship contribution statement

S.R. Petrousek: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **G.S. Kronemberger:** Methodology, Writing – review & editing. **S.A. O Rourke:** Methodology, Writing – review & editing. **L.C. Shanley:** Methodology, Writing – review & editing. **A. Dunne:** Methodology, Writing – review & editing. **D.J. Kelly:** Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – review & editing. **D.A. Hoey:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Visualization, Writing – original draft, Writing – review & editing.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

[1] Z. Julier, A.J. Park, P.S. Briquez, M.M. Martino, Promoting tissue regeneration by modulating the immune system, Acta Biomater. 53 (2017) 13–28, https://doi.org/10.1016/j.actbio.2017.01.056.

[2] S. Chen, A. Saeed, Q. Liu, Q. Jiang, H. Xu, G.G. Xiao, L. Rao, Y. Duo, Macrophages in immunoregulation and therapeutics, Signal Transduct. Targeted Ther. 8 (2023) 207, https://doi.org/10.1038/s41392-023-01452-1.

[3] A.B. Aurora, E.N. Olson, Immune modulation of stem cells and regeneration, Cell Stem Cell 15 (2014) 14–25, https://doi.org/10.1016/j.stem.2014.06.009.

[4] C. Schlundt, H. Fischer, C.H. Bucher, C. Rendenbach, G.N. Duda, K. Schmidt-Bleek, The multifaceted roles of macrophages in bone regeneration: a story of polarization, activation and time, Acta Biomater. 133 (2021) 46–57, https://doi.org/10.1016/j.actbio.2021.04.052.

[5] S. Fan, X. Sun, C. Su, Y. Xue, X. Song, R. Deng, Macrophages-bone marrow mesenchymal stem cells crosstalk in bone healing, Front. Cell Dev. Biol. 11 (2023) 1193765, https://doi.org/10.3389/fcell.2023.1193765.

[6] S. Piatnitskaia, G. Rafikova, A. Bilyalov, S. Chugunov, I. Akhatov, V. Pavlov, J. Kzhyshkowska, Modelling of macrophage responses to biomaterials in-vitro: state-of-the-art and the need for the improvement, Front. Immunol. 15 (2024) 1349461, https://doi.org/10.3389/fimmu.2024.1349461.

[7] C. Franceschi, M. Capri, D. Monti, S. Giunta, F. Olivieri, F. Sevini, M.P. Panourgia, L. Invidia, L. Celani, M. Scurti, E. Cevenini, G.C. Castellani, S. Salvioli, Inflammaging and anti-inflammaging: a systemic perspective on aging and longevity emerged from studies in humans, Mech. Ageing Dev. 128 (2007) 92–105, https://doi.org/10.1016/j.mad.2006.11.016.

[8] W. Chanput, J.J. Mes, H.J. Wichers, THP-1 cell line: an in-vitro cell model for immune modulation approach, Int. Immunopharm. 23 (2014) 37–45, https://doi.org/10.1016/j.intimp.2014.08.002.

[9] H. Shiratori, C. Feinweber, S. Luckhardt, B. Linke, E. Resch, G. Geisslinger, A. Weigert, M.J. Parnham, THP-1 and human peripheral blood mononuclear cell-derived macrophages differ in their capacity to polarize in-vitro, Mol. Immunol. 88 (2017) 58–68, https://doi.org/10.1016/j.molimm.2017.05.027.

[10] S. Tedesco, F. De Majo, J. Kim, A. Trenti, L. Trevisi, G.P. Fadini, C. Bolego, P. W. Zandstra, A. Cignarella, L. Vitiello, Convenience versus biological significance: are PMA-differentiated THP-1 cells a reliable substitute for blood-derived macrophages when studying in-vitro polarization? Front. Pharmacol. 9 (2018) 71, https://doi.org/10.3389/fphar.2018.00071.

[11] A. King, S. Balaji, L.D. Le, T.M. Crombleholme, S.G. Keswani, Regenerative wound healing: the role of interleukin-10, Adv. Wound Care 3 (2014) 315–323, https://doi.org/10.1089/wound.2013.0461.

[12] M. Bartneck, K.H. Heffels, Y. Pan, M. Bovi, G. Zwadlo-Klarwasser, J. Groll, Inducing healing-like human primary macrophage phenotypes by 3D hydrogel coated nanofibres, Biomaterials 33 (2012) 4136–4146, https://doi.org/10.1016/j.biomaterials.2012.02.050.

[13] H. Hong, X.Y. Tian, The role of macrophages in vascular repair and regeneration after ischemic injury, Int. J. Mol. Sci. 21 (2020), https://doi.org/10.3390/ijms21176328.

[14] C. Loebel, E.M. Czekanska, M. Bruderer, G. Salzmann, M. Alini, M.J. Stoddart, In-vitro osteogenic potential of human mesenchymal stem cells is predicted by Runx2/Sox9 ratio, Tissue Eng. 21 (2015) 115–123, https://doi.org/10.1089/ten.TEA.2014.0096.

[15] J.Y. Hsieh, T.D. Smith, V.S. Meli, T.N. Tran, E.L. Botvinick, W.F. Liu, Differential regulation of macrophage inflammatory activation by fibrin and fibrinogen, Acta Biomater. 47 (2017) 14–24, https://doi.org/10.1016/j.actbio.2016.09.024.

[16] Y. Miyamoto, K. Kubota, Y. Asawa, K. Hoshi, A. Hikita, M1-like macrophage contributes to chondrogenesis in-vitro, Sci. Rep. 11 (2021) 21307, https://doi.org/10.1038/s41598-021-00232-7.

[17] O.R. Mahon, D.C. Browe, T. Gonzalez-Fernandez, P. Pitacco, I.T. Whelan, S. Von Euw, C. Hobbs, V. Nicolosi, K.T. Cunningham, K.H.G. Mills, D.J. Kelly, A. Dunne, Nano-particle mediated M2 macrophage polarization enhances bone formation and MSC osteogenesis in an IL-10 dependent manner, Biomaterials 239 (2020) 119833, https://doi.org/10.1016/j.biomaterials.2020.119833.