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Original Article

Preparation of human primary macrophages to study the polarization from monocyte-derived macrophages to pro- or anti-inflammatory macrophages at biomaterial interface *in vitro*



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Background/purpose: Testing of dental materials when in contact with innate immune cells has been so far hindered by the lack of proper *in vitro* models. Human primary monocyte-derived macrophages (MDMs) would be an excellent option to this aim. However, the inability to detach them from the tissue culture plates contrast the possibility to culture them on biomaterials. The goal of the present work is to present and validate an innovative protocol to obtain MDMs from peripheral blood monocytes, and to reseed them in contact with biomaterials without altering their viability and phenotype.

Materials and methods: We differentiated MDMs on ultra-low attachment tissue culture plastics and recovered them with specific detachment solution in order to be reseeded on a secondary substrate. Therefore, using biological assays (RT-PCR, Western blot, and immunofluorescence) we compared their phenotype to MDMs differentiated on standard culture plates.

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Results: Transferred MDMs keep their differentiated M0 resting state, as well as the ability to be polarized into M1 (pro-inflammatory) or M2 (anti-inflammatory) macrophages.

Conclusion: These data provide the dental material research community the unprecedented possibility to investigate the immunomodulatory properties of biomaterials for dental application.

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Introduction

Over the past decades, the use of biomaterials has become increasingly popular in reconstructive dentistry.¹ For example, dental implants are commonly used therapeutic options for the rehabilitation of the masticatory, aesthetic, and phonetic functions in partially or totally edentulous patients;² while bone grafting materials can be employed in pre-implant reconstructive procedures to gain enough tissue before implant placement.³ Notably, the combined use of more and more performant solutions has been described to speed the healing process, promoting longer success rate after implant placement.⁴ Yet, the molecular mechanisms behind this phenomenon are poorly understood, although it has been well established that biomaterials have a profound impact on the host immune cells response, which are further known to play a pivotal role in bone healing and repair.^{5,6} Although *in vitro* settings do not completely resemble the *in vivo* situations, they can be informative and provide important insights into the mechanisms underlying biological events. However, while an extensive *in vitro* characterization of the osteogenic, osteoconductive and osteoinductive properties of the above-mentioned biomaterials is available in the literature,^{7–12} less is known regarding immune cells activation and response.

Macrophages are monocyte-derived phagocytic cells, which are essential components of the innate immune system.¹³ They can arise from circulating blood monocytes (monocyte-derived macrophages, MDMs) and play a pivotal role during the resolution of tissue inflammation. Moreover, already differentiated macrophages (also derived from an ancestral myeloid progenitor) are present in adult tissues as tissue resident M0 macrophages, which are the first cells to meet biomaterials after implantation.^{14,15} In this second scenario, according to the physicochemical properties of the implanted biomaterial, M0 macrophages can skew their resting phenotype and polarize into classically activated pro-inflammatory (M1) or alternatively activated anti-inflammatory (M2) macrophages.^{16–19} Notably, macrophage polarization is related to the bone remodeling phase following dental implant placement, with specific effects on function and differentiation of osteoclasts and osteoblasts.^{6,20} Hence, a complete *in vitro* characterization of the response of already-differentiated macrophages when in contact with dental biomaterials would be informative to elucidate the molecular mechanisms behind their therapeutic success.

In vitro research on the role played by M0 macrophages in the tissue interactions with biomaterials is challenging

due to the lack of proper models. At present, knowledge is mostly based on the use of murine macrophages.^{21–24} However, several discrepancies exist between murine and human macrophages metabolism. For example, murine macrophages have a stronger and less restricted activation of the NO₂ pathway if compared to human counterparts.²⁵ Thus, not every finding in mice can be transferred to real-life human situations. On the other hand, *in vitro* studies have also been performed by using human immortalized cell lines,^{26,27} which have, however, the limit to be of neoplastic origin and to require pharmacological treatments to achieve macrophage-like competences. This aspect may lead to changes in their phenotype over time and to the risk of obtaining inconsistent and unreliable results. To overcome these issues, the use of primary macrophages would be recommended. The ideal scenario would be the isolation of primary blood monocytes, their differentiation into differentiated MDMs (M0) and their seeding in contact with the target biomaterial. However, at present, this approach also has some caveats that need to be addressed. Indeed, MDMs are usually differentiated on standard tissue culture dishes. Given their strong adhesive properties, MDMs cannot be easily detached and, thus, should stand on the same surface for the duration of the whole experiment. This aspect clearly limits the use of these cells in combination with biomaterials. Moreover, also the direct seeding of primary blood monocytes on biomaterials before differentiation into MDMs is not optimal, since it might prematurely affect their polarization, thus, as previously mentioned, not reflecting the chronological order of cellular events *in vivo*. Indeed, although monocytes come in contact with the surface of biomaterials after blood invasion, their differentiation into macrophages would require at least 3–4 days *in vitro*, while tissue resident macrophages would experience the surface of the biomaterial immediately after its implantation. Therefore, to properly understand the mechanisms behind the interaction of primary macrophages with dental materials, an ideal protocol should allow to i) differentiate primary blood monocytes into MDMs on tissue culture dish, ii) detach them without causing significant damage and alteration and iii) transfer them on the surface of the target biomaterial for experimental studies.

Herein, we developed a protocol to fulfill these premises. Using functional and biological assays, we demonstrated that this new protocol (Test) does not affect the phenotype of M0 macrophages, which kept a morphology and CD68 expression comparable with M0 macrophages differentiated from primary blood monocytes using the

standard protocol (Control). Furthermore, we also showed that M0 responsiveness to polarizing stimuli was not affected. Indeed, M1 and M2 skewing was comparable between M0 differentiated with either the Control or the Test protocol.

Materials and methods

The methodology followed in this report has been fully illustrated in Fig. 1.

Ethics statement

This work was performed according to the Ethical Principles for Medical Research Involving Human Subjects as stated in the Declaration of Helsinki by the World Medical Association.

Isolation, characterization, and differentiation of human peripheral blood monocytes has been approved by the

Comitato Etico dell'Area Vasta Emilia Nord, Italy (protocol #3182 24.01.2018). Written informed consent was obtained from the subjects involved in this study.

Human peripheral blood monocytes isolation and differentiation into macrophages

Human peripheral blood monocytes were isolated from whole human blood. Thirty ml of blood were collected by a trained nurse and immediately transferred to the laboratory to be processed within 8 h. Blood samples were diluted 1:3 in Phosphate Buffered Saline (PBS), and 22 ml of the suspension were carefully distributed over 15 ml of Ficoll–Paque™ Plus (GE Healthcare Life Science, Pittsburgh, PA, USA). Samples were centrifuged at 400g for 30min at room temperature (RT) in a swinging-bucket rotor without brakes to obtain a buffy-coat fraction of peripheral blood mononuclear cells, which were harvested, resuspended in 50 ml of PBS, pelleted at 200×g for 10min at RT

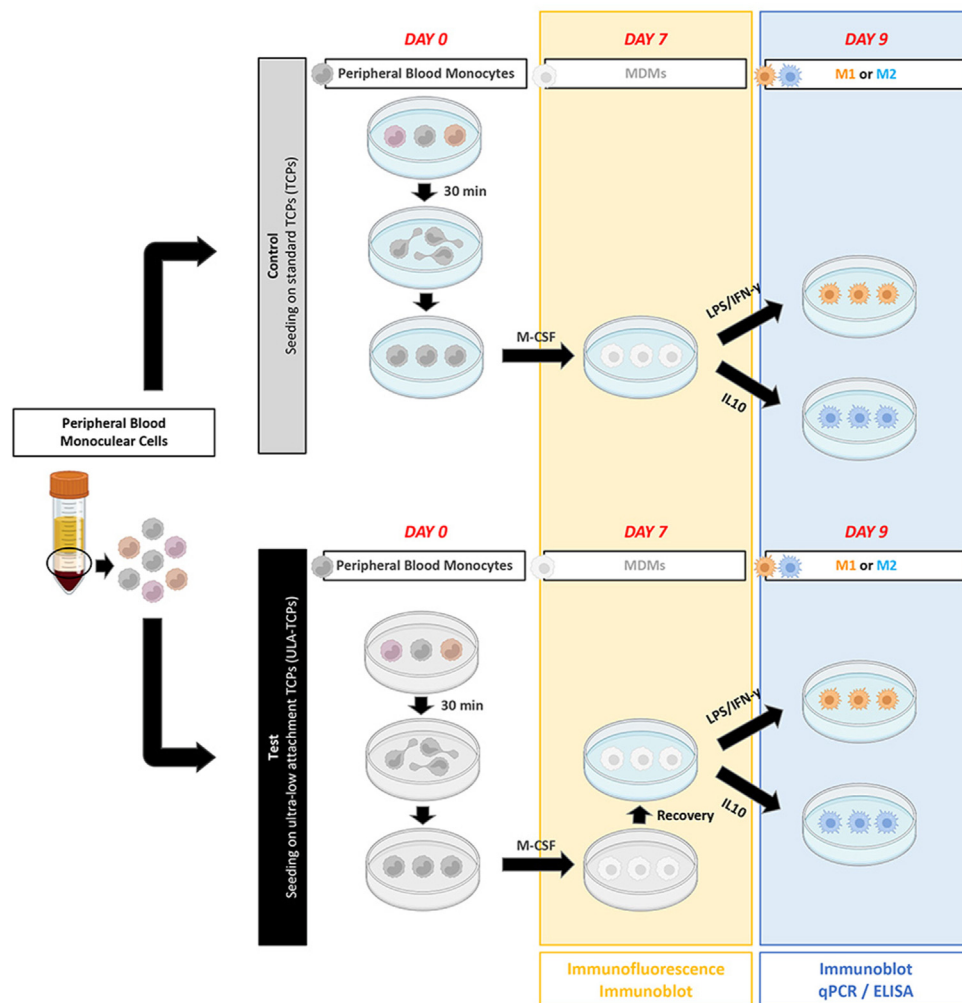


Figure 1 Diagram of the experimental procedure followed to obtain and polarize MDMs. After purification, human blood monocytes are seeded on standard (Control, Blue Petri dishes) or ULA-TCPs (TCPs, Grey Petri dishes) and cultured with M-CSF 50 ng/ml for seven days. At day seven, MDMs differentiation is evaluated by immunofluorescence and immunoblot. Afterwards, MDMs are exposed to cocktails of polarizing stimuli to drive their M1 (LPS 5 ng/ml and IFN- γ 25 ng/ml) or M2 (IL10 20 ng/ml) polarization for 2 days. At the last time point, gene and protein expressed are analyzed by RT-PCR, immunoblot and ELISA, respectively.

and washed two more times in PBS with the same procedure. Subsequently, cells were resuspended in RPMI medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with five percent of pooled AB human serum (Sigma–Aldrich, St. Louis, MO, USA) and seeded at a final density of 3×10^5 cells/ml either in standard tissue culture plates (TCPs) or in ultra-low attachment surface flasks (ULA-TCPs, both from Corning Inc., Corning, NY, USA). After 30min of incubation in standard culturing conditions (37°C , 5% CO_2), cultures were rinsed three times in PBS to purify adherent monocytes from residual suspended cells. Culture medium was finally renewed and supplemented with 50 ng/ml of macrophage colony-stimulating factor (M-CSF) (R&D Systems, Bio-Techne, Milan, Italy) to trigger macrophage differentiation and to obtain MDMs. Medium was exchanged every other day until day seven, prior to 24 h of resting in M-CSF-free RPMI medium.

MDMs detachment and reseeding

MDMs, differentiated from monocytes seeded in ULA-TCPs, were collected, and reseeded on standard TCPs seven days after their isolation. In brief, poorly adherent MDMs were mechanically collected by rinsing with PBS. To detach tightly adherent MDMs, the Macrophage Detachment Solution DXF (MDS, PromoCell GmbH, Heidelberg, Germany) was then applied for 20min at four degrees on gentle shaking. Detached MDMs were collected, mixed with those recovered mechanically, diluted 1:1 with washing buffer (PBS, 2 mM EDTA, 0.1% human serum albumin), centrifuged at 350g for 15min at RT, resuspended in M-CSF-free RPMI medium at a final density of 3×10^5 cells/ml and reseeded on standard TCPs for 24 h to allow cell adhesion. Cell morphology was daily monitored using a Nikon Eclipse TE300 inverted microscope equipped with a digital camera Nikon SIGHT DS-U1 (Nikon Europe BV, Burgerweeshuispad, Amsterdam, Netherlands).

M1/M2 polarization

To skew MDMs (M0) toward an inflammatory (M1) or anti-inflammatory (M2) phenotype, cells were incubated 48 h in RPMI supplemented with lipopolysaccharide (LPS) 5 ng/ml and interferon- γ (IFN- γ) 25 ng/ml, or with interleukin 10 (IL10) 20 ng/ml (all from Sigma-Aldrich), respectively. As a control, MDMs were maintained in fresh complete RPMI.

Immunofluorescence (IF)

For staining, cells were fixed in four percent paraformaldehyde for 15min at RT, rinsed twice in PBS, permeabilized with Triton-X-100 for 5 min at RT, blocked in 10% Bovine Serum Albumin (BSA, Sigma-Aldrich) and two percent normal goat serum (Thermo Fisher Scientific), and incubated overnight at four degrees in the presence of the mouse monoclonal anti-CD68 antibody (SantaCruz Biotechnology, Dallas, TX, USA) diluted 1:100 in blocking solution. Afterwards, cultures were washed three times in PBS and incubated with the Alexa Fluor 488 anti-mouse IgG antibody diluted 1:400 in blocking solution (Thermo Fisher Scientific) for 1 h at RT. Secondary antibody incubation was

followed by three washings in PBS and nuclei counterstaining with 1 $\mu\text{g/ml}$ DAPI (Sigma-Aldrich) for 20min at RT. Samples were finally mounted on coverslips using the Glycergel mounting medium (DAKO, Agilent Technologies, Santa Clara, CA, USA). Samples were analyzed under a confocal microscope (LSM900 Airyscan—Carl Zeiss, Jena, Germany).

RNA extraction, cDNA synthesis and real-time polymerase chain reaction (RT-PCR)

Total RNA was isolated with the GeneJET RNA Purification Kit (Thermo Fisher Scientific) following manufacturer's instructions. RNA concentration was measured with a spectrophotometer NanoDrop™ 1000 (Thermo Fisher Scientific) and stored at -80°C . Two-hundred-fifty nanograms of total RNA were used as a template for cDNA synthesis using a RevertAid RT Reverse Transcription kit (Thermo Fisher Scientific). Gene expression was detected by RT-PCR using a Power Up SYBR Green Master Mix (Thermo Fisher Scientific) on a Step One Plus apparatus (Applied Biosystems, Thermo Fisher Scientific). Data analysis was performed applying the dC_T method, when absolute mRNA normalized to *RPL15* levels are reported, or by ddC_T method, when mRNA normalized to *RPL15* levels are further referenced to a control sample set to one.

The sequences of the RT-PCR primers used are listed in the Suppl. Table 1. Sequences were designed with the NCBI primer designing tool (<http://ncbi.nlm.nih.gov/tools/primer-blast>) and tested for specificity and efficiency using cDNA standard curves.

Immunoblotting

Whole cell lysates were obtained by recovering sample with Laemmli buffer (250 mM Tris-HCl pH6.8, 8% SDS, 40% glycerol and 0.4 M DTT) diluted at its working concentration with lysis buffer (20 mM Tris-HCl pH7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β -glycerophosphate, 1 mM Na_3VO_4 , 1 mM NaF, 2 mM imidazole) supplemented with a protease inhibitor cocktail (Complete Mini EDTA-free, Roche, Basel, Switzerland). Cell lysates were subsequently heated 10min at 95°C , and proteins determined by the Lowry method. Twenty micrograms of total protein were loaded on a 10% gel and separated under reducing conditions at 100 V for 1 h and a half by SDS-PAGE. Proteins were blotted onto PVDF membranes overnight at four degrees. Blotted membranes were washed in Tris-buffered saline (50 mM Tris Base, 150 mM NaCl, pH7.5) containing Tween-20 (TBS-T), blocked in 10% blocking solution for 1 h, incubated with primary antibody overnight at four degrees on a shaker, washed three times in TBS-T and incubated with horseradish peroxidase-conjugated anti-mouse IgG (1:10,000). Finally, membranes were washed three more times in TBS-T before being exposed to the ECL Chemiluminescent HRP Substrate (Sigma Aldrich) for 1 min. Protein visualization was finally performed using the iBright™ FL1500 automated system (Thermo Fisher Scientific). Primary antibodies used for immunoblotting were the mouse monoclonal anti-CD68 (1:500, sc-17832, SantaCruz Biotechnology), rabbit

polyclonal anti-NF- κ B (1:1000, Cell Signaling, Danvers, MA, USA), rabbit polyclonal anti-pNF- κ B (1:1000, Cell Signaling), mouse monoclonal anti- β -actin (1:4000, Sigma-Aldrich).

Multiplex cytokine/chemokine array

At the end of culturing time, media were collected, centrifuged for 5 min at 1000g to remove any cell debris, and stored at -80°C until multiplex analysis of the secreted cytokines. The Human Procarta Plex Seven-plex (Thermo Fisher Scientific) was customized for the titration of the pro-inflammatory cytokines interleukin 1 β (IL-1s), 8 (IL8), 6 (IL6) and tumor necrosis factor (TNF) and of the anti-inflammatory cytokines interleukin 4 (IL4) and transforming growth factor β 1 (TGF β 1). Cytokine detection was performed following manufacturer's recommendation and quantification was carried out with the Bioplex MAGPIX[®] (BioRad Instrument, Hercules, CA, USA).

Statistical analysis

Data were analyzed with Prism6.1 (GraphPad, La Jolla, CA, USA). Values have been reported as means \pm standard deviation (SD) of three experiments performed in multiple replicates. Differences between the groups were evaluated with t-test or with one-way ANOVA, as specified for each figure. Differences were considered significant when $P < 0.05$.

Results

Differentiation of human peripheral blood monocytes into resting MDMs M0 macrophages

MDMs obtained in standard culture conditions are endowed with a spread morphology. Furthermore, they strictly adhere to the culture substrate and possess little protrusions, which are used to explore the surrounding milieu.²⁸ Live imaging of MDMs (Fig. 2A) show that our experimental (Test) protocol for macrophage

differentiation did not affect either the morphology or the adhesion properties of the cells to the culture plate notwithstanding detachment and reseeding. Indeed, resting M0 MDMs obtained with both protocols are plastic adherent, pancake-shaped and with extending pseudo-podia. To confirm resting M0 macrophages phenotype, the expression of CD68 was evaluated. CD68 is a well-known and established surface marker of the MDMs lineage, which is maintained also after M1 or M2 polarization (Suppl.Fig.1).²⁹ Its expression has been investigated in resting M0 MDMs by IF (Fig. 2B). While the abundance of CD68-positive cells was comparable between the two groups, the Test group showed an increased and more intense signal in some cells. However, when the expression of CD68 was studied by immunoblot (Fig. 2C), no significant difference in CD68 expression was detected between the groups, although a tendency to an increased expression was observed in the Test group. Overall, these data indicate that detachment and reseeding of MDMs do not substantially affect their M0 phenotype.

MDMs M0 macrophages skewing into pro- M1 or anti-inflammatory M2 macrophages

Next, we wished to ascertain whether our Test protocol affected the capacity of MDMs to be polarized into M1 or M2 macrophages, when exposed to specific stimuli (Fig. 3A). First, we studied the capacity of MDMs M0 macrophages to be activated. To this purpose, we analyzed the expression of NF- κ B and its phosphorylated form (pNF- κ B) by immunoblot before and after polarization (Fig. 3B). In both cases (Control vs. Test), while the expression of NF- κ B was comparable among all the groups, the pNF- κ B signal was 2.5-fold increased after M1-M2 skewing if compared to the resting M0 counterparts, indicating responsiveness of the cells to external stimuli. Then, we studied the induction of pro- and anti-inflammatory specific markers in M1 and M2 polarized macrophages. RT-PCR analysis for IL8 mRNA levels (gene *CXCL8*), *IL6* and *TNF* (pro-inflammatory cytokines) revealed a clearly induced expression of these genes for the M1 phenotype in both the experimental groups (Fig. 3C).

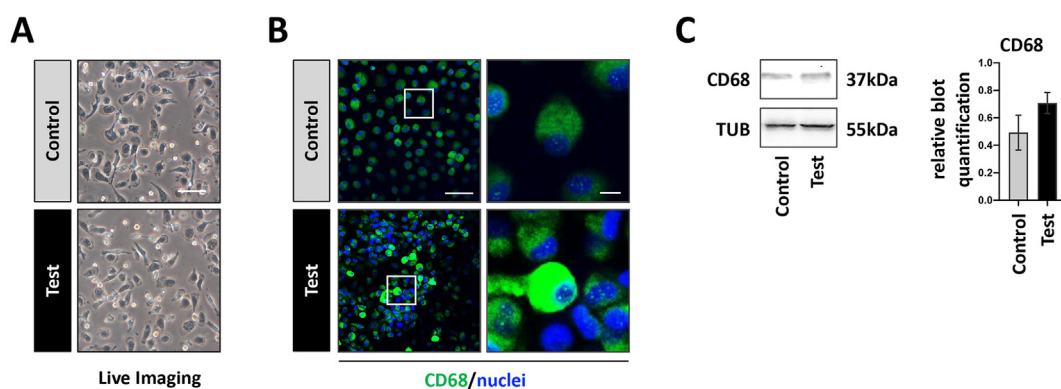


Figure 2 MDMs (M0) differentiation (A) Live imaging of MDMs (resting M0) after differentiation (Control) or after differentiation, detachment, and reseeding (Test). Scale bar: 50 μm (B) IF staining of CD68 (green) confirms monocyte differentiation into macrophages with both the Control and the Test protocols. White boxes indicate close-ups. Scale bars: 50 μm (IF); 10 μm (close-up). Nuclei (blue) (C) CD68 protein expression was further confirmed by immunoblot. Histograms at the right indicate CD68 bands quantification. kDa: kilo Dalton.

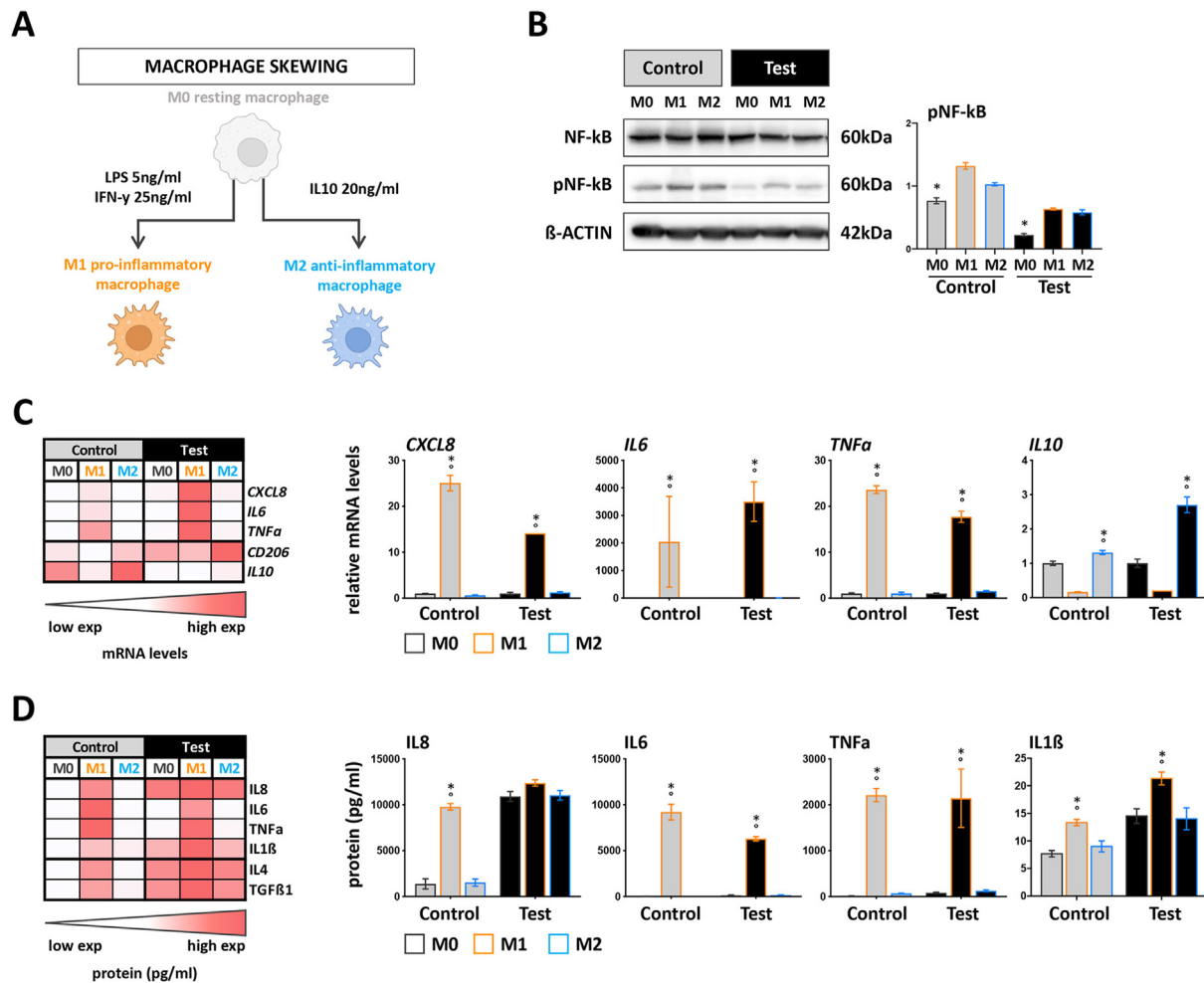


Figure 3 M1-M2 polarization (A) Diagram of the strategy adopted to differentiate resting M0 macrophages into pro-inflammatory M1 or anti-inflammatory M2 macrophages (B) Immunoblot showing NF- κ B and pNF- κ B expression in resting M0 and M1 or M2 polarized macrophages. Histograms at the right indicate pNF- κ B bands quantification. * = $P < 0.05$ M0 vs. M1 or M2. kDa: kilo Dalton (C) Heatmap showing absolute mRNA levels (white, low expression; red, high expression) of M1 (CXCL8, IL6 and TNF) and M2 (CD206 and IL10) markers expressed by resting M0, pro-inflammatory M1 or anti-inflammatory M2 macrophages obtained with either Control or Test protocol. CXCL8, IL6, TNF and IL10 relative mRNA expression levels are also reported in the histograms at the right. mRNA levels have been normalized for each group (Control vs. Test) to the levels expressed in the resting M0 macrophages (set to one). * = $P < 0.05$ M1 or M2 vs. M0; \circ = $P < 0.05$ M1 vs. M2 (D) Heatmap showing amount (white, low expression; red, high expression) of pro- (IL8, IL6, TNF and IL1 β) and anti-inflammatory (IL4 and TGF β 1) cytokines secreted in the medium by resting M0, pro-inflammatory M1 or anti-inflammatory M2 macrophages obtained with either Control or Test protocol for monocyte isolation and differentiation. IL8, IL6, TNF and IL1 β quantifications are also reported in the histograms to the right. * = $P < 0.05$ M1 or M2 vs. M0; \circ = $P < 0.05$ M1 vs. M2.

Conversely, CD206 and IL10 expression were enhanced in the M2 phenotype. The same tendency was also confirmed at the protein level for IL8, IL6 and TNF in the MDM conditioned medium (Fig. 3D). Since IL10 was used as a polarizing cytokine for M2 state, its quantification at protein level has not been reported. The only substantial difference between macrophages cultured with the Control method and those cultured with the Test protocol was the increased secretion of IL8 detected in M0 and M2 macrophages in the Test group. Additionally, the secretion of the pro-inflammatory cytokine IL1 β was also found to be more expressed in the M1 phenotypes in both the groups (Fig. 3D). Surprisingly, also the expressions of IL4 and of TGF β 1, which are normally identified as anti-inflammatory

cytokines, have been found to be enhanced in the two groups in the M1, rather than in the M2 phenotypes.

Discussion

The availability of reliable *in vitro* models to explore how biomaterial characteristics influence the local inflammatory response is a relevant biological end point, which might be of utmost importance to predict their integration. Herein, we provide the dental materials research community a suitable protocol to allow, for the first time, the use of primary human MDMs studies on changes induced by biomaterials. MDMs are a common and validated research

tool in other fields of basic research, such as cancer biology and hematology.^{30,31} However, their use in dental materials field has been so far hindered by their own nature. Indeed, MDMs, which are obtained by differentiation of human blood monocytes on standard culturing substrates, are tightly adherent cells.³⁰ This contrasts the possibility to detach and reseed them on the target biomaterials, without causing mechanical cell damage and rupture before testing. By the combined use of ULA-TCPs and a solution optimized to detach macrophages (Macrophage Detachment Solution DXF, Promega) we were able to successfully recover MDMs from the first culturing substrate and to reseed them on standard TCPs without altering their phenotype and their capacity to be activated and polarized. To validate the suitability of our method, we first investigated the capability of the macrophages differentiated with the Test protocol to adhere to conventional TCPs and to keep their differentiated M0 state after their recovery from ULA-TCPs (Fig. 2). Our method allowed to obtain a good number of viable cells, which adhere to the experimental culture plate after collection. Most importantly, this evidence indicates that our protocol does not affect the integrity of the adhesion molecules complex. Moreover, it was also possible to observe that the morphology of the cells obtained with our Test protocol was completely conserved and comparable with that shown by macrophages differentiated and maintained on conventional TCPs. Besides maintaining a proper morphology, detached and reseeded MDMs also continued to exhibit CD68 expression. However, while in the Test group CD68 expression was heterogeneous and strongly detectable in some cells, its signal in the Control group was more homogeneous, mostly likely due to a complete redistribution of the signal. This evidence could be explained by the fact that cells of the Control group were let undisturbed on standard culture plates throughout the period of differentiation. This hypothesis is also supported by immunoblot that did not show evident differences of average CD68 expression between cells obtained with the two methods, confirming that the two populations are comparable. We then investigated the functionality of the M0 resting macrophages obtained with either protocol (Fig. 3). To this purpose, MDMs obtained with the Test or the Control protocol were exposed to specific polarizing stimuli. Macrophage polarization into M1 or M2 involves the activation of the master regulator of the inflammatory response NF- κ B. After its phosphorylation, NF- κ B (pNF- κ B) migrates in the cell nucleus and promotes the transcription of several inflammatory or anti-inflammatory genes coding for several cytokines that are secreted during macrophage activation.³² The NF- κ B dependent responses were comparable in cells obtained with the two methods. Of note, pNF- κ B levels were higher after polarization compared to M0 resting macrophages, indicating the expected responsiveness of the cells to the polarization. Consistently, either mRNA abundance or the secretion of specific pro- and anti-inflammatory cytokines was increased by the M1 and M2 stimuli, respectively. The magnitude of the response was comparable between the cells differentiated with the Control or the Test method, with the exception of IL8.

We acknowledge that enzymatic or non-enzymatic approaches might also be proposed for the detachment and

reseeding of MDMs. However, the use of strong enzymatic strategies, such as trypsin or accutase, have been shown to affect the integrity of surface markers, therefore, likely affect the biological properties of the cells.³³ On the other hand, methods to achieve macrophage detachment without involving enzymatic approaches have been based on the prolonged use of solutions, containing calcium-chelating substances. In this case, challenges are due to the excessively long incubation times, which are required to obtain a sufficient cell recovery, and which may trigger apoptosis after reseeding of the cells.³⁴

Another critical issue that might arise from our work is the fact that after their implantation, biomaterials are immediately soaked with patient's own blood. The consequent conditioning of the surface with autologous protein and macromolecules makes virtually impossible the direct interaction of the biomaterial surface with cells.^{35,36} Although this was not our focus, this aspect needs to be clearly addressed and considered in future studies. Additionally, also the monocytes present in the blood stream interact with the biomaterial surface and thus differentiate into macrophages. Consequently, the direct seeding of monocytes on biomaterials would also be a possibility to study the immunoregulative properties of biomaterials. However, since monocytes require at least 3–4 days for completely differentiate into macrophages, it is highly improbable that their response would be more relevant than that of already differentiated tissue resident macrophages (M0), which experience the implantation of the foreign material immediately after its placement.

In conclusion, we believe that our non-conventional protocol, consisting of i) culturing MDMs on ULA-TCPs, ii) maximizing their recovery avoiding enzymatic treatments and iii) re-seeding them in contact with the target substrate, is a reliable method to prepare cells for the study of the response of human primary innate immune cells to biomaterials *in vitro*.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jds.2023.01.020>.

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