Macrophage interactions with polylactic acid and chitosan scaffolds lead to improved recruitment of human mesenchymal stem/stromal cells: a comprehensive study with different immune cells

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Despite the importance of immune cell–biomaterial interactions for the regenerative outcome, few studies have investigated how distinct three-dimensional biomaterials modulate the immune cell-mediated mesenchymal stem/stromal cells (MSC) recruitment and function. Thus, this work compares the response of varied primary human immune cell populations triggered by different model scaffolds and describes its functional consequence on recruitment and motility of bone marrow MSC. It was found that polylactic acid (PLA) and chitosan scaffolds lead to an increase in the metabolic activity of macrophages but not of peripheral blood mononuclear cells (PBMC), natural killer (NK) cells or monocytes. PBMC and NK cells increase their cell number in PLA scaffolds and express a secretion profile that does not promote MSC recruitment. Importantly, chitosan increases IL-8, MIP-1, MCP-1 and RANTES secretion by macrophages while PLA stimulates IL-6, IL-8 and MCP-1 production, all chemokines that can lead to MSC recruitment. This secretion profile of macrophages in contact with biomaterials correlates with the highest MSC invasion. Furthermore, macrophages enhance stem cell motility within chitosan scaffolds by 44% but not in PLA scaffolds. Thus, macrophages are the cells that in contact with engineered biomaterials become activated to secrete bioactive molecules that stimulate MSC recruitment.

1. Introduction

Implantation of tissue-engineered scaffolds triggers simultaneously two types of host response: an immune reaction against the foreign material and activation of tissue repair mechanisms at the injured area. Although the nature of these biological processes is very distinct, they share inflammation as a common denominator. If perpetuated, inflammation can be detrimental, leading to tissue damage and fibrotic encapsulation of the biomaterial, but at the same time inflammation is essential to promote progenitor cell recruitment and initiate healing mechanisms [1]. The
requirement of fine-tuning inflammation for a beneficial regenerative outcome prompted the design of ‘immuno-informed’ biomaterials to control the host response [2]. The great majority of regenerative medicine strategies aim for spatial and temporal control of stem cell differentiation. But for effective tissue repair, it is also necessary to tightly control the coordinated movement of different cell types to the injury site. Thus, strategies that modulate the immune response to attract endogenous stem cells are appealing, as we start to understand that recruitment, and not only proliferation and differentiation of progenitor cells is important for effective regeneration.

Mesenchymal stem/stromal cells (MSC) have a pivotal role in supporting the regenerative process due to their ability to migrate to inflamed tissues, to differentiate in different lineages and to their paracrine and immunomodulation properties [3]. Importantly, it was shown in a murine model that upon biomaterial subcutaneous implantation there is first recruitment of inflammatory cells, which is then correlated with recruitment of MSC [4–7]. MSC recruitment can then lead to subsequent modulation of inflammation and is essential to promote the constructive remodelling of the tissue [8,9]. Nevertheless, the specific contribution of different immune cell populations interacting with implanted biomaterials for MSC recruitment remains unexplored.

In the first 48 h upon implantation, natural killer (NK) cells and polymorphonuclear neutrophils become the predominant cells at the tissue–implant interface [10,11]. NK cells are lymphocytes crucial for remodelling of the endometrium during pregnancy, and which can promote MSC recruitment, possibly through secretion of different chemokines [12]. Monocyte recruitment from the blood to the implant site thrives under its inflammatory environment, where they gradually differentiate into macrophages that replace the short-lived neutrophils and NK cells. Macrophages are master regulators of the foreign body response and tissue regeneration through secretion of bioactive molecules, which can impact inflammation, angiogenesis, extracellular matrix remodelling and stem cell recruitment and differentiation [2]. And indeed, macrophages are potent inducers of MSC recruitment, mainly through the production of the chemokine RANTES [12,13]. The remarkable plasticity of macrophages allows them to adopt a dynamic profile between M1 pro-inflammatory and M2 pro-regenerative functional programmes, which can shift the regenerative outcome. Essentially, these over-simplistic functional phenotypes are not exclusive and the overall response is the net result of a combination of distinct macrophage phenotypes dictated by the specific microenvironment, cell–cell interaction and biomaterial properties [14,15].

Even though biomaterials impact immune cells, few studies compared the response of different populations to three-dimensional matrices. But more importantly, its consequence on the dynamical behaviour of stem cells is not understood. Here, we analysed the ability of human immune cells interacting with different scaffolds to promote human stem cell recruitment. Thus, two different types of materials were used as models for biomaterials with distinct physicochemical properties: polylactic acid (PLA), a US Food and Drug Administration (FDA) approved synthetic polymer used for bone repair or as internal fracture fixation device [16]; and chitosan which is a natural polysaccharide, currently explored for many applications, including wound dressings and bone regeneration [17,18]. Firstly, the effect of PLA and chitosan scaffolds on the behaviour of primary human peripheral blood mononuclear cells (PBMC), NK cells, monocytes and macrophages isolated from peripheral blood of healthy donors was characterized. Then, it was determined how the responses of these immune cell populations affected human bone marrow MSC recruitment and motility within three-dimensional matrices (figure 1).

2. Material and methods

2.1. Scaffold fabrication

2.1.1. Polylactic acid scaffolds

Poly-96 L/4DL-lactic acid (from PURAC, The Netherlands) was used. PLA scaffolds were fabricated by solvent casting and particle leaching as previously described [19]. A 5% (w/v) PLA solution in

Figure 1. Schematic representation of the study performed. Experiments were performed with three-dimensional PLA or chitosan scaffolds on a well of a 24-well plate, and, for invasion assays, a Matrigel-coated transwell chamber on top of it, with a spacer to compensate for scaffold height. Different immune cell populations, namely, PBMC, NK cells, monocytes and macrophages, were seeded on the scaffolds to analyse cell number, metabolic activity, cell morphology, secretory profile and MSC recruitment. Furthermore, motility of MSC seeded on scaffolds was also analysed. (Online version in colour.)
chloroform was mixed with sieved sodium chloride (NaCl) measuring between 75 and 150 μm. The slurry was cast into 24-well Teflon moulds until complete chloroform evaporation. NaCl particles were dissolved by immersing the cylinders in distilled water.

2.1.2. Chitosan scaffolds
Chitosan (France-Chitine) was purified as described [20]. Chitosan sponges were prepared by freeze-drying using a 2% solution of chitosan (degree of acetylation: 12.00 ± 2.35%, molecular weight: 324 ± 27 × 10^3) as in [20,21]. Briefly, chitosan was hydrated overnight at 4°C and dissolved by adding acetic acid (Panreac) to a final concentration of 0.2 M under strong vortex agitation. The chitosan solution was incubated for 24 h at 4°C, was centrifuged at 4165 g for 5 min and then 800 μl was added to each well of a 48-well plate. Plates were placed at ~20°C and freeze-dried at ~80°C for 48 h to produce scaffolds.

Both PLA and chitosan scaffolds were cut into a cylinder shape of 11 mm diameter and 2 mm height (20.2 ± 0.5 and 5.8 ± 0.5 mg average weight for PLA and chitosan, respectively) and disinfect as in [21].

2.2. Scanning electron microscopy characterization of three-dimensional scaffolds
Cross-sections of 2 mm thickness were cut in liquid nitrogen and mounted with carbon tape for scanning electron microscopy (SEM) analysis. Samples were sputter-coated with gold and observed with a JEOL JSM-6301F SEM, at 1 kV and amplifications of 1000× to 2500×. Bone diameter was measured with ImageJ software.

2.3. Measurement of endotoxin levels
PLA and chitosan extracts were prepared by cutting the scaffolds into small pieces that were suspended in 40 ml endotoxin-free water per gram of dry polymer, and incubated for 24 h at 50°C under continuous shaking (250 rpm), as described elsewhere [22]. Endotoxin detection was performed by Analytical Services Unit of iBET, Oeiras, Portugal using a Charles River endotoxin detection kit.

2.4. Cells
Human bone marrow MSC (Lonza) were cultured in MSC growth medium (DMEM with low glucose supplemented with Glutamax plus 10% MSC selected inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin (all from Invitrogen)). Cells were incubated at 37°C/5% (v/v) CO2 and dissolved by adding acetic acid (Panreac) to a final concentration of 0.2 M under strong vortex agitation. The chitosan solution was incubated for 24 h at 4°C, was centrifuged at 4165 g for 5 min and then 800 μl was added to each well of a 48-well plate. Plates were placed at ~20°C and freeze-dried at ~80°C for 48 h to produce scaffolds.

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2.5. Cell seeding
To understand how distinct materials affected immune cells, PBMC, NK cells or monocytes isolated from the same donor were re-suspended in DMEM without serum and seeded on two-dimensional TCPS or in PLA or chitosan three-dimensional scaffolds. For that, 25 μl of cell suspension was added to each side of the scaffold with a total of 6 × 10^5 immune cells per scaffold. Then, the seeded scaffolds were incubated for 4 h at 37°C/5% (v/v) CO2 to promote cell adhesion before adding 750 μl of DMEM without serum. Cell culture proceeded for 48 h. For macrophages, 6 × 10^3 monocytes were seeded as described and allowed to differentiate in the materials for 7 days in 750 μl of RPMI medium supplemented with 1% penicillin/streptomycin and 10% inactivated FBS. Then, this culture medium was carefully removed and washed with phosphate-buffered saline (PBS) before adding 750 μl DMEM medium without serum and culturing for another 48 h.

2.6. Metabolic activity
The metabolic activity of PBMC, NK cells and monocytes that had interacted for 48 h with the materials, or macrophages that had been in culture for 9 days with the scaffolds was assessed with a resazurin assay as described elsewhere [23]. Values obtained with scaffolds incubated in the absence of cells were subtracted in the final analysis.

2.7. DNA quantification
PLA and chitosan scaffolds were washed in PBS, thinly cut and incubated in 1% Triton X-100 (Sigma) at 4°C under agitation for 1 h. To reduce the effects of Triton X-100 (Sigma) in DNA quantification, the solutions were diluted in PBS for a final concentration of 0.1% Triton X-100. Then, scaffolds were vortexed and Quant-iT™ PicoGreen dsDNA Reagent (Invitrogen) was used according to the manufacturer’s protocol to quantify DNA content. To estimate the number of cells on the scaffolds, a standard curve was prepared using samples with a known number of PBMC, NK cells and monocytes.

2.8. Nuclei and actin staining
For analysis of cellular morphology, cells were stained for actin and nuclei. After 48 h (for PBMC, NK cells and monocytes) or 9 days (for macrophages) of culture in the scaffolds, cells were washed with PBS and fixed with 4% paraformaldehyde for 30 min at 4°C and washed with PBS. Cells were incubated with phalloidin–AlexaFluor®633 (50 μl, Invitrogen) in PBS (1:40) for 1 h on ice and washed with PBS. Nuclei were then counterstained with propidium iodide (20 μg ml−1) for 10 min followed by washing with PBS. Scaffolds were imaged on a laser scanning confocal microscope (Leica TCS SP5 II, model DMi6001B-CS, Bioimaging Center for Biomaterials and Regenerative Therapies, b.IMAGE, Porto).

2.9. Protein arrays
Monocyte-derived macrophages were cultured for 7 days on two-dimensional TCPS or in three-dimensional chitosan or PLA scaffolds in RPMI medium supplemented with 1% penicillin/streptomycin and 10% inactivated FBS. Cells were cultured in the absence of any additional growth factors/cytokines such as M-CSF or GM-CSF.

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Array III (Raybiotech) was used to analyse secretion of 40 cytokines according to the manufacturer's instructions. Supernatants from chitosan scaffolds incubated without cells were used as a control.

2.10. Enzyme-linked immunosorbent assays

Enzyme-linked immunosorbent assays (ELISAs) were performed to quantify secreted cytokines. Supernatants were collected after 48 h of immune cell–material interaction, centrifuged and kept at −80°C until analysis. IL-6, IL-8, MIP-1α, MIP-1β, MCP-1 and RANTES levels were quantified according to the manufacturer's instructions (Mini ELISA Development Kits, PeproTech). Concentrations were determined for five donors per condition and values were corrected with the amount determined for materials incubated in the absence of cells.

2.11. Invasion assay

Studies on invasion of MSC were performed using a modified transwell chamber system. Membrane filters with a pore size of 8 μm that had been coated with Matrigel (BD Biosciences) were used for the top compartment. Matrigel mimics the extracellular matrix that cells need to invade through in order to arrive to the implant site. The lower compartments of the invasion chamber contained 750 μl of DMEM medium, as a negative control, or immune cells (PBMC, NK cells, monocytes and macrophages) seeded on two-dimensional TCPS or PLA and chitosan three-dimensional scaffolds as already described. Custom spacer rings with 2 mm height were used to maintain the distance between the seeded scaffolds and the transwell insert. Matrigel-coated inserts were pre-incubated for 1 h with serum-free DMEM before adding MSC at 4 × 10^5 cells in 500 μl serum-free DMEM medium. The invasion chambers were incubated for 24 h at 37°C/5% CO₂. After incubation, inserts were washed with PBS and cells were fixed in 4% parafomaldehyde for 20 min at room temperature (RT). Inserts were washed with PBS and kept at 4°C until analysis. Cells on the top surfaces of filters were wiped off with cotton swabs and the membrane was carefully cut and mounted on a slide with Vectashield and DAPI. Cells that had migrated into the lower compartment and attached to the lower side were counted in ten 200x fields of view for each membrane. The number of migrated cells was estimated by taking into account the area of a field of view and the total area of the membrane.

2.12. Photoswitching and imaging Dendra2+ mesenchymal stem/stromal cells to measure cell motility

MSC motility was estimated by following the behaviour of photo-switched Dendra2+ MSC for up to 7 days, as previously described [24]. Dendra2- MSC can be converted from green to red fluorescence in defined regions, being then easier to track the cells over long incubation periods that require placing the cells in the incubator between time points. Briefly, cells were transfected by electroporation with Dendra2 plasmid DNA (pDendra2, Clontech) using an X-Cell Gene Pulser (BioRad), with 80% efficiency, as described in [24]. MSC were then seeded on PLA or chitosan scaffolds with a square shape of 4 × 4 mm and 2 mm height. When mentioned, scaffolds had been previously seeded with 6 × 10^6 human monocytes, which were allowed to differentiate into macrophages for 7 days. Then, 2 × 10^6 MSC in 25 μl of DMEM medium were added to each side (top and bottom) of a scaffold in non-treated 24-well tissue culture plates. Cells were allowed to adhere to the scaffold for 4 h at 37°C/5% CO₂ and then 400 μl of medium was added. MSC to macrophage ratio was 1:3. Green-to-red photoconversion was performed with a laser scanning confocal microscope (Leica TCS SP5 II, model DMi6000B-CS, Bioimaging Center for Biomaterials and Regenerative Therapies, bIMAGE, Porto). The reference red cell population was then used to centre the scaffold, which was imaged 1, 4 and 7 days after conversion (three to five spots per scaffolds in each individual experiment). After imaging, cells were returned to the incubator and cultured at 37°C/5% CO₂ until the next imaging time-point.

2.13. Dendra2 imaging data analysis

Matlab plugin: an automatic tool named Dendra2Imaging was implemented in Matlab™. This tool enabled analysis of the infiltrated area covered by photo-marked cells in microscopy images acquired in different days. The algorithm defines a threshold value used to bin the input image in order to extract the objects (cells) from their background [25]. For standardization purposes, the same threshold value was applied to images of the same series in distinct experimental conditions, allowing unbiased comparison of cell motility. Based on the segmented image the algorithm automatically estimates the region occupied by cells as well as the convex hull contour of that same region. The region occupied by photo-marked cells was then normalized to the area measured at time-point 0.

2.14. Statistical analysis

Statistical analysis was performed using Passw statistics, v. 5.01. Friedman matched pair test followed by Dunns comparison test was used to compare more than two samples. For grouped samples, two-way ANOVA test was used followed by Bonferroni post-test. Data are mean ± s.e.m. unless stated otherwise. Values of *p < 0.05, **p < 0.01 and ***p < 0.001 were considered statistically significant.

3. Results

3.1. Scaffold characterization

To compare the behaviour of immune cell populations incubated with materials with a different chemistry, we prepared PLA and chitosan scaffolds with approximately the same pore size (electronic supplementary material, figure S1), which can be instrumental in defining the inflammatory response towards biomaterials. Both types of scaffolds showed a homogeneous porosity with the large pores having an average diameter of 141 ± 44 μm for PLA and 125 ± 25 μm for chitosan, in agreement with previous reports [21,26]. PLA had a rougher surface compared with chitosan and displayed micropores with a mean diameter of 25 ± 10 μm against 42 ± 24 μm for chitosan. Both types of scaffold had endotoxin levels lower than 0.2 EU ml⁻¹, which are below the recommended FDA limit for implanted biomaterials.

3.2. Metabolism, cell number and morphology

Upon biomaterial implantation, distinct immune cell populations are recruited and activated in a highly regulated manner over the acute and chronic phases of inflammation. Here, the behaviour of PBMC, NK cells and monocytes was evaluated after 2 days in contact with the materials while macrophages were analysed after differentiation through 9 days of contact with the scaffolds. Although not statistically significant, there was a tendency for the metabolic activity of macrophages, but not monocytes, to increase in contact with PLA and chitosan scaffolds as opposed to two-dimensional TCPS. By contrast,
PBMC and NK cell metabolism was significantly reduced upon interaction with PLA or chitosan scaffolds (figure 2a).

Immune cells metabolism was not correlated with cell number as DNA quantification suggests that NK cells showed a significantly higher number of cells when incubated with PLA. This indicates that there was cell proliferation even in the absence of any exogenous stimulus apart from the scaffold, but that the metabolic activity per cell is diminished (figure 2b). The number of macrophages was maintained in both types of three-dimensional scaffolds but the cells disappeared on TCPS. Therefore, caution must be taken when comparing data from macrophages differentiated on TCPS with cells in biomaterials. No major differences were observed with monocytes. Visualization of the cells upon actin staining revealed that in all cases cells were distributed throughout the scaffolds (electronic supplementary material, figure S2). A more detailed observation suggests that NK cells adopted a more elongated morphology, with polarized actin, when in chitosan scaffolds than in PLA (figure 2c). Furthermore, an irregular morphology with extensive actin protrusions was frequently observed for macrophages in PLA but not in chitosan. Some cell fragmentation was noted, consistent with minor cell death within the scaffolds, as anticipated from the results obtained when estimating the number of cells by DNA quantification (figure 2b). Very few giant cells were observed for either PLA or chitosan scaffolds.

**Figure 2.** Modulation of immune cell behaviour by distinct materials. (a) Metabolic activity, (b) cell number and (c) morphology were determined for immune cells incubated for 48 h or 9 days (macrophages) on two-dimensional TCPS or in three-dimensional scaffolds of chitosan (Ch) or PLA. n = 3. (a) Metabolic activity was estimated with a resazurin assay. The background fluorescence intensity obtained in the presence of each material was subtracted to experimental values. (b) Cell number was estimated by quantifying the amount of DNA with Quant-IT PicoGreen. The dashed line indicates the number of cells initially seeded. (c) To visualize cells, scaffolds were fixed and stained for actin (green) and the nuclei (red) before being visualized by laser scanning confocal microscopy. Chitosan scaffolds can be observed in green due to their autofluorescence. Scale bar, 12.5 μm. Data are represented as floating bars with line at the mean ± maximum to minimum value. (Online version in colour.)
3.3. Cytokine secretion

As macrophages showed the highest metabolic activity in the presence of three-dimensional biomaterials, their supernatants were analysed for the presence of 40 inflammatory mediators with antibody arrays. Importantly, materials had a profound effect on the secretion profile of these macrophages (figure 3a). ELISA was performed to quantify the inflammatory mediators that achieved quotients of signal intensity higher than 0.3: IL-6, IL-8, MIP-1α, MIP-1β, MCP-1 and RANTES (figure 3b). Distinct immune populations were cultured in the different materials (figure 4) and it was found that PBMC secreted low amounts of IL-6, MIP-1α and MCP-1 and expressed high levels of IL-8 with no statistical differences when incubating with the different materials. NK cells secreted more RANTES when cultured on two-dimensional TCPS than in three-dimensional chitosan or PLA scaffolds. Monocytes showed a trend to express more IL-6 and MIP-1α when in chitosan and PLA scaffolds than when with TCPS while their culture in PLA induced a significantly higher expression of IL-8 than on two-dimensional TCPS or in three-dimensional chitosan scaffolds. No differences were found for MCP-1 between materials and MIP-1β was not detected in monocyte supernatants. Most interestingly, when these cells were allowed to differentiate into mature macrophages there was secretion of IL-6, IL-8 and MCP-1 for PLA while the interaction with chitosan scaffolds led to secretion of IL-8, MIP-1α, MIP-1β, MCP-1 and RANTES. There was no correlation between the purity of the immune cell populations and the concentration of secreted cytokines (electronic supplementary material, figure S3).

Thus, the secretion profile of PBMC was not affected by the material, while NK cells showed decreased production of RANTES in the three-dimensional biomaterials. And of significant importance, differentiation of monocyte-derived macrophages on PLA and chitosan scaffolds led to secretion of inflammatory mediators known to stimulate MSC recruitment.

3.4. Mesenchymal stem/stromal cell recruitment and motility in three-dimensional microenvironments

Different materials impacted on different immune cells, with PLA and even more strongly chitosan stimulating macrophage activity and chemokine secretion. Therefore, we sought to understand the functional consequences of this material–cell interaction on human stem cell recruitment. To investigate this, a modified Boyden chamber invasion assay was performed. PLA or chitosan scaffolds were placed on a well of a 24 well plate, and a Matrigel-coated transwell chamber was inserted on top of it. Human MSC were allowed to migrate for 24 h towards the bottom well with the scaffolds seeded with different immune populations (PBMC, NK cells, monocytes or macrophages). To ensure that immune cells had time to produce paracrine factors, immune cells were cultured in DMEM without serum for 24 h and only then was the invasion assay performed. To analyse the role of macrophages, monocytes were allowed to differentiate in the different materials for 7 days.

Chitosan scaffold by itself showed a trend to recruit more MSC compared with TCPS and PLA negative controls (figure 5). PBMC were not capable of relevant MSC recruitment.
independently of the substrate, with the number of recruited MSC being very similar to the negative controls. On the other hand, NK cells on two-dimensional TCPS were able to recruit 4.5 times more MSC than in the control. This value decreased to 1.9 and 2.1 times in chitosan and PLA three-dimensional scaffolds, respectively. Monocytes on two-dimensional TCPS showed a 5.8-fold increase in MSC recruitment compared with the negative control. However, monocyte interaction with chitosan and PLA scaffolds hindered this increase to 1.2- and 1.5-fold, respectively. Importantly, when these cells were differentiated into macrophages the number of recruited MSC was decreased on two-dimensional TCPS but was markedly higher when in contact with chitosan and PLA scaffolds. Indeed, macrophages were able to recruit 3 and 2.1 times more MSC for chitosan and PLA against 1.9-fold observed for TCPS. Thus, NK cells and monocytes on two-dimensional TCPS led to a high number of recruited MSC but when differentiated in the presence of either chitosan or PLA scaffolds macrophages were the most effective in recruiting MSC (figure 5), in agreement with the secretion profile found for these cells.

To understand whether the presence of macrophages would encourage not only MSC recruitment but also MSC motility within the three-dimensional scaffolds, the dynamical behaviour of the cells was imaged throughout 7 days as previously described [24]. Tracking Dendra2 labelled and photo-marked MSC revealed that three-dimensional MSC motility in the absence of immune cells was similar for PLA and chitosan scaffolds. But interestingly, there was a 44% increase in the cell motility within chitosan scaffolds at day 7 when MSC were co-cultured with macrophages (figure 6a,b). The same co-culture led to a 17% decrease in cell motility in PLA scaffolds (figure 6c). Thus, interactions between macrophages and chitosan scaffolds led to the highest increase in both MSC recruitment and motility.

Figure 4. Cytokine secretion profile of PBMC, NK cells, monocytes and macrophages cultured on materials. Cells were cultured for 48 h on TCPS or in three-dimensional chitosan and PLA scaffolds. Macrophages were allowed to differentiate for 7 days, washed and cultured in serum free medium for 48 h. The amounts of IL-6, IL-8, MIP-1α, MIP-1β, MCP-1 and RANTES present in the supernatants from five donors were quantified by ELISA. *p < 0.05 and ***p < 0.001.
4. Discussion

Here, we investigated which immune cells are activated by PLA and chitosan scaffolds in such a way that promotes stem cell recruitment and motility. While the type of scaffold influenced the extent and type of immune cell responses, it was the cell type that led to major differences in promoting MSC recruitment.

In this study, to avoid interference of unknown molecules in the secretion profile and cell recruitment assays, experiments were performed after washing and in the absence of serum. However, immune responses can depend on the proteins adsorbed to the material surface and this should be addressed in future studies. Also, provisional matrix formation includes dynamic adsorption and desorption of several serum proteins, the Vroman effect, and this can lead to another level of complexity: activation of complement and coagulation systems, which are difficult to mimic.

Here, the number of NK cells increased when cultured on three-dimensional materials for 48 h without any other stimuli, especially on PLA, suggesting that these biomaterials can trigger NK cell proliferation. Indeed, cell culture in a three-dimensional spatial arrangement has been reported to affect a range of cellular functions, including cell proliferative index and gene expression, compared with two-dimensional substrates [27,28]. Moreover, similar observations were found between three-dimensional matrices with distinct properties [29]. Nevertheless, the cell metabolism was reduced when in contact with the scaffolds, indicating that individual cells are less active. As resazurin conversion rate depends on the cell metabolic pathway, which is modulated by oxygen availability [30], the decreased metabolic activity in the scaffolds could be related to hypoxic gradients created by the matrix architecture and culture density in these three-dimensional biomaterials [31,32]. Similarly to other studies, NK cells on two-dimensional TCPS secreted high levels of RANTES and IL-8 and low amounts of MIP-1α and MIP-1β [33]. It has also been recently shown that NK cells secrete NAP-2 when on TCPS but its production in the presence of biomaterials remains unknown [34]. However, secretion of MIP-1α, MIP-1β and RANTES, which can stimulate MSC recruitment, was abrogated when NK cells were cultured with chitosan or PLA scaffolds. In agreement with this, NK cells were able to recruit MSC when on TCPS but not in the three-dimensional biomaterials. Thus, the role of these cells as promoters of stem cell recruitment may be hindered during the acute response to biomaterials.

As for monocytes, their number and metabolic activity were maintained upon incubation with the different materials. However, monocytes secreted higher levels of IL-8 in PLA while RANTES was expressed on TCPS but absent in the biomaterials. Similar to NK cells, monocytes had an improved capacity to promote MSC recruitment on TCPS but lost this ability in the presence of the three-dimensional biomaterials. RANTES can impact on MSC chemotaxis and also on other key functions linked with their regenerative potential. Indeed, it was recently shown that the immunosuppressive ability of MSC relies on STAT-1 signalling [35], which in these cells can be specifically activated by RANTES [36,37]. Therefore, interactions of monocytes and NK cells with biomaterials hamper expression of key chemokines and, as a consequence, MSC recruitment. It will be of interest to find strategies to modify biomaterials to stimulate NK cells and monocyte-mediated MSC recruitment.

The purity of the enriched population is an important issue to consider when interpreting the results of secreted...
inflammatory mediators. The percentage of NK cells and monocytes used specifically for the ELISA measurements was higher than 81%, being on average 89% and 90%, respectively. The contaminant population was CD3⁻CD2⁰ and thus probably includes B cells, dendritic cells and monocytes or NK cells, respectively. It is unlikely that these contaminant cells are the contributors to the cytokine levels because there was no correlation between the percentage of cells of each population and the levels of secreted mediators (electronic supplementary material, figure S3).

Contrary to the other immune cell populations analysed, macrophages differentiated in the presence of PLA or chitosan scaffolds showed high levels of metabolic activity. This difference was not perceived for their precursor cell, suggesting that monocyte to macrophage differentiation is needed to yield this response. There was probably an increased conversion of resazurin to resorufin due to abundant mitochondrial reactive oxygen species production by macrophages interacting with the scaffolds. Indeed, we noticed that macrophage culture in three-dimensional biomaterials lowered the pH of culture...
has revealed that MSC motility within PLA and chitosan is
interacting with the three-dimensional materials. Interestingly,
ability of monocyte-derived macrophages to recruit MSC when
inflammatory cytokines and chemokines that have been reported
to exacerbate the expression of CCR2, CCR3 and CCR4 receptors
action macrophages can produce an array of pro-
have all been implicated as potent chemotactic agents for
increased IL-8, MCP-1, MIP-1α. Distinct biomaterials had a
differentiation and survival. This will have an impact on the levels of cytokines being produced by mono-
cells with a reasonable expression of IL-8 that
very similar but that macrophages stimulated MSC movement when in chitosan but not in PLA. MSC movement in three-
dimensional microenvironments might be favoured by increased local concentrations of collagenases and gelatinases, which is
promoted by pro-inflammatory environments. In fact, a strong increase in expression of MMP-1, -3, -9 and -13 has been reported
for MSC upon treatment with inflammatory cytokines [37,45]. Moreover, it has been previously demonstrated that
chitosan leads to enhanced activity of MMP-9 produced by macrophages [46]. Therefore, differences in the inflammatory milieu could explain why macrophages encouraged MSC
teraction and motility of primary human responses triggered by different three-
mediation might be more useful [40]. Distinct biomaterials had a
proficient effect on macrophage secretion profile, with PLA sta-
mulation of secretion of IL-6 and IL-8 while chitosan led to
increase of IL-8, MIP-1α and MIP-1β and RANTES produ-
tion. IL-6, IL-8, MIP-1α, MIP-1β, MCP-1 and RANTES
have all been implicated as potent chemotactic agents for
MSC recruitment [37,41–44]. Also, upon biomaterial inter-
action macrophages can produce an array of pro-
flammatory cytokines and chemokines that have been reported
to exacerbate the expression of CCR2, CCR3 and CCR4 receptors
on MSC [36,37]. Thus, this synergism could be the basis for
the ability of monocyte-derived macrophages to recruit MSC when
interacting with the three-dimensional materials. Interestingly,
analysis of the cell motility within three-dimensional matrices
Figure 7. Summary of PBMC, NK cell, monocyte and macrophage behaviour when in contact with two-dimensional TCPS or three-dimensional chitosan and PLA scaffolds and their impact on MSC recruitment. Cell number is compared with the initial number of seeded cells. Metabolic activity of cells in two-dimensional TCPS was considered baseline (—) for comparisons with chitosan and PLA scaffolds. Recruitment of MSC in relation to each negative control is indicated. (Online version in colour.)

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<td>low; IL-8, MIP-1α; MIP-1β</td>
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<td>medium; IL-8, RANTES</td>
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<tr>
<td>low; IL-6, MCP-1</td>
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<tr>
<td>high; IL-6, IL-8</td>
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<tr>
<td>low; MIP-1α; MCP-1</td>
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<tr>
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5. Conclusion
In summary, this work provides a comprehensive analysis of primary human responses triggered by different three-
dimensional biomaterials and the functional consequence of those responses on recruitment and motility of primary
human MSC (figure 7). PBMC and NK cells showed an increased cell number in PLA and expressed an unfavourable secretion profile when interacting with chitosan or PLA scaffolds, and did not stimulate MSC recruitment. Importantly, monocyte-derived macrophages but not monocytes yielded the highest MSC recruitment when interacting with these biomaterials. This may imply that stem cell recruitment is only signalled at later stages of host response and that it is promoted by mature macrophages. Moreover, the impact of macrophages on MSC motility was biomaterial-dependent. This stem cell dynamical behaviour can be essential for the regenerative switch from detrimental inflammatory response to constructive remodelling.

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Competing interests. We declare we have no competing interests.

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Authors’ contributions. H.C.R. designed the experiments, acquired and analysed the data and wrote the manuscript; T.E. and P.Q. developed Dendra2imaging plugin; M.N. designed the experiments, assisted in PLA scaffold fabrication and analysed the data; M.A.B. designed the experiments and analysed the data; C.R.A. designed and supervised the experiments, analysed the data and wrote the manuscript. All authors gave final approval for publication.

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