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Isolation and characterization of porcine macrophages and their inflammatory and fusion responses in different stiffness environments

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Abstract

Evaluating the host immune response to biomaterials is an essential step in the development of medical devices and tissue engineering strategies. To aid in this process, *in vitro* studies, whereby immune cells such as macrophages are cultured on biomaterials, can often expedite high throughput testing of many materials prior to implantation. While most studies to date utilize murine or human cells, the use of porcine macrophages has been less well described, despite the prevalent use of porcine models in medical device and tissue engineering development. In this study, we describe the isolation and characterization of porcine bone marrow- and peripheral blood-derived macrophages, and their interactions with biomaterials. We confirmed the expression of the macrophage surface markers CD68 and F4/80 and characterized the porcine macrophage response to the inflammatory stimulus, bacterial lipopolysaccharide. Finally, we investigated their inflammatory and fusion response of porcine macrophages cultured on different stiffness hydrogels, and we found that stiffer hydrogels enhanced inflammatory activation by more than two-fold and promoted fusion to form foreign body giant cells. Together, this study establishes the use of porcine macrophages in biomaterial testing and reveals a stiffness-dependent effect on biomaterial-induced giant cell formation.

Graphical Abstract

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Ethical Statement

This study was IACUC exempt because no live animals were used. Blood and carcasses were purchased from Premier BioSource. The vendor collected blood from the jugular vein from deceased animals within 6 hours postmortem.

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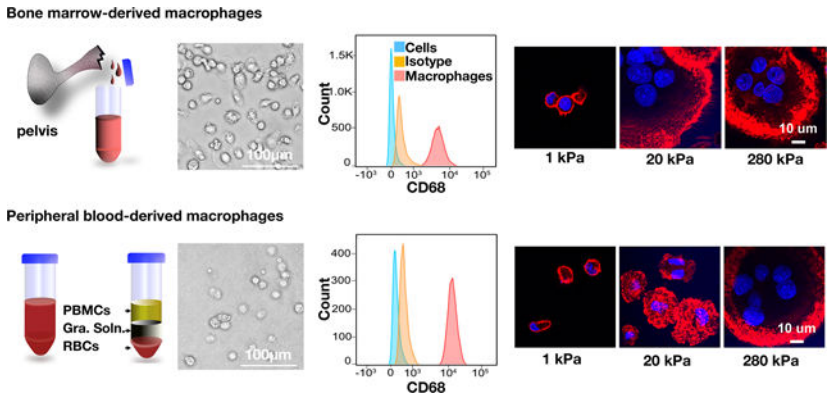

Introduction

Porcine models are essential tools for the translation of tissue engineering and regenerative medicine research to the clinic (1, 2). Their use has increased over the years, particularly for musculoskeletal tissue engineering applications, where it is critical for the animal model to mimic the biomechanical environment present in humans (1). Despite some anatomical and morphological differences, porcine and human musculoskeletal tissues have many similarities in their mechanical loading profiles and biochemical properties, and, thus, porcine models have been widely chosen for studies of the cartilages, ligaments, and bones in joints such as the knee and temporomandibular joint (TMJ) (3, 4). In the context of injury response, porcine and human wound healing are also thought to occur through similar processes, and responses to wound therapies in porcine models are reported to be 78% consistent with responses in humans (5, 6). While there have been many advances in the field of tissue engineering, one of the major challenges hindering their success has been adverse innate and adaptive immune responses that result from implantation of engineered materials. The host immune response involves an acute inflammatory response followed by tissue fibrosis around the implant or infiltrating the construct, which can lead to altered function and device failure (7–10). Therefore, the ability to evaluate immune responses to engineered tissues in a porcine model is critical for developing new tissue-engineered and regenerative therapies

Cell culture platforms are a valuable tool for assessing the host response to biomaterials or implants, bridging the design and fabrication of new materials with *in vivo* pre-clinical studies. *In vitro* culture of cells on biomaterials can be completed more rapidly and at a higher throughput compared to *in vivo* studies, where materials are implanted into animals, often individually, and the inflammatory or fibrotic response is assessed at various time points afterwards. Culture models also allow the study of basic mechanisms underlying immune cell-biomaterial interactions, which can lead to new immunomodulatory strategies. Previous work from our laboratory and others has shown a robust correlation between the extent of inflammatory cytokine secretion by macrophages cultured on biomaterials with the inflammatory responses elicited by these materials after implantation in animals (11–13). These include studies evaluating libraries of new chemistries (11) as well as of materials with different physical and topographical properties (12, 13), suggesting that the methods

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are broadly applicable to a wide range of materials. However, these findings have largely been established using rodent models, where material implantation and biocompatibility studies are common and immune cells are easily accessible through bone marrow harvest. To date, few studies have used cells derived from large animals, such as porcine sources, despite the established use of these models in tissue engineering.

Biomaterial stiffness has become widely appreciated for its role in regulating many cellular behaviors in healthy and pathological states (14–17), including immune activation and foreign body responses. In the context of medical devices, compliance mismatch between the implant and the surrounding tissue is thought to be a major driver of the foreign body response (18, 19). At the cellular level, our laboratory has demonstrated that macrophages cultured on soft fibrin or polyacrylamide (PA) hydrogels suppress the inflammatory activation compared to stiffer PA, polystyrene, or glass (12, 20). Similar observations were made when the macrophages were cultured on soft PEG hydrogels, leading to significantly less expression of tumor necrosis factor (TNF-α), interleukin 1 beta (IL-1β), and interleukin 6 (IL-6) (21). Furthermore, subcutaneous implantation of softer hydrogels recruited significantly fewer macrophages to the implant surface and led to a less severe foreign body reaction when compared to a stiff hydrogel (12, 21), suggesting that stiffness can regulate tissue repair responses *in vivo*. Taken together, material stiffness can play a critical role in immune cell activation and foreign body response to biomaterials, but the role of stiffness in regulating porcine immune cells has not yet been examined.

Here, we describe the isolation and characterization of porcine bone marrow-derived and peripheral blood-derived macrophages, as well as their responses in different stiffness environments. We identify candidate bones to consistently isolate maximal number of cells and confirm the expression of commonly expressed macrophage cell surface markers after differentiation including CD68 and F4/80. Both bone marrow-derived and peripheral blood-derived macrophages demonstrated an increased secretion of TNF-α with increased lipopolysaccharide (LPS) doses, as well as characteristic changes in inflammation-associated cell shape. Finally, we investigated the role of substrate stiffness on porcine macrophage behavior and found that culture on stiffer substrates increases their inflammatory activation, similar to our previous studies using murine and human cell systems (12, 20). Interestingly, fusion to foreign body giant cells was also enhanced in higher stiffness environments, specifically for bone-marrow derived macrophages. Together, this study will aid in the evaluation of new biological and synthetic biomaterials for tissue engineering.

Results

Isolation of porcine bone marrow- and peripheral blood-derived macrophages

A schematic for isolation of bone marrow-derived and peripheral blood-derived macrophages is shown in Figure 1. To determine the optimal method of harvesting macrophages from porcine bone marrow, different bones including the radius, ulna, humerus, scapula, and pelvis were harvested from the minipig and cleaned of muscle and soft tissues to harvest bone marrow cells. Quantities of bone marrow cells harvested were analyzed to determine the ideal bones to use for future harvest. We found that

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the pelvis yielded more than five times greater number of cells compared to any of the other bones tested and proceeded with isolating cells from only the pelvis in subsequent harvests. The cells were cultured in differentiation media containing recombinant porcine granulocyte macrophage colony stimulating factor (rpGM-CSF) to differentiate monocytes to macrophages, which are adhesive and can be isolated by removing nonadherent cells (22). We also attempted culture with human macrophage colony stimulating factor (hM-CSF)-containing media, as has been previously reported (23), but found that cells did not adhere to the tissue culture plate in this culture medium. We isolated ~28×10^7 bone marrow cells, which yielded ~64×10^6 macrophages after differentiation, and therefore, approximately 20% of the cells differentiated over seven days. We further confirmed this differentiation efficiency in two subsequent minipig donors, totaling three donors for later experiments. In conclusion, pelvises were determined to be the optimal bone for efficient isolation of macrophages, and rpGM-CSF was appropriate for cell differentiation.

To isolate peripheral blood mononuclear cells (PBMCs), blood was processed within 24 h of collection and stored in either sodium ethylenediaminetetraacetic acid (EDTA) or sodium citrate as anti-coagulants. PBMCs were then isolated using Sepmate-50 tubes for density gradient centrifugation. The cells were cultured in rpGM-CSF-containing media for seven days with fresh media added on day 3. We isolated ~10×10^7 PBMCs from 75 ml blood, which yielded ~14×10^6 macrophages after 7 days of differentiation. Further, we found that the blood stored in sodium citrate as an anti-coagulant resulted in less red blood cell contamination (Supp Fig. 1), and, thus, we continued using sodium citrate for future isolations.

Finally, we found that it was possible to freeze down differentiated macrophages for later experimentation, alleviating the need to perform experiments on the day of cell harvest. Of note, we found that bone marrow cells that were frozen prior to differentiation, in either 90% heat-inactivated fetal bovine serum (FBS) and 10% dimethyl sulfoxide (DMSO) or 45% FBS and 10% DMSO, did not survive thawing and failed to adhere to the plate. However, both peripheral blood- and bone marrow- derived cells that had been cultured in differentiation media for seven days were successfully frozen using media supplemented with 20% FBS and 10% DMSO and exhibited greater than 90% viability upon thawing. Together, these results describe a method to isolate and store porcine macrophages for downstream biological studies.

Porcine macrophages express F4/80 and CD68 on the cell surface

Following differentiation, macrophages were dissociated from the tissue culture plate for further analysis. The transmembrane protein cluster of differentiation 68 (CD68) is a marker highly expressed by macrophages and other mononuclear phagocytes, and it is often used to detect macrophages by flow cytometry and immunostaining (24, 25). Flow cytometry was performed to evaluate CD68 expression in porcine macrophages using a porcine-specific CD68 antibody, clone BA4D5 (26). Staining the cells with BA4D5 antibody after 7 days of differentiation showed substantial CD68 staining in both bone marrow-derived and peripheral blood-derived macrophages (Fig. 2A) compared to isotype control. We confirmed the protein expression of CD68 by immunofluorescence (Fig. 2C and D) in the bone

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marrow- and peripheral blood-derived macrophages. Another marker, F4/80 protein, which is encoded by the *ADGRE1* gene, has been widely used as a macrophage marker in mice. However, recent RNA-seq analysis identified *ADGRE1* gene expression in monocyte-derived and alveolar macrophages of eight different mammalian species including pig (27). Therefore, we confirmed *ADGRE1* gene expression by quantitative real time polymerase chain reaction (qRT-PCR) (Fig. 2B) and expression of F4/80 protein in both bone marrow and peripheral blood-derived porcine macrophages by immunostaining (Fig. 2C and D). Secondary antibody only controls showed no positive staining (Supp Fig. 2). Together, our data show that porcine peripheral blood- and bone marrow-derived macrophages express CD68 and F4/80, two well-documented macrophage-specific markers.

Porcine macrophages respond to LPS and increase the expression of inflammatory genes.

To demonstrate the effect of inflammatory stimuli on the differentiated macrophages, cells were seeded onto glass or polystyrene for 24 h, and then stimulated with varied concentrations of bacterial LPS, a potent agonist of Toll-like receptor 4 (TLR-4), for 6 h. We found that both bone marrow- and peripheral blood-derived macrophages showed dose-dependent secretion of the inflammatory cytokine TNF-α in response to LPS (Fig. 3A). Expression of inflammatory genes *iNOS, TNFα* and *IL6* in bone marrow- and peripheral blood-derived macrophages also increased compared to unstimulated cells (Fig. 3B and C). In bone marrow-derived macrophages, *iNOS* expression was 1.25 fold higher than the unstimulated cells with a p- value of 0.001. Expression of other inflammatory genes *TNFα* and *IL6* were at least 1.65 fold higher, and significantly different, compared to the unstimulated cells, with a p-value of 0.026 and 0.027, respectively. In blood-derived macrophages *iNOS, TNFα,* and *IL-6* expression were at least 1.8 fold higher, and significantly different, than the unstimulated cells with p-values of 0.003, 0.04 and 0.05, respectively. In addition, we performed immunostaining using an antibody targeting the inflammatory marker inducible nitric oxide synthase (iNOS) (Fig. 3C). We chose iNOS as an inflammatory marker in our study. iNOS is an enzyme that synthesizes nitric oxide from L-arginine. Its expression is enhanced with M1 (LPS and IFNγ) stimulation in mouse and rat macrophages and plays a critical role in systemic inflammation and sepsis (28), although the levels have been reported to be varied in porcine models (29–31). Nonetheless, detection of iNOS is feasible and valuable because it is intracellular across different cell types (32–34). On the contrary, TNF-α and IL-6 are secreted cytokines, and their levels as detected through immunofluorescence staining may not represent the true expression. We observed the expression of iNOS, both in unstimulated and LPS stimulated macrophages, suggesting that this marker does not show changes in inflammation at this time point (6 h post stimulation). Together, our data show that inflammatory cytokine gene expression and protein secretion can be used to evaluate responses to agonists such as LPS.

Substrate stiffness influences porcine macrophage inflammatory activation and fusion

Our previous work has shown that culture of murine and human macrophages on stiffer substrates enhances their inflammatory activation, whereas culture on soft substrates reduces their response to LPS (12, 20). To examine whether stiffness also plays a role in the inflammatory activation in porcine macrophages, we cultured bone marrow- and peripheral blood-derived macrophages on PA gels with stiffnesses of 1, 20, or 280 kPa, coated with

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20 μg/ml of fibronectin. After 24 h of culture, cells were stimulated with 10 ng/ml LPS for 6 h, and the supernatants were collected and analyzed for TNF-α secretion. Irrespective of the PA gel stiffness LPS stimulation enhanced the section of TNF-α secretion. Macrophages cultured on 20 and 280 kPa PA gels and stimulated with LPS, secreted at least two-fold higher inflammatory cytokine TNF-α compared to the cells cultured on soft (1 kPa) PA gels ($p \leq 0.002$) (Fig 4A). However, TNF-α secretion from cells cultured on 20 kPa was not significantly different from the secretion by cells on 280 kPa PA gels. These results are consistent with what we have previously observed in human and murine macrophages (12).

To evaluate the effects of stiffness on cell morphology, we stained cells with phalloidin to visualize their actin cytoskeleton. We found that macrophages cultured on 1 kPa PA gels were rounded with intense cortical actin staining, whereas cells cultured on 20 and 280 kPa exhibited significantly higher spread area compared to cells cultured on 1 kPa both with and without LPS stimulation, with cytoplasmic actin staining, along with membrane ruffles. (Fig. 4B and C). Analysis of spread area of cells cultured on different stiffness PA gels showed heterogeneity with respect to cell size, particularly for the cells cultured on 20 and 280 kPa gels (Fig. 4C). In addition, LPS stimulation did not significantly increase the cell area of bone marrow-derived and peripheral blood-derived macrophages on any of the stiffness tested (Fig. 4C). Interestingly, we also observed a striking increase in cell fusion, with the presence of many giant cells containing up to 50 nuclei on 20 and 280 kPa, whereas greater than 95% of cells on 1 kPa surfaces remained as single cells, although sometimes clustered together (Fig. 4B and C). Multinucleated giant cells were observed in both bone marrow-derived and peripheral blood-derived macrophages, although giant cells with two or more nuclei were more abundant in bone marrow-derived macrophages compared to peripheral blood-derived macrophages (Fig. 4B and C). In addition, LPS stimulation enhanced cell fusion, increasing the number of nuclei per giant cell on 20 kPa, but not on 280 kPa, in bone marrow-derived macrophages, and on 280 kPa, but not 20 kPa, in peripheral blood-derived macrophages. The fusion responses may at least in part explain the increases in cell area, since the well spread cells tended to have multiple nuclei (indicated by the blue dots in the cell area plot). Together, these data not only show that increased substrate stiffness enhances the inflammatory response of porcine macrophages to LPS, similar to human and murine macrophages, but also reveal that stiffness causes an increase in cell fusion and giant cell formation.

Discussion

Large animal models have been increasingly used for medical device development, tissue engineering, and regenerative medicine (1, 35). Porcine models in particular offer better homology with humans in terms of their anatomy and biomechanics of musculoskeletal tissues and are also thought to exhibit more similar immune responses to wound healing therapies (6, 36), However, large animals are costly, particularly for long term studies, and *in vitro* testing can offer a lower cost and expedient alternative for screening materials and developing tissue engineering strategies prior to studies in animals. This motivated our current study to isolate porcine macrophages and to characterize their responses to different biomaterial environments. We determined the pelvis yields the highest number of bone marrow cells and differentiated macrophages, and optimal differentiation occurs with

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rpGM-CSF. While L929–conditioned media, human M-CSF, and porcine GM-CSF have all been used as differentiating factors for porcine macrophages (22, 23, 37), we found that recombinant human M-CSF did not result in monocyte differentiation to macrophages, and cells remained in suspension, whereas rpGM-CSF yielded many adherent macrophages. After differentiation for seven days, we analyzed the macrophages for the expression of CD68 and F4/80 and found that both markers were highly expressed. While F4/80 (*ADGRE1*) is often thought to be a mouse-specific macrophage marker, a recent study also reported its expression in other species including porcine (27). Taken together, we successfully isolated and differentiated bone marrow cells and PBMCs to bone marrow- and peripheral blood-derived macrophages, respectively.

Inflammation is a key aspect of the tissue repair process. It is caused by injury to the tissue and presence of a foreign biomaterial and is also needed to initiate wound healing responses. However, chronic inflammation is associated with poor healing and fibrosis (16). We tested the inflammatory response to LPS, a bacterial component and agonist of TLR4, and found that bone marrow- and peripheral blood-derived porcine macrophages responded to LPS by secreting the inflammatory cytokine TNF-α, consistent with an earlier study (22). In addition, we found a dose-dependent increase and saturation of the response at approximately 5 ng/ml of LPS. We also observed that LPS induced expression of inflammatory genes including *iNOS, IL6, and TNFα,* although iNOS protein analyzed by immunofluorescence staining appeared to be expressed regardless of LPS stimulation. Dynamic changes in nitric oxide (NO) synthesis has been observed in porcine macrophages in response to LPS (30). Another study also showed that regardless of *iNOS* gene expression after LPS treatment, cells did not produce any detectable NO or iNOS protein, contrary to what we show here (38, 39). Using TNF-α secretion to measure inflammation, we also examined porcine macrophage response to substrate stiffness. We found that porcine macrophages stimulated with LPS cultured on stiffer substrates secreted significantly higher TNF-α levels than the softer substrate, with cells cultured on 1 kPa exhibiting significantly less inflammation compared to cells on 20 or 280 kPa, consistent with what we have observed in human and murine macrophages (12).

Macrophages often exhibit characteristic cell shape changes in response to their biochemical and biophysical environment, and we found that porcine macrophages indeed exhibit a flattened, “fried-egg” morphology, when stimulated with LPS. In addition, we observed a profound increase in cell fusion and the presence of multi-nucleated giant cells, particularly in bone marrow-derived macrophages cultured on stiffer PA hydrogels, which was not observed in cells cultured on soft polyacrylamide hydrogels. Fusion responses are common during the foreign body response to biomaterial implant, during which macrophages can exhibit “frustrated phagocytosis” as they are unable to engulf large materials (40). *In vitro* studies have demonstrated that macrophage fusion requires stimulation with IL-4 and CCL2/MCP-1 (41–43). A recent study has also shown that this response occurs in different biomaterial contexts (44). Here, we observed fusion of up to 50 cells after only 24 h of culture on stiffer PA hydrogels in the presence of rpGM-CSF, and a further increase with LPS stimulation. Interestingly, fusion occurred the most in cells cultured on 20 kPa hydrogels, to a lesser extent on 280 kPa, and was nearly absent in cells on 1 kPa in bone marrow-derived macrophages. Moreover, while fusion was less prominent

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in peripheral blood-derived macrophages, the most occurred in cells cultured on 280 kPa gels. The differential response between bone marrow- and peripheral blood-derived macrophages may be caused by differences in cell origin, and thus diverse experiences in their respective mechanical environments (45, 46). Nonetheless, fusion of cells is thought to require fusogens, cell surface proteins such as integrins and ion channels, as well as cytoskeletal rearrangements (47–52), and further studies will be needed to elucidate the molecular underpinnings of stiffness-dependent porcine macrophage fusion.

In summary, we describe here an efficient method to isolate porcine macrophages from peripheral blood and bone marrow and characterize their response to LPS and the stiffness of the environment. Since macrophages are recruited abundantly to biomaterial implants and tissue-engineered constructs, it is crucial to understand the macrophage response to the integrative effects of chemical and physical stimuli. The findings from this study will assist in studying the immunomodulatory properties of new tissue-engineered constructs and biomaterials used in medical devices.

Methods

Isolation of bone marrow- and peripheral blood derived-macrophages and differentiation

Pelvis, scapula, radius, ulna, and humerus from 5 to 8 month old Yucatan minipigs were obtained within 6 hours of postmortem. The bones were cleaned of muscle and other soft tissues. Using a sterile chisel and hammer, the bone marrow was exposed and flushed with phosphate buffered saline (PBS) or un-supplemented 1X RPMI-1640 media. The cells were then passed through a 70 μm filter, centrifuged, rinsed with PBS, treated with ACK lysing buffer to remove any red blood cells, and subsequently washed with PBS. Cells were then seeded at approximately 10 million cells per 100 × 25 mm petri dishes or 0.176 million per cm^2 in differentiation culture media composed of RMPI-1640 (Fisher Scientific), L-glutamine (Fisher Scientific), and 1% penicillin-streptomycin (Fisher Scientific), supplemented with 10% FBS and 20 ng/mL recombinant porcine granulocyte-macrophage colony-stimulating factor (rpGM-CSF, R&D Systems) or human macrophage colony stimulating factor (M-CSF, PeproTech) to differentiate cells to macrophages. Cells were fed with the same media on day 3 and dissociated from the culture plate on day 7 for experiments or frozen down in 1X RPMI media with 20% FBS and 10% DMSO for future use.

Blood from the jugular vein was collected in sodium citrate as an anti-coagulant and stored at 4° C until use. The PBMCs from the blood were isolated by density gradient centrifugation using SepMate™-50 tubes (Stem Cell Technologies) following the manufacturer's protocol. The isolated PBMCs were incubated with ACK lysing buffer to remove red blood cells and subsequently washed with PBS. Finally, the cells were resuspended and differentiated using the differentiation media and protocol described above.

Flow cytometry

After 7 days of differentiation with rpGM-CSF, the cells were dissociated from the plate using dissociation buffer (ThermoFisher) and blocked using anti-CD16 (clone 2.4G2, Tonbo

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Biosciences) on ice. The cells were stained with mouse anti-pig macrophage antibody, clone BA4D5, specific for porcine CD68 (Bio-Rad) and IgG2b isotype control. The unbound and excess antibody was washed thoroughly using 1X PBS. Flow cytometry was performed on a BD LSRII flow cytometer using BD FACSDiva software (BD Biosciences). Data acquisition was performed until at least 10,000 events were collected, and post processing of the date was performed in FlowJo (Tree Star).

Assessment of cytokine secretion by ELISA and immunofluorescence staining of the cells

After 7 days of cell culture with rpGM-CSF, the cells were dissociated from the plate using cell dissociation buffer and seeded on tissue culture polystyrene or cover glass. Cells were seeded at a density of 0.1 million cells/well in 24 well plates. After 24 h of culture, the cells were stimulated with 10 ng/ml ultrapure LPS (InvivoGen). Supernatants were collected 6 h after stimulation for assessment of cytokine secretion by enzyme-linked immunosorbent assay (ELISA) following the manufacturer's protocol (R&D Systems). Further, the cells were immediately fixed in 4% PFA (Electron Microscopy Sciences) for 10 min at room temperature (RT). The cells were washed 3 times with PBS and permeabilized using 0.3% Triton X-100 in PBS. Samples were then blocked with 2% bovine serum. The samples were incubated in the following primary antibodies overnight at 4 °C: F4/80 (Thermo Fisher Scientific, BM8) or CD68 monoclonal antibody (KP1; MA5–13324, Thermo Fisher Scientific). Cells were then washed with 2% bovine serum albumin (BSA; 0219989880, MP Biomedicals) in PBS and incubated with secondary antibody anti-rat IgG-488 (for F4/80; Jackson ImmunoResearch laboratories, Inc.) and anti-mouse Alexa fluor 488 (for CD68; ab150113, abcam) at RT for 1 h. Nuclei and actin were stained using Hoechst and Alexa fluor 594-phalloidin (Invitrogen), diluted in 2% BSA in PBS for 30 min at RT. Finally, the cells were washed with PBS and mounted on glass slides using Fluoromount G (Southern Biotech). Images were acquired at 40X using the Olympus FV3000 laser scanning confocal microscope.

Polyacrylamide hydrogel synthesis

Polyacrylamide (PA) hydrogels with tunable mechanical properties were synthesized on glass coverslips according to the previously described protocol (53). The PA coated glass coverslips were conjugated with 20 μg/ml fibronectin using sulfo-SANPAH (Thermo Scientific) overnight at 4°C. Cells were cultured for 24 h on the gel and stimulated with 10 ng/ml LPS for 6 h and the supernatant was collected for ELISA, and cells were fixed immediately for immunostaining.

RNA isolation, cDNA preparation, and qRT-PCR analysis

After the collection of supernatants, cells were lysed using TRI Reagent (Sigma), and RNA was isolated following the manufacturer's protocol. The pellet was briefly air-dried and the RNA was dissolved in DEPC treated water. cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit from Applied Biosystems (Cat. no. 4368814) with 1 μg of total RNA following the manufacturer's protocol. PerfeCTa® SYBR® Green SuperMix Reaction Mixes from QuantaBio was used for quantitative real-time PCR, and a total of 40 cycles were performed on Bio-Rad's CFX-96 real-time PCR system. Relative gene expression was analyzed by $2^{-\Delta\Delta CT}$ method and expressed relative to the housekeeping

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gene *GAPDH* and normalized to the unstimulated condition. The primers used for qPCR in this study are in Supp. Table 1.

Statistical Analysis

One -way ANOVA with Tukey's *post hoc* test was used for bone marrow- and peripheral blood-derived macrophages to assess LPS dose response and the response to substrate stiffness. Student *t*-tests were performed to compare the gene expression of inflammatory genes in bone marrow- and peripheral blood-derived macrophages. For cell spread, Kruskal-Wallis test with Dunn's multiple comparisons were performed for both bone marrow- and peripheral blood-derived macrophages. For all the statistical tests, p value less then or equal to 0.05 were considered significant. The determined p value is reported with in the graph for each comparison made. Values presented here are mean ± standard deviation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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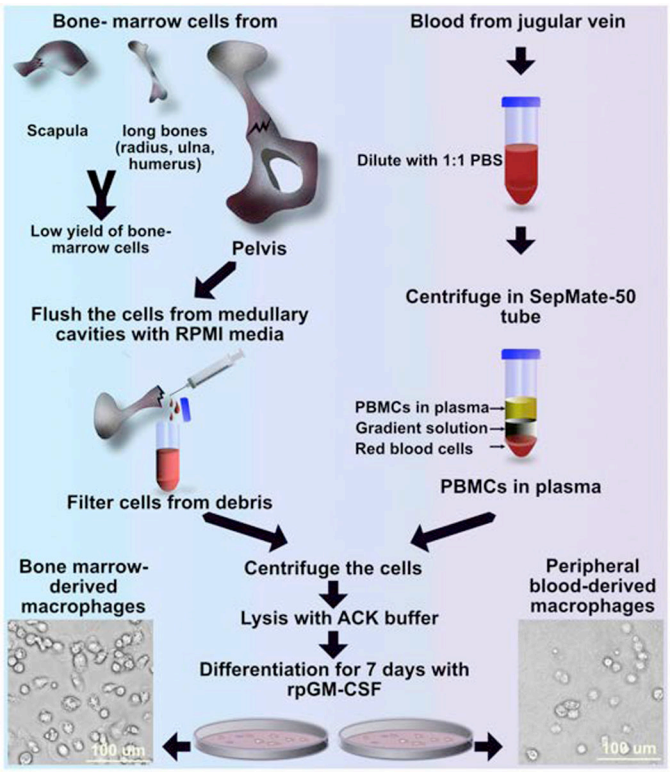

Figure 1: Schematic representation of the protocol used to isolate and differentiate bone marrow and peripheral blood- derived macrophages.

Bone marrow-derived macrophages were isolated from the pelvises and differentiated for seven days using rpGM-CSF. Peripheral blood was used to isolate PBMCs using SepMate™-50 tube with density gradient solution. Isolated PBMCs were differentiated for seven days using rpGM-CSF.

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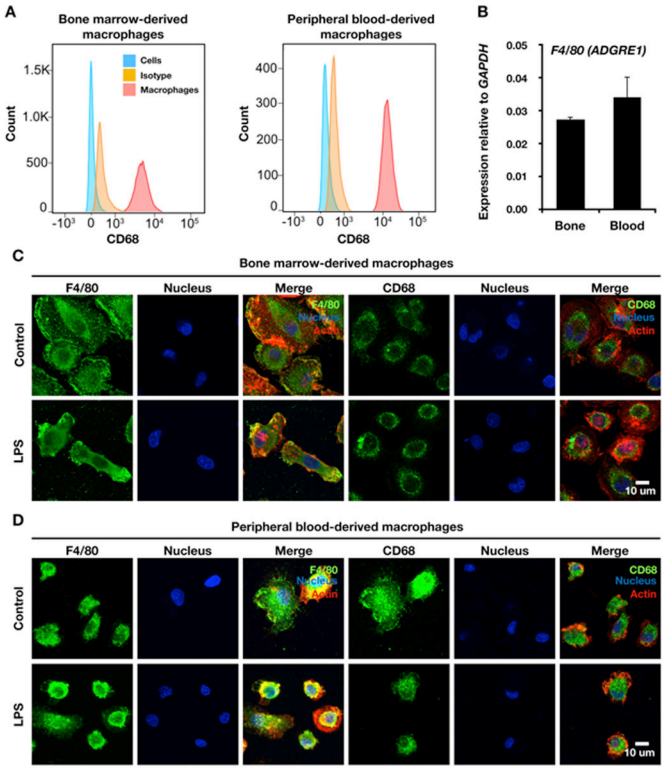

Figure 2: Bone marrow- and peripheral blood derived-macrophages express CD68 and F4/80 upon differentiation

(A) Representative flow cytometry histograms with unstained macrophages in light blue curves, isotype controls indicated using orange, and CD68 with red curves for bone marrow- (left) and peripheral blood-derived (right) macrophages after 7 days of differentiation using rpGM-CSF on tissue culture-treated polystyrene. (B) Expression of *ADGRE1* (F4/80) relative to *GAPDH* assessed by quantitative PCR in bone marrow- and peripheral blood-derived macrophages after 7 days of differentiation. The values are the mean ± SEM from three porcine donors. Immunofluorescence confocal images of F4/80 (left) and CD68 (right) of bone marrow- (C) and peripheral blood-derived macrophages (D) after 24 h adhesion and 6 h stimulation with LPS.

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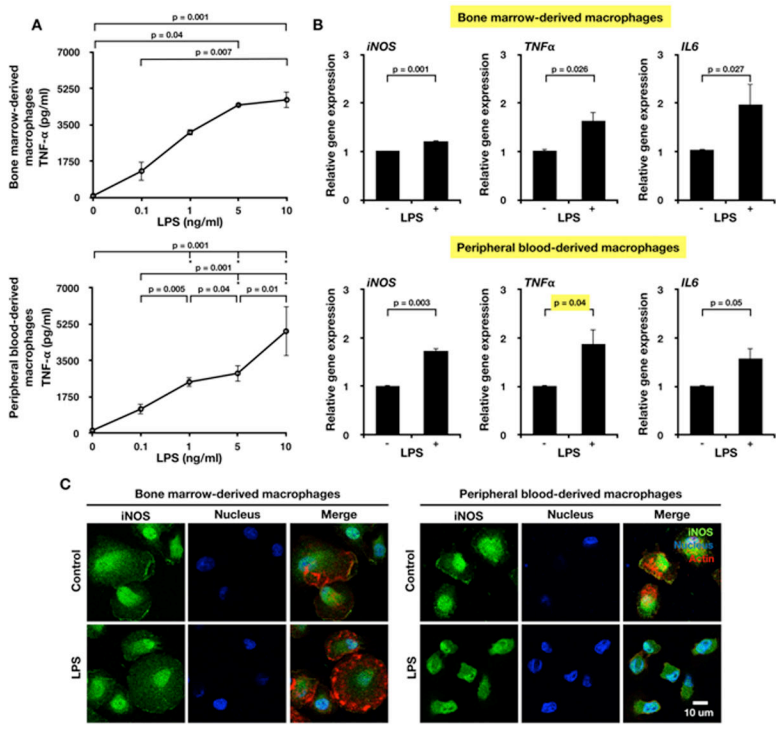

Figure 3: Differentiated bone marrow- and peripheral blood-derived macrophages are activated upon LPS stimulation

(A) Secretion of TNF-α by bone marrow- (top) and blood- (bottom) derived macrophages after 24 h of adhesion and 6 h of stimulation with increasing concentrations of LPS. (B) Relative expression of *iNOS, TNFα* and *IL6* genes in bone marrow- and peripheral blood-derived macrophages when stimulated with 10 ng/ml LPS, analyzed by qRT-PCR, and normalized to M0 (no LPS) condition. (C) Immunofluorescence confocal images of iNOS in bone and peripheral blood-derived macrophages cultured on glass for 24 h and stimulated with LPS for 6 h. The values are the mean ± SEM. and from three donors. Statistics: p values are depicted in the graph for each comparison, assessed by one-way ANOVA with Tukey's multiple comparisons for LPS titration experiment and two-tailed Student's t-test for qRT-PCR analysis.

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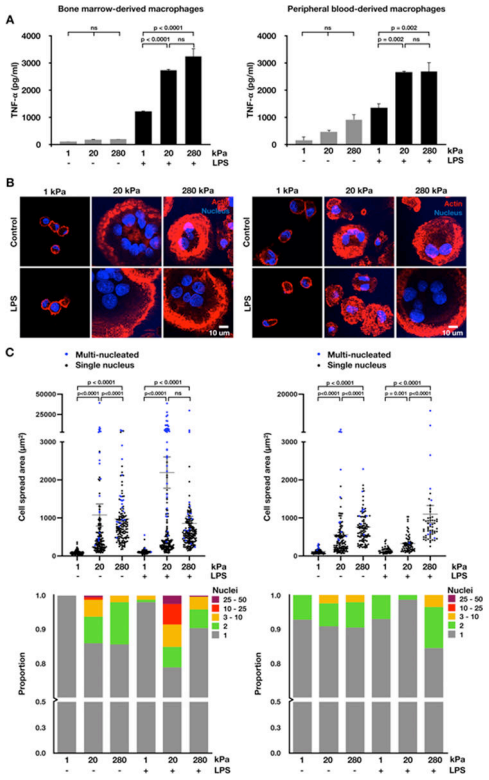

Figure 4: Substrate stiffness enhances inflammatory activation and fusion of macrophages.

(A) Secretion of TNF-α by bone marrow- (left) and peripheral blood- (right) derived macrophages after 24 h of adhesion to PA gels of varying stiffness and 6 h of stimulation with 10 ng/ml LPS. (B) Immunofluorescence confocal images of F-actin (phalloidin, red) and nuclei (blue) in bone marrow- and peripheral blood-derived macrophages cultured on PA gels of varying stiffness for 24 h and stimulated with 10 ng/ml LPS for 6 h. The values are the mean ± SEM. and from three donors. Statistics: One-way ANOVA with Tukey's multiple comparisons test was used and the determined p value is reported in the graph. (C) Cell spread (top) and proportion of multinucleated cells (bottom) in bone marrow-derived macrophages (left) or peripheral blood-derived macrophages (right) cultured on PA gels of different stiffness. Each condition had 50–100 cells analyzed. Statistics: For the cell spread the determined p value is reported in the graph, assessed by Kruskal-Wallis test with Dunn's multiple comparisons. ns: not significant.