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FULL-LENGTH ARTICLE
Mesenchymal Stromal Cell Therapy

Mesenchymal stromal cells can block palmitate training of macrophages via cyclooxygenase-2 and interleukin-1 receptor antagonist

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ABSTRACT

Innate training of macrophages can be beneficial for the clearance of pathogens. However, for certain chronic conditions, innate training can have detrimental effects due to an excessive production of pro-inflammatory cytokines. Obesity is a condition that is associated with a range of increased pro-inflammatory training stimuli including the free fatty acid palmitate. Mesenchymal stromal cells (MSCs) are powerful immunomodulators and known to suppress inflammatory macrophages via a range of soluble factors. We show that palmitate training of murine bone-marrow-derived macrophages and human monocyte-derived macrophages (MDMs) results in an increased production of TNFα and IL-6 upon stimulation with lipopolysaccharide and is associated with epigenetic remodeling. Palmitate training led to metabolic changes, however, MSCs did not alter the metabolic profile of human MDMs. Using a transwell system, we demonstrated that human bone marrow MSCs block palmitate training in both murine and human macrophages suggesting the involvement of secreted factors. MSC disruption of the training process occurs through more than one pathway. Suppression of palmitate-enhanced TNFα production is associated with cyclooxygenase-2 activity in MSCs, while secretion of interleukin-1 receptor antagonist by MSCs is required to suppress palmitate-enhanced IL-6 production in MDMs.

Introduction

Since the discovery that the bacille Calmette-Guérin (BCG) vaccine can induce memory in macrophages, innate immune training has received ever-increasing attention [1,2]. Various pro-inflammatory training stimuli have been investigated including β-glucan, zymosan and chitin, which are ligands for dectin-1 [3–8]. In the context of BCG-induced trained immunity, innate immune cells are better equipped to clear infections [9]. However, trained immunity can also lead to detrimental effects associated with overzealous innate immune responses in chronic disease. Training with β-glucan can increase disease severity in experimental autoimmune encephalomyelitis, a mouse model of multiple sclerosis [10], rheumatoid arthritis (RA) [11,12] and systemic lupus erythematosus (SLE) [13]. Additionally, it has been hypothesized that the aberrant production of pro-inflammatory cytokines in SLE may also serve as a training stimulus itself and result in ever-increasing disease severity [14]. This hypothesis is supported by the fact that SLE is associated with high levels of the same pro-inflammatory cytokines observed in innate training: TNFα, IL-6 and IL-1β [15–17]. Furthermore, macrophages derived from SLE patients exhibit a metabolic switch toward glycolysis [18] and distinctive epigenetic modifications consistent with innate training [19,20].

Abbreviations: 2-DG, 2-deoxy-D-glucose; BCG, bacille Calmette-Guérin; BMDM, bone marrow-derived macrophage; BSA, bovine serum albumin; COX-2, cyclooxygenase-2; cRPMI, complete RPMI; DPBS, Dulbecco's phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; EP, E-type prostanoid receptor; FAO, fatty acid oxidation; FBS, fetal bovine serum; HDM, house dust mite; hIL-6, human IL-6; HSPCs, hematopoietic stem progenitor cells; hTNFα, human TNFα; IL-1RA, interleukin-1 receptor antagonist; LPS, lipopolysaccharide; M-CSF, macrophage colony-stimulating factor; MDM, monocyte-derived macrophage; MSC, mesenchymal stromal cell; MTA, methylthioadenosine; mTNFα, mouse TNFα; NLRP3, NLR family pyrin domain containing 3; oxLDL, oxidized cholesterol; OXPHOS, oxidative phosphorylation; Palmitate, palmitate-BSA; PBMCs, peripheral blood mononuclear cells; PGE2, prostaglandin E2; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus

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In the context of chronic inflammatory conditions, the chronic pro-inflammatory environment concomitant with obesity has been associated with trained immunity. Oxidized cholesterol (oxLDL) is elevated in patients with obesity, has been associated with innate training [21–24], and oxLDL-trained macrophages have been linked to exacerbated atherosclerosis [25,26]. Saturated free fatty acids are also highly elevated in obesity and have been linked to innate training [27,28]. Interestingly, Western diet, characterized by a high percentage of saturated fat, led myeloid progenitor cells to be reprogrammed to a more pro-inflammatory state on an epigenetic level, which persisted even when the mice were switched back to a standard diet [22]. In a different study, mice fed a diet high in the saturated free fatty acid, palmitate, exhibited a hyper-inflammatory lipopolysaccharide (LPS) response and increased endotoxemia severity and mortality [27]. The pro-inflammatory effects of a high-fat diet were shown to persist in a mouse model even after weight loss, indicating that they are indeed connected to innate training and not simply inflammatory priming of the immune cells [28].

Mesenchymal stromal cells (MSCs) are powerful modulators of the immune response, especially of macrophages. MSCs have been shown to decrease macrophage secretion of TNFα, IL-6 and IL-1β in response to LPS via both cell contact-dependent and independent mechanisms, and promote macrophage-driven tissue repair [29–32]. In a mouse model where intraperitoneal LPS administration was followed 1 month later by the induction of ischemic stroke, human umbilical cord MSCs were able to reduce the training effect of LPS in microglia that led to a worse stroke outcome [33]. Dunbar et al. [34] show that MSCs can block trained immunity induced by house dust mite (HDM), both *in vitro* and *in vivo* in a mouse model of allergic asthma.

Based on this existing knowledge, we used both murine and human samples to determine if palmitate effects epigenetic changes in hematopoietic stem progenitor cells (HSPCs) and monocytes that persist throughout their differentiation into macrophages. Human bone marrow MSCs were tested for their ability to suppress palmitate training of macrophages *in vitro*, and their effects on the metabolic phenotype of the mature macrophages were measured. Finally, we elucidated two pathways involved in the suppression of innate training by human bone marrow MSCs.

Materials and Methods

Ethics approval

Ethical approval for use of mouse bone marrow cells and human work was granted by the biological research ethics committee of Maynooth University (BRESC-2022-2482563).

Human MSC culture

Human bone marrow-derived MSCs (three different donors) were purchased from RoosterBio (Frederick, MD, USA). Initially, MSCs were expanded in RoosterBio expansion medium (RoosterBasal and RoosterBooster) for passages 1 and 2 according to the manufacturer's instructions. After, MSCs were cultured and maintained up to passage 6 in low glucose Dulbecco's modified Eagle medium (Sigma-Aldrich, Wicklow, Ireland) supplemented with 10% (v/v) fetal bovine serum (FBS; Thermo Fisher Scientific, Dublin, Ireland) and 1% (v/v) penicillin/streptomycin (Sigma-Aldrich, Wicklow, Ireland). MSCs were seeded at 1×10^6 cells per T175 flask and cultured at 37°C in 5% CO_2. Medium was replenished every 2–3 days and cells were passaged at 80% confluency.

Isolation and culture of murine bone marrow-derived macrophages (BMDMs)

Male and female wildtype C57BL/6 mice were humanely euthanized via cervical dislocation. Bone marrow was isolated from femur and tibia, centrifuged at 300 g for 5 min, and red blood cells were lysed using 1X RBC lysis buffer (eBioscience/Thermo Fisher Scientific, Dublin, Ireland). Cells were centrifuged at 300 g for 5 min before seeding at a density of 4.95×10^5 cells per well in tissue culture 24 well plates. Cells were cultured for 6 days in RPMI 1640 (RPMI; BioSciences, Dublin, Ireland) supplemented with 10% (v/v) heat-inactivated FBS (Thermo Fisher Scientific, Dublin, Ireland), 1% (v/v/) penicillin/streptomycin (Sigma-Aldrich, Wicklow, Ireland) (complete RPMI [cRPMI]), and 20% L929 conditioned medium as source of macrophage colony-stimulating factor (M-CSF). On day 5, cells were detached by gentle scraping with the tip of a Pasteur pipette, collected and centrifuged at 300 g for 5 min. BMDMs were then seeded into 96 well flat bottom plates at a density of 2×10^4 cells per well for further analysis.

Isolation and culture of human monocyte-derived macrophages (MDMs)

Human peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats received from the Irish Blood Transfusion Service (Saint James' Hospital, Dublin, Ireland). PBMCs were seeded at a density of 2×10^6 cells/well in tissue culture 24-well plates or 10×10^6 cells/well in 6 well plates and allowed to adhere for 60 min. Cells were washed with Dulbecco's Phosphate-Buffered Saline (DPBS; Merck, Cork, Ireland) to remove any nonadherent cells and medium was replaced. Cells were cultured in 500 μL cRPMI, supplemented with 5% human male AB plasma (Merck, Cork, Ireland) for 6 days, to differentiate monocytes into MDMs. On day 5, cells were detached by first washing them with DPBS, then adding 300 μL per well of lidocaine detachment buffer (0.5% bovine serum albumin (BSA; Merck) and 5 mg/mL lidocaine HCL (Fluorochem, Cork, Ireland) for 20–30 min at 37°C. Cells were gently collected in a centrifugation tube. An amount of 400 μL DPBS was added to wells, remaining cells were gently dislodged with the tip of a Pasteur pipette, collected in the same tube, and centrifuged at 300 g for 5 min. MDMs were then seeded into 96 well flat bottom plates at a density of 1×10^4 cells per well for further analysis.

MDM training

PBMCs were isolated from buffy coats from 4 different donors and seeded in TC-coated 6 well plates at 10×10^6 cells/well in serum-free RPMI and monocytes were allowed to adhere for 60 min. Nonadherent cells were washed off with warm PBS and medium was replaced with 1 mL abRPMI (RPMI + 10% heat inactivated FBS + 1% P/S + 5% human AB serum). For training, medium was supplemented with 0.3 mM palmitate-BSA. 2×10^5 MSCs from 3 donors were seeded in transwells and pretreated with 10 μM NS-398 for 24 h, thoroughly washed with PBS, and then added to monocyte wells during training period. For interleukin-1 receptor antagonist (IL-1Ra) neutralization experiments, 10 μg/mL human IL-1ra/IL-1F3 antibody or an equal concentration of antigoat IgG control were utilized. After 24 h of training, supernatants were collected for cytokine array and monocytes were washed three times with warm PBS. An amount of 700 μL abRPMI were added. After 24 h, wells were topped up with another 300 μL abRPMI. Monocytes were differentiated into macrophages. On day 6, cells were stimulated with 100 ng/mL LPS for 24 h.

Cytokine array

A Proteome Profiler Human Cytokine Array Kit was purchased from R&D Systems and supernatants from untrained monocytes, palmitate-trained monocytes, palmitate + MSC-trained monocytes, and MSCs exposed to palmitate were analyzed following the manufacturer's instructions. An amount of 1 mL supernatant was used for each sample and all four samples were processed, imaged and analyzed in parallel. Analysis was performed using ImageJ.

BMDM training

Mouse bone marrow containing HSPCs were isolated and seeded as described above. To study epigenetic modifications, 1 mM of the methyltransferase inhibitor methylthioadenosine (MTA; Sigma) or DMSO as a vehicle control were added 30 min prior to any stimulus, including the L-929 conditioned medium (M-CSF) for differentiation. Without removing the MTA cells were treated with either a medium control (cRPMI + 20% M-CSF), 0.3 mM palmitate-BSA (palmitate; Cayman Chemicals, MI, USA) or 0.3 mM palmitate-BSA and 4×10^4 MSCs in a transwell for 24 h. The transwell and medium were then removed and the well thoroughly washed with PBS. The murine HPSCs needed to undergo multiple centrifugation steps to ensure a proper washout of the palmitate and were then seeded back into their wells. The cells then underwent a rest and differentiation period until day 5, at which point they were detached, counted and seeded into 96 well flat bottom plates at a density of 2×10^4 (BMDMs) cells per well and allowed to adhere overnight. On day 6, the medium was replaced with fresh medium containing 100 ng/mL LPS for 24 h. Finally, supernatants were collected for analysis by enzyme-linked immunosorbent assay (ELISA).

Flow cytometry

MDM surface phenotype was analyzed after the 24 h LPS stimulation using the Attune Nxt flow cytometer. Gating was performed on live (live/dead stain, near-IR fluorescent reactive dye, Invitrogen), $CD14^+$ (PE) cells using antibodies for CD206 (Pacific Blue), HLA-DR (FITC), CD11b (PE-Cy7), CD86 (APC) and CD163 (PerCP). Data were analyzed using floreada.io.

Metabolic phenotyping

To determine metabolic dependence of cytokine production in response to LPS, human MDMs were trained with 0.3 mM palmitate with or without MSCs in a 0.4 μm transwell as described before. After training, cells of each treatment group were split into a vehicle control and an inhibitor treatment, and then treated with either a metabolic inhibitor or its corresponding vehicle control (Supplementary Table 1) for 1 h, followed by a 24 h stimulation with 100 ng/mL LPS in continuing presence of the inhibitors.

Enzyme-linked immunosorbent assay (ELISA)

Levels of mouse TNFα (mTNFα), mouse IL-6, human TNFα (hTNFα) and human IL-6 (hIL-6) (all BioLegend, CA, USA) in cell culture supernatant were determined using ELISA kits following the manufacturer's instructions. Samples were diluted as necessary to be within standard range. Analysis was carried out in corning 96-well half-area plates (Thermo Fisher Scientific, Dublin, Ireland).

Analysis of gene expression

Total RNA was extracted from naïve, or palmitate-exposed human bone marrow MSCs using TRIzol (Ambion Life Sciences, Cambridgeshire, UK) following the manufacturer's instructions. RNA concentrations were measured via spectrophotometry (Nanodrop 2000, Thermo Fisher Scientific, DE, USA). For cDNA synthesis, 500 ng RNA were used following the manufacturer's instructions (Quantabio, MA, USA). Real-time PCR was carried out using PerfeCta SYBR Green FastMix (Quantabio, MA, USA). Prostaglandin-endoperoxide synthase 2 (*PTGS2*) and *IL1RN* (primer sequence information is available in Supplementary Table 2) was qualified in relation to the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (*HPRT*), using the ΔCT method. The fold change in gene expression relative to the control was determined via calculating the $2^{-\Delta\Delta CT}$ values.

RNA-Seq data analysis

A publicly available RNA sequencing dataset (GEO: GSM4748241) of human CD14+ monocytes, that had been exposed to 200 mM palmitate for a period of 30 min, 2, 4, or 12 h was utilized to investigate the downstream signaling cascade in the cells in response to palmitate. Processed datasets were downloaded, genes (2 h) in the hypothesized pathway were identified using their Entrez ID and fragments per kilobase of transcript per million mapped reads (FPKM) of the differentially expressed genes were plotted. A heat map was further generated using Flourish software, to highlight the Log 2 Fold Changes (Log2FC) between genes in the IL-1 signaling pathway, and other targets of interest from the cytokine array. Data is a representation of 3 PBMC donors.

Statistical analysis

An ordinary One-Way ANOVA with Tukey's multiple comparisons test was performed to test for statistical significance between multiple experimental groups, and an unpaired *t*-test with Welch's correction was performed to test for statistical significance between two experimental groups. GraphPad Prism version 10.1.0 was used for statistical computations and graphing.

Results

Methyltransferases play a crucial role in palmitate training of macrophages

Innate training has been associated with epigenetic modifications [1,35,36], including DNA and histone methylation [37,38] by methyltransferases. To investigate if methylation plays a role in palmitate training of macrophages, bone marrow cells containing HSPCs were isolated from the femurs and tibias of healthy mice and exposed to the methyltransferase inhibitor MTA for 30 min prior to the addition of the training stimuli palmitate (0.3 mM) for 24 h (Figure 1A). Both untrained and trained BMDMs did not produce any detectable amounts of mTNFα (Figure 1B) or IL-6 (Figure 1C) at baseline. In response to LPS, mTNFα and mouse IL-6 expression by trained BMDMs significantly exceeded that of the untrained controls, but inhibition of methyltransferase activity in the MTA group reduced cytokine production back to baseline, while the DMSO vehicle control had no effect.

Studies investigating the training effects of palmitate on macrophages have so far only been carried out in animal models [27,28,39,40]. Thus, we sought to investigate if palmitate can train human monocyte-derived macrophages (MDMs). palmitate-trained human MDMs produced significantly increased levels of hTNFα (Figure 1D) and hIL-6 (Figure 1E) compared to the untrained cells, with MTA suppressing this training effect. To confirm that MDMs were undergoing a rest period and did not continue to express pro-inflammatory cytokines, supernatants were taken each time medium was changed (24 h, 5 days, 6 days) and analyzed for hTNFα and hIL-6 (Supplementary Figure 1). Both quickly returned to undetectable levels, with IL-6 levels remaining elevated on day 5 due to the medium not being changed between 24 h and 5 days.

Human bone marrow MSCs can suppress palmitate training of macrophages

MSCs are known for their immunosuppressive characteristics, including suppression of macrophage function [30,41–43], but there is little research on their effects on innate immune cell training [33,44]. Based on a recent paper showing that MSCs can suppress training by HDM [34], our hypothesis was that MSCs are also able to block the palmitate training of BMDMs if present during the training

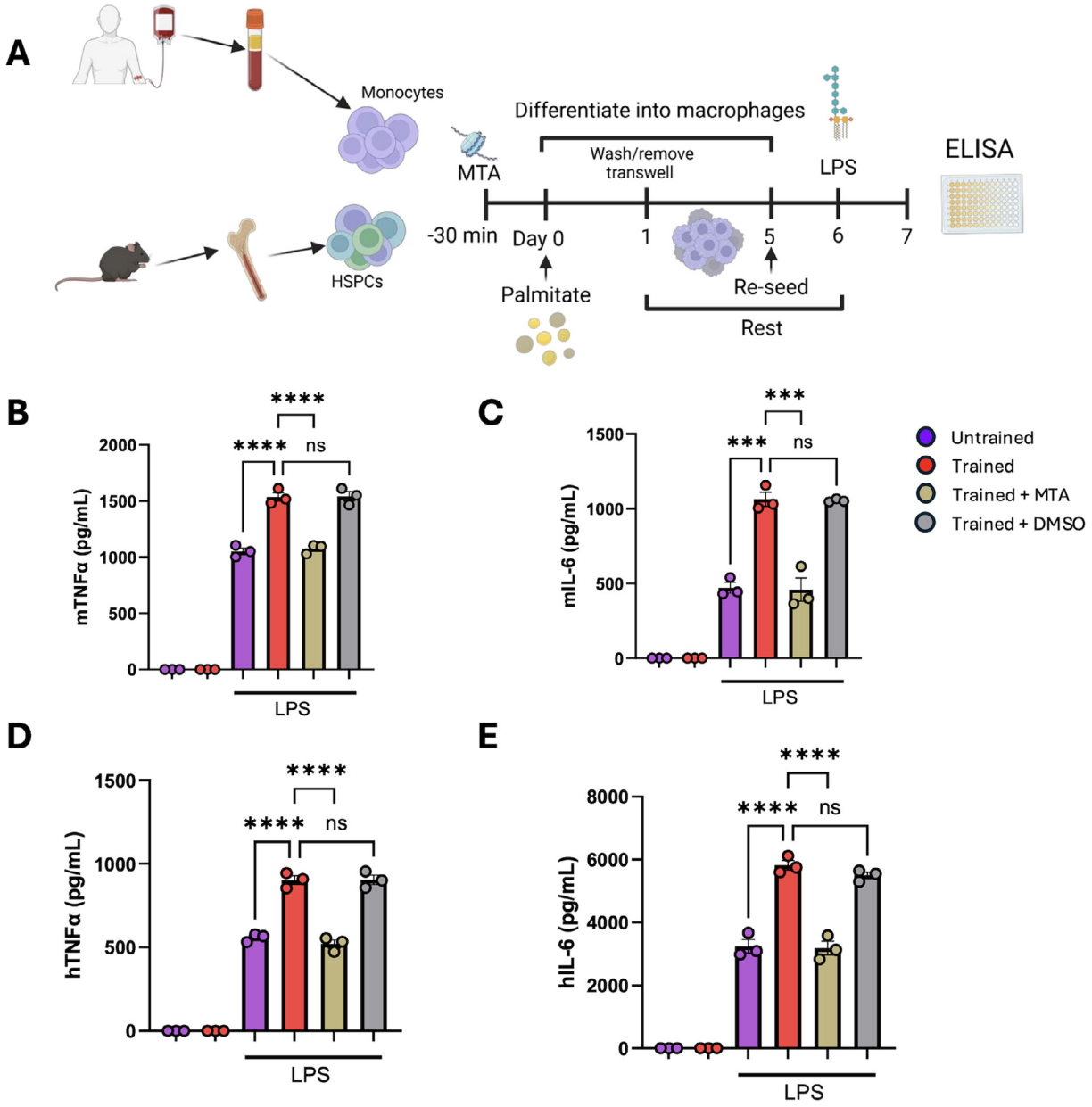

Fig. 1. Methyltransferases are required for palmitate training of macrophages (A) Experimental design: training assays were performed starting with whole bone marrow containing hematopoietic stem progenitor cells (HSPCs) isolated from the femurs and tibias of C57BL/6 mice and human monocytes derived from peripheral blood mononuclear cells (PBMCs) isolated from buffy coats. Mouse bone marrow cells or human monocytes were treated with 1 mM methylthioadenosine (MTA) 30 min before 0.3 mM palmitate were added. After 24 h, cells were thoroughly washed with DPBS and differentiated from HSPCs into bone marrow-derived macrophages (BMDMs) or from monocytes into monocyte-derived macrophages (MDMs). This differentiation period also served as a rest period from the training stimulus. On day 5, BMDMs and MDMs were re-seeded and allowed to adhere overnight, before they were stimulated with 100 ng/mL LPS for 24 h and the supernatants analyzed via ELISA. Concentrations of mouse (B) TNFα (C) IL-6, and human (D) TNFα and (E) IL-6 were measured. $n = 3$ (3 mice/3 PBMC donors). Data are presented as mean ± SEM. $^{***}P < 0.001$, $^{****}P < 0.0001$. Statistical test: Ordinary one-way ANOVA with Tukey's multiple comparisons test. (Color version of figure is available online.)

period. MSCs were seeded in a transwell and removed after the 24-h training period with the wash step (Figure 2A). Our data demonstrates that the training effect for both TNFα and IL-6 in BMDMs (Figure 2B,C) and human MDMs (Figure 2D,E) was blocked by transwell co-culture with MSCs during the training period, indicated by a reduction of the cytokine concentrations back to the levels of the untrained control.

IL-1RA is produced by MSCs in coculture with palmitate-stimulated human monocytes.

We performed a preliminary human cytokine proteome profiler array to determine what secreted factors are released by MSCs in response to being cocultured in the presence of monocytes stimulated with palmitate for 24 h. We compared the cytokine proteome profile of supernatants from monocytes, monocytes + palmitate, monocytes + palmitate + MSCs and MSCs + palmitate. This was used as a screening approach with an $n = 1$ to identify potential mediators produced by MSCs that might be responsible for the MSC suppression of cytokine production in Figure 2. With acknowledgment of the limitation of this $n = 1$ screening approach, a number of potential candidates were identified including IL-1RA, IL-16, IL-8, ICAM-1, CXCL10 and IL-6 as levels of these cytokines were higher in the monocyte + palmitate + MSC group when compared to the monocytes + palmitate group or to the MSC + palmitate group. The proteome array also demonstrated that palmitate led to increases in production of IL1α, IL1β, TNFα, CXCL1, MIP1α/MIP1β and CCL5 in monocytes (Figure 3).

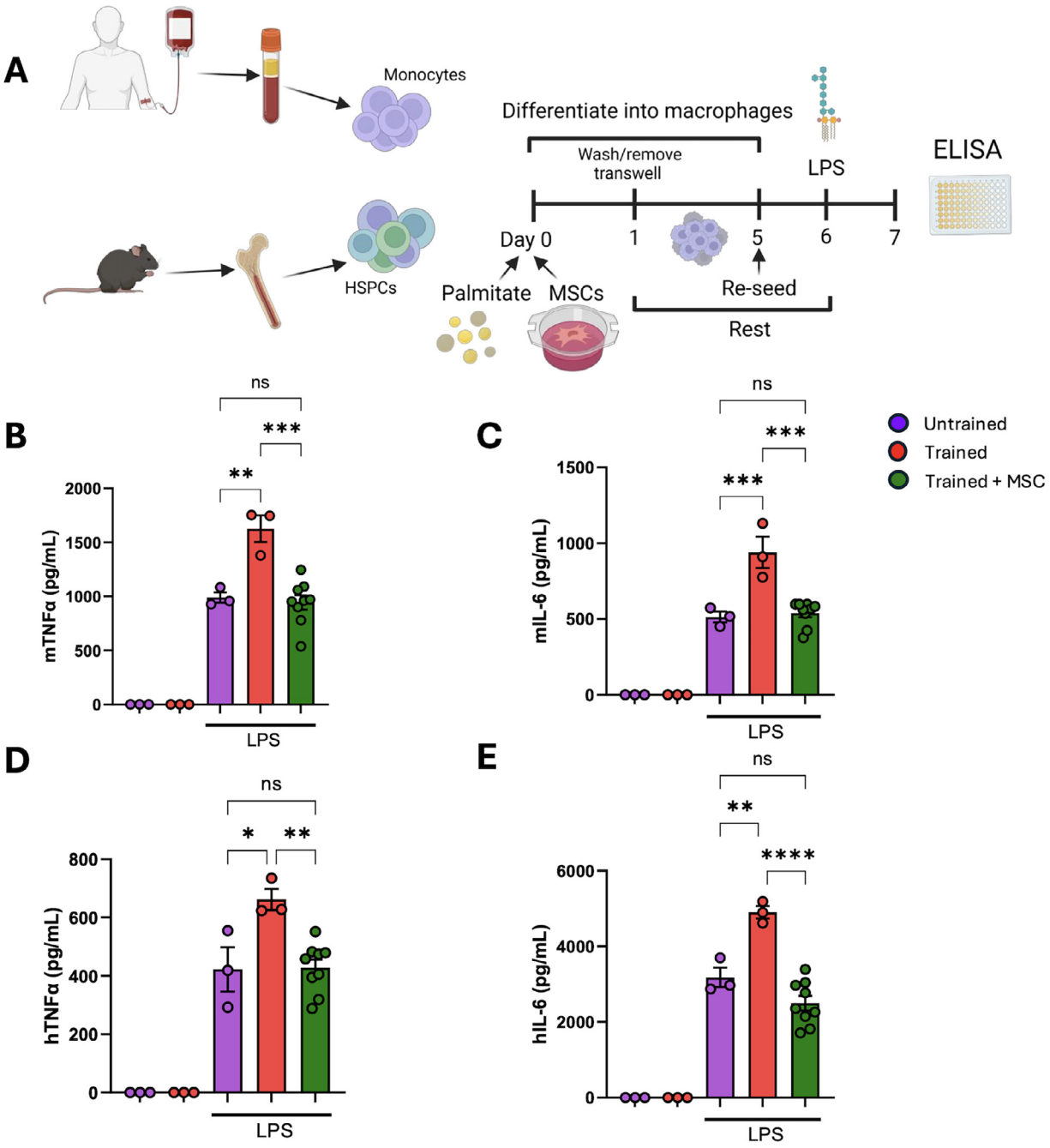

Fig. 2. MSCs block palmitate training of macrophages. (A) Experimental design: training assays were performed starting with whole bone marrow containing hematopoietic stem progenitor cells (HSPCs) isolated from the femurs and tibias of C57BL/6 mice and human monocytes derived from peripheral blood mononuclear cells (PBMCs) isolated from buffy coats. Mouse bone marrow cells or human monocytes were exposed to 0.3 mM palmitate and human bone marrow MSCs in a transwell. After 24 h, the transwell was removed, cells were thoroughly washed with DPBS and differentiated from HSPCs into bone marrow-derived macrophages (BMDMs) or from monocytes into monocyte-derived macrophages (MDMs). This differentiation period also served as a rest period from the training stimulus. On day 5, BMDMs and MDMs were re-seeded and allowed to adhere overnight, before they were stimulated with 100 ng/mL LPS for 24 h and the supernatants analyzed via ELISA. Concentrations of (B) mouse TNFα, (C) mouse IL-6, (D) human TNFα and (E) human IL-6 were measured. $n = 3–9$ (3 mice/PBMC donors, 3 MSC donors). Data are presented as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. Statistical test: Ordinary one-way ANOVA with Tukey's multiple comparisons test. (Color version of figure is available online.)

Palmitate training drives an M2 MDM phenotype which is not altered by MSCs

In addition to enhancing proinflammatory cytokine production, training of MDMs with various stimuli can lead to changes in phenotype [45]. Studies have shown that palmitate exposure drives an M2 phenotype with increased CD206 expression and decreased HLA-DR expression [46,47]. In our study, untrained human MDMs stimulated with LPS expressed significantly increased levels of M1 markers CD86 and HLA-DR while the frequency of cells expressing the M2 marker CD163 significantly decreased and the mean fluorescence intensity (MFI) of the M2-associated marker CD11b significantly decreased (Supplementary Figure 2). Palmitate training in MDMs led to a phenotypic switch from M1–M2 following stimulation with LPS with significantly increased CD206+ frequency and MFI and significantly decreased expression of CD86 and HLA-DR MFI. Presence of MSCs in a transwell co-culture during palmitate training did not alter the phenotype of MDMs following LPS stimulation (Supplementary Figure 2). This is aligned with studies investigating trained immunity that show elevated expression of

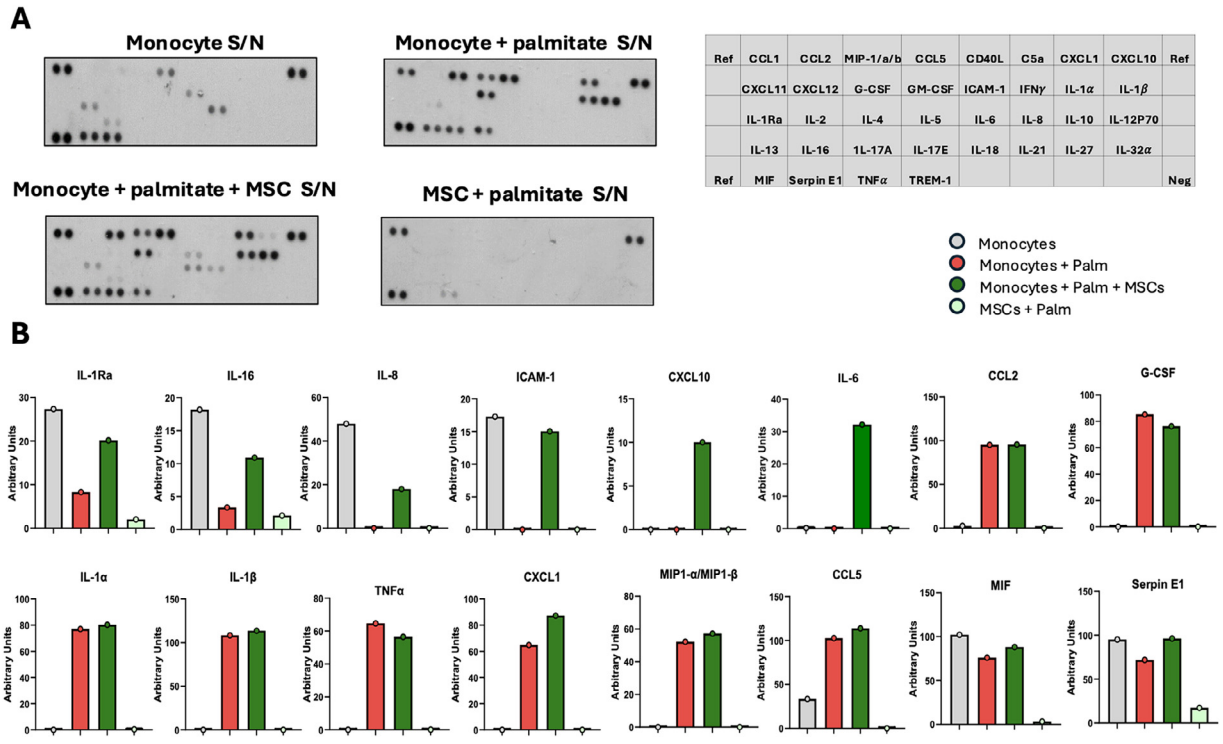

Fig. 3. IL1RA is produced by MSCs in coculture with palmitate-stimulated human monocytes. Human peripheral blood monocytes were isolated, trained with 0.3 mM palmitate with or without the presence of MSCs in a transwell. After 24 h, supernatants were collected from the four groups (monocytes; monocytes + palmitate; monocytes + palmitate + MSC; MSC + palmitate) and probed using a human cytokine proteome profiler array. (A) Image of blot of cytokine array. All four samples were imaged at the same time to avoid different exposure times. Exposure time was 10 min ($n = 1$ per group). A total of 36 different cytokines were probed as outlined in the key (top right). (B) The arbitrary units for each cytokine detected (IL-1Ra, IL-16, IL-8, ICAM-1, CXCL10, IL-6, CCL2, G-CSF, IL-1α, IL-1β, TNFα, CXCL1, MIP1a/MIP1B, CCL5, MIF and Serpin E1) were plotted comparing each of the four groups. (Color version of figure is available online.)

CD206 following training in the context of BCG and exercise-induced trained immunity [48,49]. At the same time others studies confirm our findings that palmitate exposure/training leads to elevated production of pro-inflammatory cytokines including TNF-α, IL-1β, IL-6 and IL-8 [50,51]. Therefore, it seems in the case of palmitate-induced trained immunity the elevated production of pro-inflammatory cytokines and the macrophage phenotype may be distinctly or differentially regulated.

RNA-Seq identification of pro-inflammatory signaling pathway involved in palmitate-trained monocyte activation of MSCs

The preliminary proteome profiler array suggested that monocytes produce elevated levels of IL-1α, IL-1β, TNF-α, CXCL1, MIP1a/MIP1b, CCL2, CCL5 and G-CSF (Figure 3). We confirmed these findings using a publicly available RNA-sequencing dataset on palmitate-stimulated monocyte-derived macrophages showing significant induction/elevation of CXCL1, IL-6, IL-1β, IL-1α, IL-8 and TNFα (Figure 4). Moreover, the RNA-Seq data set revealed significantly elevated levels of IL-8, TLR2 and NLR family pyrin domain containing 3 (NLRP3) (Figure 4). Published studies have shown that palmitate can induce heterodimerization of TLR2 with TLR1 in human blood monocytes. This heterodimerization leads to downstream signaling culminating in pro-IL-1β cleavage by caspase-1 and subsequent secretion of mature IL-1β [52]. Palmitate has also been shown to stimulate NLRP3 inflammasome activation [53]. Release of IL-1β has been shown to induce IL-6 production [54] and TNFα [55].

MSCs do not cause a metabolic switch in trained MDMs

Innate training with a pro-inflammatory stimulus is known to affect the metabolic phenotype of macrophages, usually switching to a more glycolytic phenotype [23,56,57]. Differentially, Lundahl et al. [56] describes how training with an anti-inflammatory stimulus can lead to a switch to oxidative phosphorylation (OXPHOS) and a higher reliance on glutamine metabolism for the expression of TNFα and IL-6. Training with palmitate has been shown to increase macrophage metabolism, with both maximal extracellular acidification rate and oxygen consumption rate being elevated [28]. In different contexts, MSCs have been shown to be able to shift the metabolism of LPS-activated macrophages toward OXPHOS via secretion of prostaglandin E2 (PGE2) [58] or lactate [59] and decrease glycolytic activity via extracellular vesicles [60]. Using metabolic inhibitors, we characterized the metabolic pathways involved in cytokine production in palmitate-trained human MDMs and investigated if MSCs alter metabolism in these trained MDMs.

Inhibition of glucose metabolism using 2-deoxy-D-glucose (Figure 5A) significantly reduced hTNFα and hIL-6 production in untrained, trained and trained with MSCs MDMs pointing toward a high dependence on glucose for the production of pro-inflammatory cytokines. Blockade of mitochondrial respiration using oligomycin (Figure 5B) resulted in an upward trend in production of hTNFα in all groups. Given that blockade of mitochondrial respiration via oligomycin blocks OXPHOS and promotes glycolysis, this data suggests that the increased usage of the glycolytic pathway promoted increased expression of hTNFα (Figure 5B). In contrast, hIL-6 expression seemed more linked to OXPHOS and dropped when mitochondrial respiration was inhibited (Figure 5B). Inhibition of fatty acid oxidation (FAO) using etomoxir did not have an effect on hTNFα expression (Figure 5C). Once again, hIL-6 expression was negatively affected by etomoxir across all groups. Finally, inhibition of glutamine metabolism using CB839 affected hTNFα expression only in the untrained control (Figure 5D) but not in the trained and trained with MSCs group. For hIL-6 expression, inhibiting glutamine metabolism again decreased the cytokine in all three groups (Figure 5D). Although MSCs are able to suppress palmitate training in MDMs,

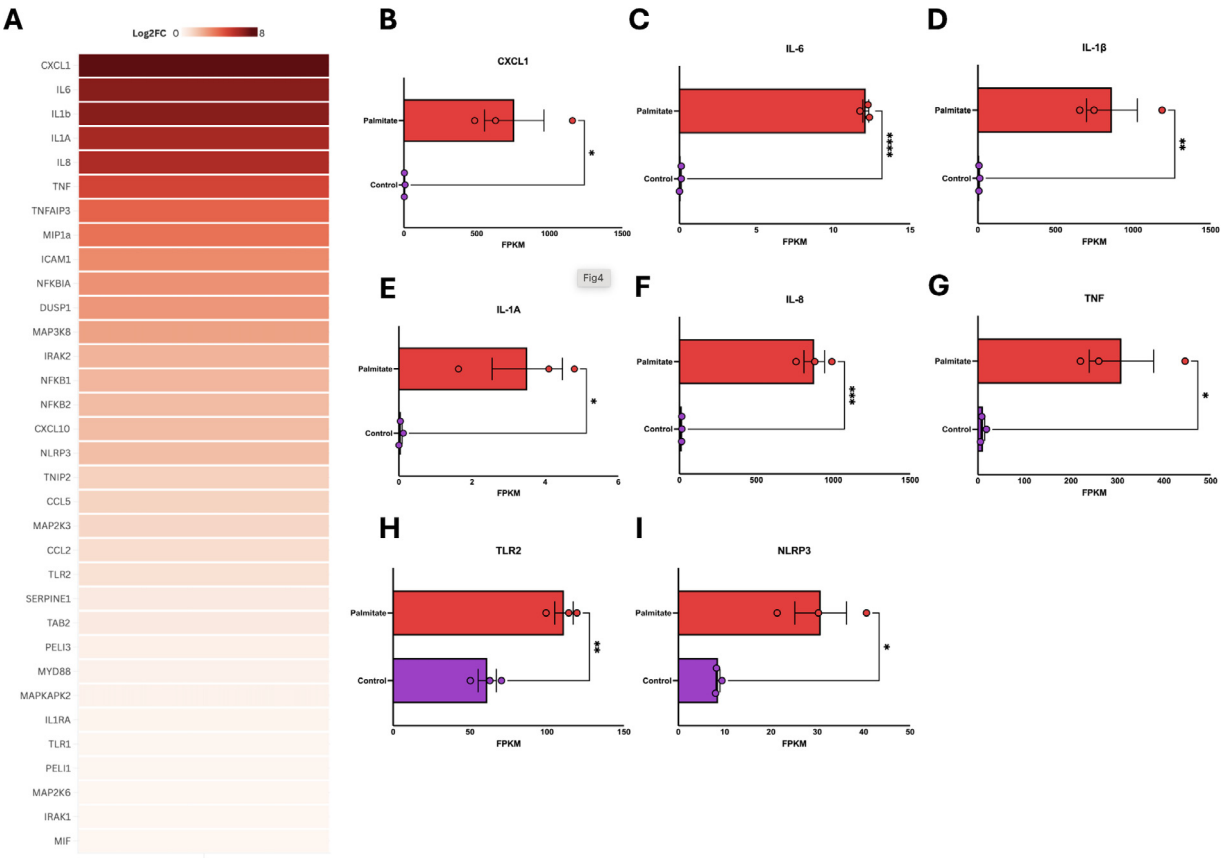

Fig. 4. RNA-Seq identification of pro-inflammatory signaling pathways upregulated in palmitate-trained monocytes. A publicly available RNA sequencing dataset (GEO: GSM4748241) of human CD14+ monocytes, that had been exposed to 200 mM palmitate for a period of 2 h was utilized to investigate the downstream signaling cascade in the cells in response to palmitate. Human monocytes derived from 3 independent PBMC donors were exposed to palmitate and sent for sequencing. The (A) Log2FC of components of the IL-1 signaling cascade were observed and plotted in heat-map format using Flourish. The FPKM from genes of interest, (B) *cxcl1*, (C) *il-6* (D) *il-1β*, (E) *il-1a*, (F) *il-8*, (G) *tnf*, (H) *tlr2* and (I) *nlrp3* were also plotted, for each individual PBMC donor. Data is presented as mean ± SEM; $n = 3$ per group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. (Color version of figure is available online.)

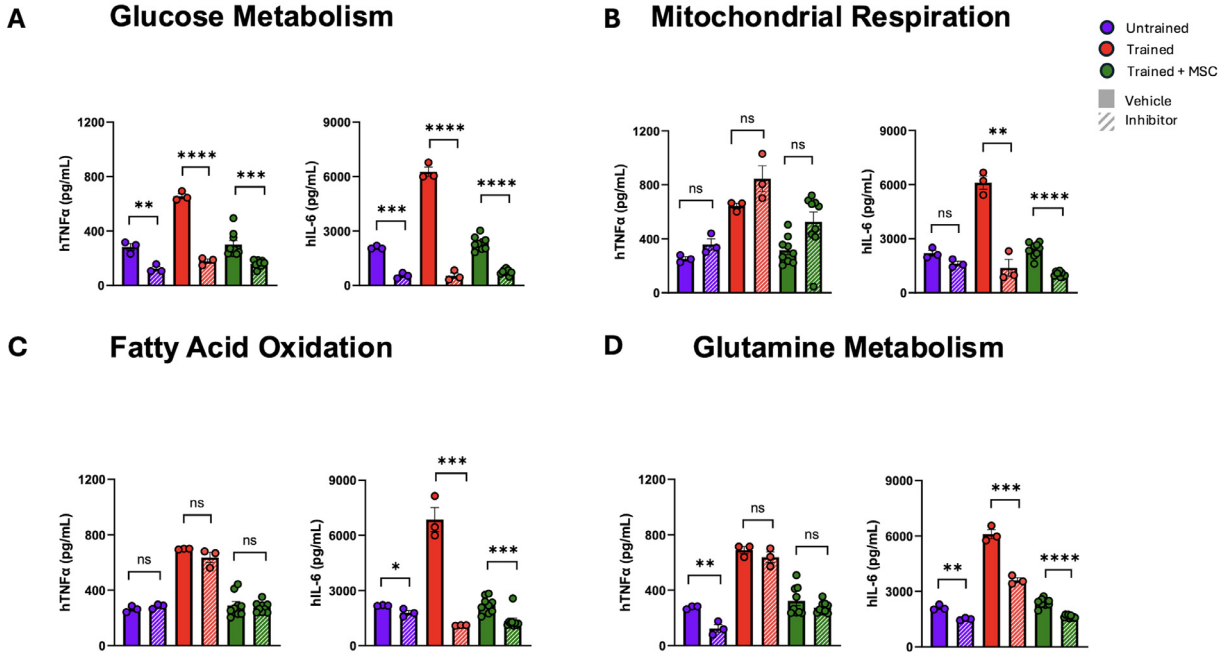

Fig. 5. The presence of MSCs during palmitate training does not affect macrophage metabolic phenotype. Human peripheral blood monocytes were isolated, trained with 0.3 mM palmitate with or without the presence of MSCs in a transwell, and differentiated into macrophages. After counting and re-seeding, MDMs were then stimulated with 100 ng/mL LPS in presence of either 5 mM 2-DG (A), 1 μM oligomycin (B), 3 μM etomoxir (C) or 10 μM CB839 (D) or the corresponding vehicle control for 24 h. Human TNFα and human IL-6 - were measured via ELISA and quantified relative to the vehicle control. $n = 3–9$ (3 PBMC donors, 3 MSC donors). Data are presented as mean ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. Statistical test: Unpaired *t*-test with Welch's correction (carried out between vehicle and inhibitor of same treatment group). (Color version of figure is available online.)

their presence during training did not alter the metabolic profile of trained MDMs.

MSCs block palmitate training of MDMs via cyclooxygenase-2 (COX-2) and IL-1RA

When it comes to the suppression of macrophage-produced TNFα and IL-6 in response to LPS stimulation by MSCs, PGE2, produced via the COX-2 pathway, is frequently identified as the primary mediator [43,58,61,62]. The signaling behind the COX-2 pathway leading to blockade of TNFα or other pro-inflammatory mediators by MSCs has been extensively documented by us and others. We have previously shown that upregulation of COX-2 leads to PGE2 production by MSCs [43]. MSC-derived PGE2 binds to monocytes via EP2/EP4 receptors [63] which can activate adenylate cyclase and increase cAMP followed by enhancement of C/EBP-β and inhibition of M1 macrophages [64]. Here, we show that palmitate leads to significantly increased levels of PTGS2 the gene that codes for COX-2 (Figure 6A).

Additionally, inflammasomes and IL-1β signaling have been implicated as being of importance in innate training [4,22,65]. The cytokine proteome profile array identified elevated levels of IL-1RA produced by MSCs + monocytes + palmitate when compared to monocytes + palmitate or MSC + palmitate (Figure 3). MSC-derived IL-1RA has been shown to promote M2 macrophage polarization *in vitro* [66] and to mediate MSC protective effects in a lung injury model [67]. TNFα dose-dependently increases MSC production of IL-1RA [68]. Indeed our findings from the proteome profiler array support the published evidence that palmitate induces the production of IL-1β and TNFα in monocytes and MSCs produce higher levels of IL-1RA when co-cultured with monocytes exposed to palmitate. Palmitate significantly increases IL-1RN gene expression in MSCs (Figure 6B). Using the COX-2 inhibitor NS-398 pretreatment of MSCs or an IL-1Ra neutralizing antibody during the training period, we investigated the role of these factors in MSC blockade of the elevated proinflammatory cytokines TNFα and IL-6 associated with palmitate induce trained immunity. Interestingly, while inhibiting COX-2 activity in MSCs restored the palmitate training response for hTNFα in MDMs, it had no tangible effect on MSC suppression of the training effect on hIL-6 expression (Figure 6C). Similarly, addition of the IL-1RA neutralizing antibody did not affect MSC suppression of the training effect on hTNFα expression, while hIL-6 was restored back to the elevated levels of the trained response (Figure 6D). This indicates that the training takes place via more than one signaling pathway, and MSCs interfere with these pathways by multiple means.

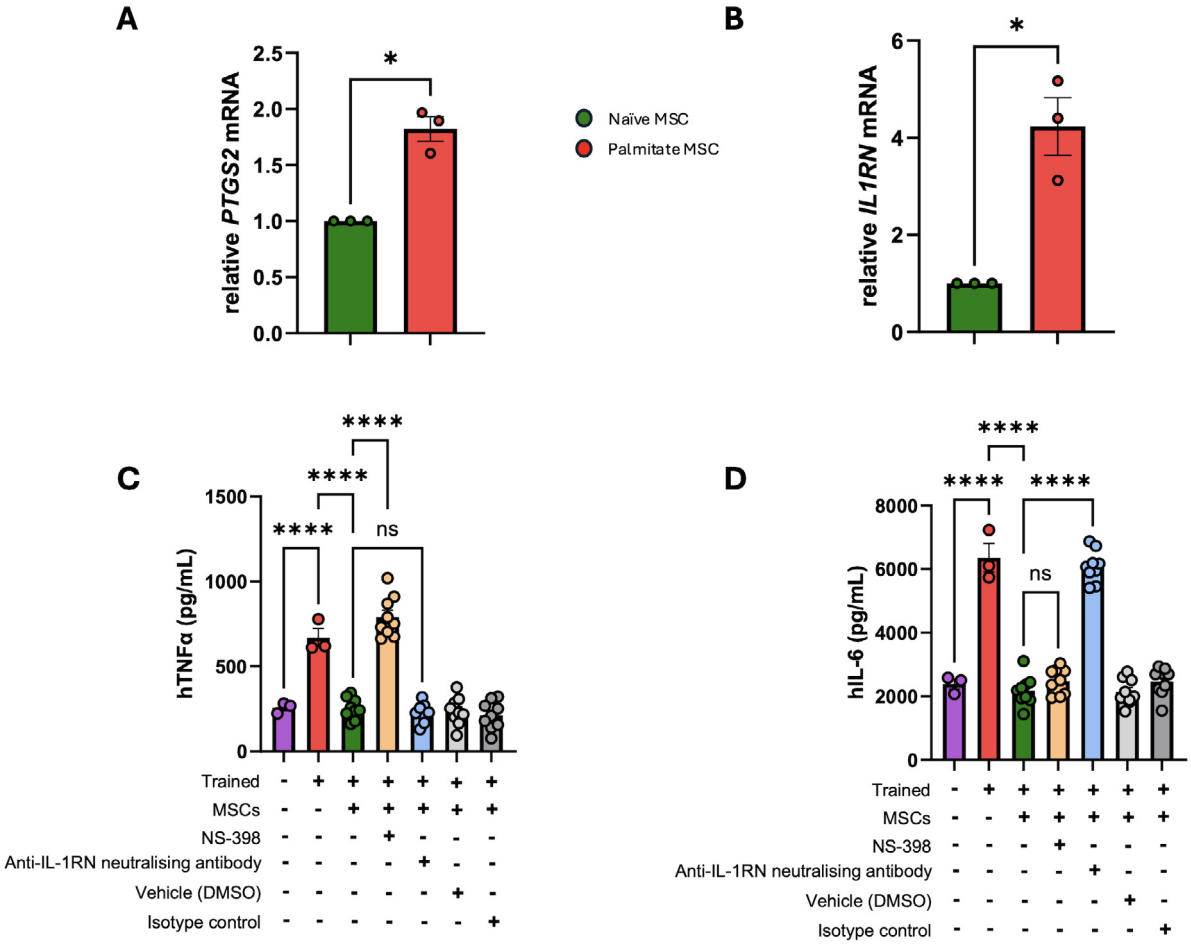

Fig. 6. MSCs block palmitate training via COX-2 and IL-1Ra. Human bone marrow MSCs were exposed to 0.3 mM palmitate for 24 h. Relative *PTGS2* (A) and *IL-1RN* (B) mRNA expression were measured via qPCR. $n = 3$ (3 MSC donors). Human MDMs were trained with 0.3 mM palmitate, with or without the presence of MSCs in a transwell. To suppress COX-2 activity, MSCs were pretreated with 10 μM NS-398 for 24 h before training. To neutralize MSC-derived IL-1Ra, 10 ng/mL anti-IL-1RA neutralizing antibody was added during the training period. On day 6 after training, MDMs were stimulated with 100 ng/mL LPS for 24 h. Human TNFα (C) and IL-6 (D) were measured. $n = 3–9$ (3 PBMC donors, 3 MSC donors). Data are presented as mean ± SEM. $^{*}P < 0.05$, $^{****}P < 0.0001$. Statistical test: Unpaired *t*-test with Welch's correction (A and B) and ordinary one-way ANOVA with Tukey's multiple comparisons test (C and D). (Color version of figure is available online.)

Discussion

Obesity is known to cause a chronic state of inflammation [69–73] and innate immune cells can retain an elevated pro-inflammatory phenotype even after weight loss, which is consistent with innate training [28,74]. OxLDL, which is typically elevated in patients with obesity [75,76], has been investigated and confirmed as a pro-inflammatory innate training stimulus in obesity. Innate training by oxLDL is associated with lasting epigenetic and metabolic changes in macrophages, specifically with an increase in glycolytic activity via mTOR and HIF-1α signaling [3,21,24]. Palmitate, a pro-inflammatory saturated free fatty acid that is also elevated in obesity and known to cause detrimental health effects [50,77–82] has so far only received very little attention regarding its ability to promote innate training. Differential findings show that palmitate can drive a pro-inflammatory [27] or immunoparalysed [40] phenotype. In our hands, both murine and human macrophages responded to 0.3 mM palmitate as a training stimulus with an increased pro-inflammatory response after re-stimulation with 100 ng/mL LPS. This is in line with the results published by Seufert et al. [27], where the authors used a concentration of 0.5 mM palmitate. Our data showed that palmitate-trained monocyte-derived macrophages display an M2 phenotype following LPS re-stimulation with increased CD206+ expression. This aligns with the published studies investigating the influence of palmitate on macrophage phenotype [46,47]. Moreover, innate immune training studies have also demonstrated a switch to a CD206 + macrophage phenotype [48,49]. Although proinflammatory cytokine production usually correlates with an M1 phenotype, there are published studies supporting our data whereby palmitate stimulation leads to significantly increased levels of pro-inflammatory cytokines [50,51] and an M2 macrophage phenotype [46,47]. While the presence of MSCs blocked the palmitate-induced elevated pro-inflammatory cytokine release in trained macrophages, MSCs did not alter the palmitate-induced M2 phenotype switch in LPS re-stimulated cells.

General nutrition and specifically cellular exposure to palmitate are known to affect DNA methylation [83–86]. The observation that inhibiting DNA and histone methylation via MTA prevents macrophage training by palmitate aligns with studies of other training stimuli, including the BCG vaccine [35] and LPS [87,88]. Our study is the first that links palmitate training to methyltransferase activity in human macrophages.

While trained immunity in macrophages has initially caught attention as a beneficial mechanism that helps clear infections [1,9], further studies have shown that trained macrophages can also have pathogenic effects that need to be addressed [10–13]. MSCs can suppress an acute inflammatory macrophage response [30,41,61] thus we hypothesized that MSCs may be capable of suppressing palmitate-mediated training through soluble factors. For our model, we first demonstrated that MSCs are able to suppress palmitate training in murine BMDMs, and then replicated the experiment in human MDMs, demonstrating a cross-species mechanism of action that is nevertheless relevant in the context of human cells.

Previously, it has been shown that pro-inflammatory innate training, including palmitate training, shifts cells toward a more glycolytic phenotype [2,28,89,90]. Meanwhile, anti-inflammatory training, for example with a combination of IL-13 and IL-4, can switch macrophages toward a more OXPHOS-heavy metabolic phenotype [56]. As our experiments demonstrated that MSCs had a suppressive effect on the pro-inflammatory training, we assumed that they had an anti-inflammatory influence on the macrophages and might thus cause a metabolic switch opposing the palmitate training. However, this was not the case. For all four metabolic pathways that were inhibited, glucose metabolism, mitochondrial respiration, FAO and glutamine metabolism, palmitate-trained macrophages and macrophages that were palmitate-trained in the presence of MSCs demonstrated the same trends when exposed to the respective inhibitors. TNFα expression heavily relied on glucose metabolism and trended upward when mitochondrial respiration was inhibited, suggesting a strong reliance on glycolysis. Meanwhile, FAO and glutamine metabolism played no significant role in the expression of this cytokine. IL-6, on the other hand, was significantly reduced in response to each of the metabolic inhibitors, indicating that the macrophages are not exclusively relying on glycolysis.

Given that MSCs did not affect the metabolic phenotype of trained macrophages, their suppressive activity must be exerted through different means, and through a secreted factor given that MSCs were separated from MDMs via a transwell. Based on the use of human MSCs in our experiments, species nonspecific metabolites were the most likely candidates. A cytokine proteome profile screen identified a number of potential factors including IL-1RA that might be involved in the MSC blockade of trained immunity. The COX-2/PGE2 pathway has been described as crucial for macrophage suppression [30,41,61,91] and has been described as a pathway via which MSCs suppress training of murine macrophages by HDM [34]. We confirmed that inhibition of COX-2 activity in the MSCs restored the increased TNFα response of the palmitate training, but not the IL-6 response. Suppression of TNFα expression via PGE2 happens primarily via signaling through the E-type prostanoid receptor (EP) 2 and EP4. PGE2 binds to EP2/EP4, leading to an increase in intracellular cyclic adenosine monophosphate, and eventual suppression of NFκB [92–97]. A second major player in innate immune training is the NLRP3 inflammasome, specifically the IL-1 signaling associated with it [3,65,98]. Palmitate and a high-fat diet, in general, are known activators of NLRP3 [22,99–101], and could thus be activating this particular training pathway. It is known that MSCs can express IL-1RA [102–104] and while cross-species reactivity between human and mouse is imperfect, human IL-1RA has been shown to be functional in rabbit articular chondrocytes [105], a murine myocardial ischemia-reperfusion model [106], and to be able to bind to both bovine and murine fibroblasts [107]. Although preliminary data (with an $n = 1$) the proteome profile array combined with this evidence provided sufficient basis to suspect MSC-derived IL-1RA as a potential cause for the inhibition of palmitate-mediated training. Indeed, addition of a human IL-1RA neutralizing antibody during the training period, but not of the isotype control, restored the training effect on IL-6 expression. As a result, we were able to demonstrate that there are two pathways involved in palmitate training of macrophages, which MSCs block through different secreted factors.

The ability of human bone marrow MSCs to suppress innate training through COX-2 expression has recently been described in a mouse model of HDM-induced allergic asthma [34], and human umbilical cord MSCs have been shown to be able to suppress the effects of LPS training in microglia 2 weeks after the training stimulus [33]. Other studies investigating MSCs in the context of neutrophil training have shown that MSCs can affect the HSPCs in the bone marrow which, in the case of neutrophils, is directly associated with altered myeloid proliferation [44,108]. These findings are particularly important given that our initial experiments involve blocking the training of murine HSPCs, as they demonstrate that human MSCs and MSC-secreted factors are able to affect the murine bone marrow niche. However, further experiments are needed to verify these changes for our model.

In summary, as a factor that is highly elevated in patients with obesity, palmitate poses a risk as a pro-inflammatory stimulus, altering macrophages on an epigenetic level. MSCs are able to block the changes in cytokine expression, via the expression of COX-2 and secretion of IL-1RA. Further research is required to determine if MSCs can successfully reverse palmitate training after it has already occurred, instead of blocking it during the process. This new knowledge may provide insights on the potential application of MSCs in chronic conditions where trained immunity may have detrimental

effects, to prevent repeated training of innate immune cells and consistent worsening of disease.

Declaration of competing interest

The authors declare that they have no competing interests.

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Author Contributions

LMB: Conception and design, generation of data, data analysis and interpretation, manuscript writing. CT: Data analysis and interpretation. AEH: Conception and design, data interpretation and manuscript writing. JAA: Manuscript writing. KE: Conception and design, manuscript writing, final approval of manuscript.

Data Availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.jcyt.2024.10.011.

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