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Low-density cultured cartilage cells expanded in platelet lysate present distinct features to develop an innovative clinical treatment for diffuse cartilage lesions

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Abstract

Purpose: Chondrocyte-based cell therapies are effective for the treatment of chondral lesions, but remain poorly indicated for diffuse lesions in the context of early osteoarthritis (OA). The aim of this study was to develop a protocol to obtain chondroprogenitor cells suitable for the treatment of diffuse chondral lesions within early OA.

Methods: Cartilage cells were expanded at low density in human platelet lysate (hPL). A test was performed to exclude senescence. The expression of surface cluster of differentiation 146, cluster of differentiation 166, major histocompatibility complex (MHC)-I and MHC-II and of genes of interest were evaluated, as well as the trophic potential of these cells, by the assessment of lubricin and matrix production. The immunomodulatory potential was assessed through their co-culture with macrophages.

Results: Cartilage cells expanded at low density in hPL showed higher proliferation rate than standard-density cells, no replicative senescence, low immunogenicity and expression of lubricin. Moreover, they presented an increased expression of chondrogenic and antihypertrophic markers, as well as a superior matrix deposition if compared to cells cultured at standard density. Cartilage cells induced on macrophages an upregulation of CD206, although a higher increase of CD163 expression was observed in the presence of low-density cells.

Conclusions: These findings lay the grounds to explore the clinical usefulness of low-density cultured cartilage cells to treat diffuse lesions in early OA joints for both autologous and allogenic use.

Level of Evidence: Not applicable.

Abbreviations: ACI, autologous chondrocyte implantation; APC, allophycocyanin; BC-PC, buffy coat-platelet concentrate; BMPR1B, bone morphogenetic protein receptor type 1B; BSA, bovine serum albumin; CD146, cluster of differentiation 146; CD166, cluster of differentiation 166; CPC, chondroprogenitor cell; DAPI, 4′,6-diamidino-2-phenylindole; DKK1, dickkopf WNT signaling pathway inhibitor 1; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; ECM, extracellular matrix; FRZB, frizzled related protein; GMP, Good Manufacturing Practice; GORILLA, Gene Ontology enRIchment anaLysis and visuaLizAtion tool; GREM1, gremlin 1; HE, hematoxylin and eosin; HLA, human leukocyte antigen; hPL, human platelet lysate; MHC, major histocompatibility complex; MMP, matrix metalloproteinase; MSC, mesenchymal stem cell; OA, osteoarthritis; PBS, phosphate-buffered saline; PRG4, proteoglycan 4; PRGF, platelet-rich growth factor; PRP, platelet-rich plasma; TIMP, tissue inhibitor of metalloprotease.

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KEYWORDS
cartilage cells, cartilage damage, early osteoarthritis, low-density culture, platelet lysate

INTRODUCTION

Osteoarthritis (OA) is the most common cause of joint degeneration, with a worldwide prevalence of approximately 528 million individuals (2019 data) and its global prevalence is anticipated to escalate as the population ages [47].

The currently available symptomatic treatments suffer of poor long-term efficacy and often lead clinicians to more invasive and permanent surgical solutions [3]. Joint replacement is a very invasive procedure that is not suitable for young patients due to the limited duration of the implant. The need for surgery can only be avoided by counteracting the degenerative progression through an effective treatment performed in the earliest stages. In this scenario, the development of alternative conservative cell-based approaches is particularly relevant.

Autologous chondrocyte implantation (ACI) [2] has been safely used for over 25 years in clinical practice for the treatment of focal chondral lesions, with very satisfactory long-term results [22, 33, 37, 41]. Despite it has been also proposed as a suitable therapeutic option in patients with early OA [10, 30], ACI remains poorly indicated for these patients [1], mainly due to the presence of a chronic degenerative microenvironment hindering the survival and healing potential of the implanted chondrocytes [1, 23]. In addition, because of the larger size of the damaged joint surface, more chondrocytes would be required and thus the donor site morbidity associated with more abundant harvesting of healthy cartilage would be an important limiting factor for the treatment of diffuse lesions [18]. Alternatively, extensive in vitro cell expansion would be needed to achieve a relevant number of chondrocytes from a limited portion of healthy cartilage. However, during the in vitro expansion, chondrocytes tend to loss their chondrogenic phenotype [18], thus compromising the proper long-term repair of the cartilage defect [27].

While mature chondrocytes have been long considered the only cell type present within articular cartilage, a population of cartilage cells termed as chondroprogenitor cells (CPCs) characterised by migratory, clonogenic ability and differentiation potential has been identified in both healthy and damaged cartilage [19, 49]. The whole population of cartilage cells isolated from OA patients has revealed a noteworthy chondrogenic potential, active secretory response and strong immunomodulatory behaviour after inflammatory priming [12] ascribable to the presence of CPCs. Because of their 'stem-like' and chondrogenic properties, CPCs are likely to have a role in cartilage repair [39]. Due to the lack of specific markers for the selective isolation of CPCs from other cartilage cells, there exists an unmet need for the development of robust protocols aimed at enriching cartilage cell cultures with CPCs. Herein, a low-density culture of cartilage cells in human platelet lysate (hPL) [6, 8, 46] has been developed to reach this goal. The rationale beyond this approach is multiple: (i) a high number of cells is required in clinical application for the treatment of diffuse lesions in OA joints; (ii) cells characterised by an active secretory response and a strong immunomodulatory behaviour are needed to restore the homeostasis of the OA joint. Our hypothesis is that cartilage cells cultured at low density in Good Manufacturing Practice-compliant hemoderivative as hPL present distinct features that would make them the perfect candidate to develop an innovative clinical treatment for diffuse cartilage lesions. Building upon this hypothesis, the aim of the present study was to prove that our protocol is able at producing cells with appropriate features for the treatment of diffuse chondral lesions within the context of early OA. To this aim, cartilage cells prepared using our protocol were compared to cells expanded at standard density, assessing proliferation rate, senescence, expression of specific markers, extracellular matrix (ECM) production and immunomodulatory ability.

MATERIALS AND METHODS

Informed consent statement

The research adhered to local laws and institutional guidelines. Donors contributing whole blood for hPL preparation are not obligated to give specific consent regarding its use or publication of derived data. Primary human macrophages are sourced from anonymized buffy coats obtained from a local blood bank, devoid of donor information. These coats are solely utilised for monocyte isolation and macrophage differentiation, excluding any diagnostic or therapeutic purposes or genetic investigations. Consequently, ethical approval is not mandated for cell isolation and use, and blood donors are not required to provide specific consent for their use or data publication. Human articular

chondrocytes are procured from patient biopsies post informed consent signature, following the approved clinical protocol by the San Raffaele Hospital Ethics Committee on 16 December 2020 (registered as number 214/int/2020).

Preparation of hPL

A platelet pool of 60 healthy donors was used to prepare each of the three hPL bags at the Blood Component Production and Validation Center, City of Health and Science of Turin, S. Anna Hospital, Italy.

Briefly, in a triple-bag system (Fresenius Kabi) containing citrate-phosphate-dextrose, 450 ± 45 mL of whole blood was collected. The blood units were separated by centrifugation with an automated separator (Compomat G5; Fresenius Kabi). The buffy coat-platelet concentrate (BC-PC) was prepared by pooling 4 O-group buffy coats with AB-group plasma. Leucocyte depletion was obtained through centrifugation and filtering by the TACSI system (Terumo BCT Europe) and pathogens inactivation through the Mirasol PRT System (Terumo BCT Europe). Platelet concentration was determined equal at 1000×10^6/μL (Sysmex XE-2100), while platelet fragmentation and growth factor release were obtained by three cycles of freezing (−35°C) and thawing (37°C) of BC-PC. Finally, BC-PC was centrifuged (5000*g*, 8 min), platelet bodies were removed and the supernatant collected and supplemented with 200 IU/mL of heparin. Each batch of hPL was then divided into aliquots and frozen at −20°C until use.

Assessment of hPL protein content

Quantibody® Human Cytokine Array 4000 Kit (https://www.raybiotech.com/quantibody-human-cytokine-array-4000/, accessed on April 2021) was used to determine the concentration of 200 soluble inflammatory and growth factors, chemokines, receptors and cytokines in the hPL, according to the manufacturers' instructions (RayBiotech). Briefly, a multiplexed sandwich-based enzyme-linked immunosorbent assay was used for the simultaneous detection of multiple cytokines in a single experiment. Each antibody, along with positive controls, is arranged in quadruplicate. To quantify cytokines, specific cytokine standards with predetermined concentrations are added to generate a standard curve for each cytokine. By comparing the signals from unknown samples to the standard curve, the cytokine concentrations in the samples were determined. Only factors detected above the detection threshold in all samples were considered. The Gene Ontology enRIchment anaLysis and visuaLizAtion tool was used to identify the process modulated by the protein found in hPL.

Isolation, cryopreservation and expansion of cartilage cells

Upon informed signed consent, waste biological material was collected by nine patients (four male and five female, age range: 41–72 years, mean age: 55 years) who underwent total hip arthroplasty.

The articular cartilage harvested from the femoral head was cut into small pieces under sterile condition and enzymatically digested with 0.15% (w/v) Type II Collagenase (Worthington Biochemical Corporation), for 22 h at 37°C, as previously reported [11]. The digested tissue was filtered with a 100 μm cell strainer to remove tissue residuals. Cells were counted and cryopreserved at −80°C in hPL with 10% dimethyl sulfoxide (DMSO) (Sigma-Aldrich).

After thawing, cells were seeded at 10,000 cells/cm^2 in high glucose Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 10% (v/v) hPL, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 1 mM sodium pyruvate, 200 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, namely expansion medium (all reagents from ThermoFisher Scientific) and incubated at 37°C. After 7 days, the cells were split and expanded at standard (S) (5000 cells/cm^2) and low density (L) (50 cells/cm^2) in expansion medium, with standardised detachment time, 7 days for standard-density cultured cells (P1 after 7 days, P2 after 14 days) and 14 days for low-density cultured cells (P1 after 14 days, P2 after 28 days). The cells were expanded up to three passages (indicated as P3, 21 days for standard-density and 42 days for low-density cultured cells) and used for the subsequent experiments. Figure 1 shows the culture method of cartilage-derived progenitor cells. Population doubling/day was calculated as follows: hour/population doublings (h/PD)/days in culture, with $h/PD = \text{Log}(N/N0)/\text{Log}2$, where N is the final number of cells and $N0$ is the initial number of cells.

Senescence assay

An assay based on the β-galactosidase activity (SA-β-Gal) was performed on cells expanded for 42 days (P3) in each density condition to exclude cellular senescence potentially induced by in vitro expansion. SA-β-Gal activity was measured by a senescence detection kit (BioVision), according to the manufacturer's instructions. Briefly, cells were fixed for 10 min and stained with a solution containing X-gal at 37°C overnight. The next day, cells were visualised using inverted Olympus IX71 microscope. Quantitative analysis of β-galactosidase signal intensity was conducted with Image J (National Institutes of Health).

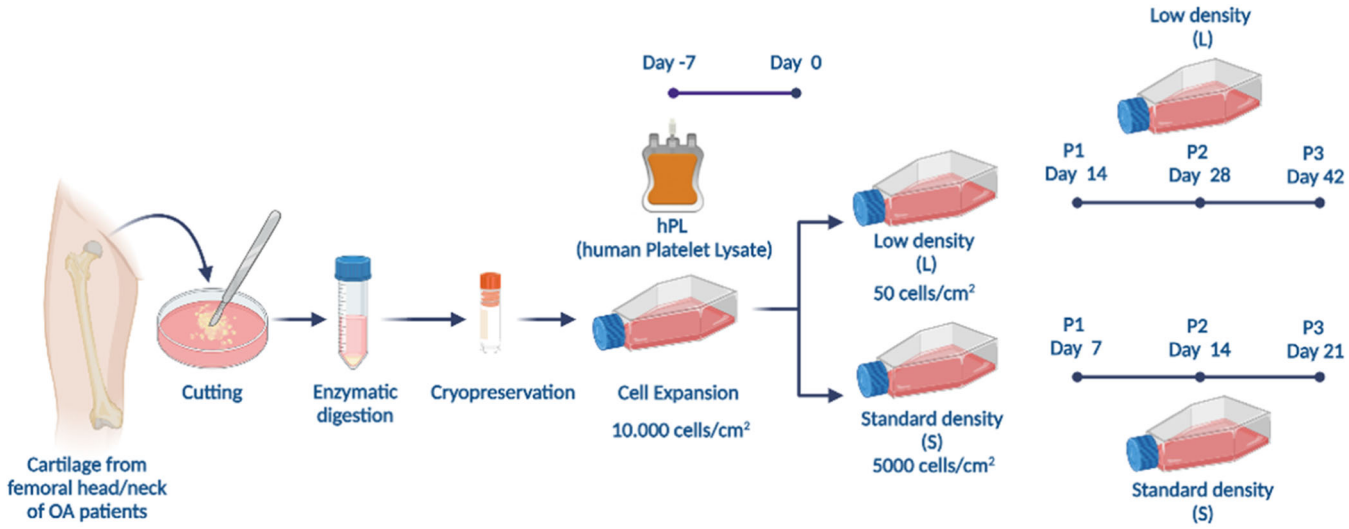

FIGURE 1 Culture method of cartilage-derived progenitor cells. After isolation, cells were cryopreserved. After thawing, cells were seeded at a density of 10,000 cells/cm^2 in culture medium with human platelet lysate (hPL). After 7 days, when confluence was reached, the cells were split and expanded at standard (S) (5000 cells/cm^2) and low density (L) (50 cells/cm^2). S cells were expanded at P1 after 7 days, P2 after 14 days and P3 after 21 days. L cells were expanded at P1 after 14 days, P2 after 28 days and P3 after 42 days. This figure has been created with BioRender.com.

Expression of surface markers

The expression of cluster of differentiation 166 (CD166), a marker which was found to correlate with the clinical success of ACI [17], cluster of differentiation 146 (CD146) a marker that denotes a perivascular phenotype [50] and major histocompatibility complex (MHC)-I and -II, markers of immunogenicity, were analysed by flow cytometry analysis. The cells were washed with cold buffer (PBS added with 2% FBS and 0.04% EDTA Tetrasodium salt) and then incubated for 30 min with CD166 conjugated FITC (Ancell Corporation), CD146 conjugated allophycocyanin (APC)/Fire, human leukocyte antigen (HLA)-A, B, C (MHC-I) conjugated APC and HLA-DR (MHC-II) conjugated peridinin-chlorophyll-protein (BioLegend) anti-human monoclonal antibodies diluted 1:50 in 100 µL of buffer. Background fluorescence was established by unstained cells as negative controls and data were acquired using a Cytoflex flow cytometer (Beckman Coulter).

Immunofluorescence

Immunofluorescence staining for proteoglycan 4 (PRG4), also known as Lubricin, a putative chondrogenic progenitor cells marker [42], was performed on low- and standard-density cartilage cell monolayers, seeded at 5×10^3 cells/cm^2 on coverslips coated with poly-L-lysine solution (Sigma-Aldrich). After 48 h in culture with expansion medium, the cell monolayer was incubated with Brefeldin-A solution, inhibitor for protein transport, 1:1000 (ab193369; Abcam) for 4 h at room temperature, fixed for 10 min with 2% paraformaldehyde (Santa Cruz Biotechnology), permeabilized for 10 min with Triton X-100 0.01% both diluted in phosphate-buffered saline (PBS) and blocked (1% bovine serum albumin [BSA]; Sigma-Aldrich) for 45 min with slow stirring at room temperature. After blocking, the slides were incubated for 45 min at room temperature with antilubricin (ab28484; Abcam) primary antibodies diluted 1:500 in PBS 5% BSA and detected using polyclonal anti-rabbit immunoglobulin G (IgG), (H + L) raised in goat (A-11008; ThermoFisher Scientific) diluted 1:500 in PBS 2% BSA. 1 µg/mL 4′,6-diamidino-2-phenylindole (DAPI) was used for nuclear staining for 10 min at room temperature. The fluorescent images were acquired by confocal microscopy (Leica SP8). Image analysis was conducted using ImageJ (National Institutes of Health). The count of nuclei was determined by applying a threshold and performing particle analysis on the DAPI channel. A separate analysis was conducted with the signal channel, including a fixed threshold and total mean intensity measurement.

Gene array analysis

After expansion for 14 days at standard- and low-density culture, cells were collected for RNA extraction. RNA was extracted using the RNeasy Mini Kit (Qiagen). For residual genomic DNA digestion, RNase-Free DNase Set (Qiagen) was used and the isolated RNA was quantified spectrophotometrically (Nanodrop; Thermo Scientific). A panel of 31 selected genes of interest was investigated through Taqman gene expression array (ThermoFisher Scientific) following the manufacturer's

protocol by using the QuantStudio Real-Time PCR system. TATA-box binding protein and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta were used as housekeeping genes for data normalisation.

Culture in pellet

After three passages in culture, 4×10^5 cartilage cells expanded at standard and low density were centrifuged (5 min at 600*g*) and maintained for 14 days in expansion medium (Control) or in serum-free chondrogenic medium supplemented with 2.50 µg/mL amphotericin B, 1.25 mg/mL human serum albumin, 10 µg/mL insulin from bovine pancreas, 5.5 µg/mL human transferrin, 5 ng/mL sodium selenite, 0.5 mg/mL BSA and 4.70 µg/mL linoleic acid, 0.1 µM dexamethasone, 0.1 mM L-ascorbic acid-2-phosphate (all from Sigma-Aldrich) and 10 ng/mL TGF-β1 (Peprotech).

Histological analysis

Pellets were fixed with 10% neutral buffered formalin (Sigma-Aldrich), rinsed in PBS, placed in 70% ethanol, then embedded in paraffin and sectioned at 4 µm thickness. Sections were stained using haematoxylin and eosin (Carlo Erba) to evaluate cell morphology or alcian blue pH 2.5 (Sigma-Aldrich) to evaluate the glycosaminoglycan content. The pellet sections were also immunohistochemically assessed for type I and II collagens. Briefly, sections were blocked with PBS 2% BSA and then incubated with a rabbit monoclonal anti-collagen type I, 1:4000 (ab138492; Abcam) or rabbit polyclonal anticollagen type II, 1:100 (ab34712; Abcam) diluted in 5% PBS BSA, for 1 h at room temperature. The incubation was followed by washing with PBS buffer (PBS 1X + Tween20) and incubation for 30 min with 1:200 anti-rabbit IgG, (H + L) biotinylated secondary antibody diluted in PBS 2% BSA (VC-BA-1000-MM15; Vector Laboratories). After further 30 min of incubation with avidin/biotin-based peroxidase system, diaminobenzidine (Vector Labs) was used as a chromogenic substrate of the peroxidase reaction. Bern Score visual grading system was used to assess the chondrogenic differentiation [15]. Additionally, immunostained sections were scored for the presence of type I and II collagen by using a semiquantitative scoring system, as follows: 0 = *absence*, 1 = *mild*, 2 = *moderate*, 3 = *marked* [9].

Evaluation of the immunomodulatory potential of the cartilage cells

Human primary white blood cells were isolated from 10 healthy donors obtained from the local blood bank by Ficoll (Ficoll® paque Plus, GE Healthcare Bio-Sciences) density gradient separation. Monocytes were then separated by positive magnetic selection using CD14 microbeads (Miltenyi Biotec) [28]. After isolation, monocytes were counted and frozen in liquid nitrogen using FBS added with 10% (v/v) DMSO. After thawing, monocytes obtained from 10 donors were pooled together and 2×10^5 cells/cm^2 were seeded in each well of six-well plates in RPMI 1640 (Sigma-Aldrich) added with 100 U/mL penicillin, 100 µg/mL streptomycin, 200 mM glutamine (ThermoFisher Scientific) and 10% (v/v) hPL.

To differentiate monocytes towards macrophages, 20 ng/mL of macrophage colony-stimulating factor (M-CSF; Peprotech Inc.) was added to the medium [14, 31, 43, 44]. After 2 days, nonadherent cells were replated and medium was refreshed.

On Day 5, cartilage cells from five donors, which had been expanded under standard and low-density conditions, were thawed and seeded onto a polycarbonate membrane within a trans-well (Merck) at a density of 1.5×10^4 cells/cm^2. Cells were then incubated in expansion medium for 48 h to allow for attachment.

On Day 7, the chondrocyte-seeded trans-wells were transferred to the macrophage-seeded six-well plates to initiate the co-culture. Cartilage cells and macrophages were co-cultured for 2 days using a mix of macrophage and chondrocyte medium (1:1). At the end of the co-culture, macrophages were analysed by flow cytometry to assess the expression of M2a (CD206) and M2c (CD163) anti-inflammatory markers. Monocytes differentiated for 7 days into M0 macrophages and treated with 10^{-7} M dexamethasone (Sigma-Aldrich) were used as anti-inflammatory control.

Briefly, macrophages were washed, detached with nonenzymatic cell dissociation buffer (ThermoFisher) and centrifuged at 500*g* for 5. Macrophages were then treated with FcR Blocking Reagent (Miltenyi Biotec) for 10 min at 4°C to block unwanted binding of antibodies to human fragment crystallizable receptor and counted. Cells were stained with anti-human CD206-FITC (Clone 15-2; Biolegend) for M2a phenotype and anti-human CD163-PE (Clone GHI/61; Biolegend) for M2c phenotype. Data were acquired using a Cytoflex flow cytometer (Beckman Coulter).

Statistical analysis

For sample size calculation, CD146 was designated as the primary endpoint due to its significance. Through preliminary experiments, we determined that the average percentage of CD146+ cells in those expanded at standard density was approximately 10%. We hypothesised that cells expanded at low density would exhibit around 20% expression of this marker. Subsequently, we calculated the sample size for a

study with two groups and a continuous primary endpoint, setting α at 0.05 and power at 90%. The calculations resulted in five donors per each group.

Statistical analysis was conducted by GraphPad Prism v.8.0.2 (GraphPad Software). All values are expressed as the mean ± SD. Normal distribution of values were assayed by the Kolmogorov–Smirnov normality test. Paired comparisons between cells cultured at low density with respect to the standard-density cultured cells were performed by using a two-tailed *t* test. In the case of not normally distributed values, repeated measures were compared with the Kruskal–Wallis test with the Dunns' correction. Differences were considered as statistically significant for $p \leq 0.05$, while $p \leq 0.1$ were defined as trends.

RESULTS

Platelet lysate protein content

The protein content of the three batches of hPL is shown in Supporting Information S1: Table 1. Cytokines and other factors involved in inflammatory and immunomodulatory process, proteins involved in cell growth and proliferation and few factors participating in angiogenesis, apoptosis and matrix remodelling were detected. Gene Ontology enrichment analysis (Table 1) revealed the presence of many proteins putatively involved in cellular response to IFNγ and monocyte chemotaxis.

Cell characterisation

Low-density cultured cells formed colonies and showed a 1.7-fold higher number of doublings/day compared to the standard-density cultured cells (Figure 2a), corresponding to a higher amount of cells compared to the standard-density cultured cells at Day 14 (Figure 2b).

After 42 days, the β-galactosidase staining was clearly increased in standard-density cultured cells, indicating the presence of more senescent cells than in low-density cultured cells (Figure 2c). The low-density cultured cells showed an increase in CD146 (fourfold higher) and in CD166 (1.2-fold higher) expression compared to the standard-density cultured cells. Gene expression analysis confirmed the higher expression of both CD146 and CD166 in low-density cultured cells (Figure 2e).

Moreover, cells expanded at both densities exhibited a low immunogenicity profile resembling the one of mesenchymal stromal cells, being almost completely negative for MHC-II (Figure 2f).

Quantification of PRG4 expression

PRG4 was produced by both low-density and standard-density cultured cells, with no statistically significant differences in the signal intensity (Figure 3a). Gene expression analysis confirmed no differences in PRG4 expression between low-density and standard-density cultured cells (Figure 3b).

Gene expression of specific markers

The expression of a panel of genes in the low- and standard-density cultured cells is shown in Figure 3b. As above mentioned, significantly higher expression of the surface marker CD146 and a trend for a higher expression of the surface marker CD166 were observed in the cells cultured at low density with respect to the control cells. Gremlin 1 (GREM1), a marker involved in the downregulation of hypertrophy, was significantly upregulated in cells cultured at low density with respect to the cells cultured at standard density. Among the other relevant markers involved in cartilage hypertrophy, frizzled related protein (FRZB) and dickkopf WNT signaling pathway inhibitor 1 (DKK1) were slightly upregulated and bone morphogenetic protein receptor type 1B (BMPR1B) was slightly downregulated in low-density cultured cells in

TABLE 1 GORILLA of proteins involved in inflammation/immunomodulation; ontology:process.

Detected proteins	Process
4-1BB, ALCAM, B7-1, BCMA, BLC, CCL13, CCL14, CCL16, CCL17, CCL18, CCL19, CCL20, CCL21, CCL22, CCL23, CCL25, CCL26, CCL27, CCL28, CCL7, CCL8, CD14, CD30, CD40, CD40L, CEACAM-1, CXCL1, CXCL10, CXCL11, CXCL12, CXCL16, CXCL5, CXCL6, DR6, Eotaxin, Eotaxin-2, E-Selectin, Fcg RIIBC, G-CSF, GDF-15, GITR, GM-CSF, gp130, HVEM, I-309, ICAM-1, ICAM-2, ICAM-3, IFNγ, IL-1 R4, IL-1 RI, IL-10, IL-10 Rb, IL-11, IL-12p40, IL-12p70, IL-13, IL-13 R1, IL-13 R2, IL-15, IL-16, IL-17, IL-17B, IL17F, IL-17R, IL18BPa, IL-1a, IL-1b, IL-1ra, IL-2, IL-2 Ra, IL-2 Rb, IL-2 Rg, IL-21R, IL-23, IL-28a, IL-29, IL31, IL-4, IL-5, IL-6, IL-6R, IL-7, IL-8, IL-9, LIF, Lipocalin-2, L-Selectin, MCP-1, MCSF, MCSF R, MICA, MICB, MIF, MIG, MIP-1a, MIP-1b, MIP-1d, MST1, PECAM-1, PF4, PPBP, RANTES, SDF-1b, Siglec-5, SPP1, TNF RII, TNFa, TNFb, TNFSF14, Trappin-2, TREM-1, VCAM-1, XCL1, XEDAR	Monocyte chemotaxis, Cellular response to IFNγ

Note: *p* Value threshold 10^{-5}.

Abbreviations: GORILLA, Gene Ontology enRIchment anaLysis and visuaLizAtion tool; IFNγ, interferon gamma.

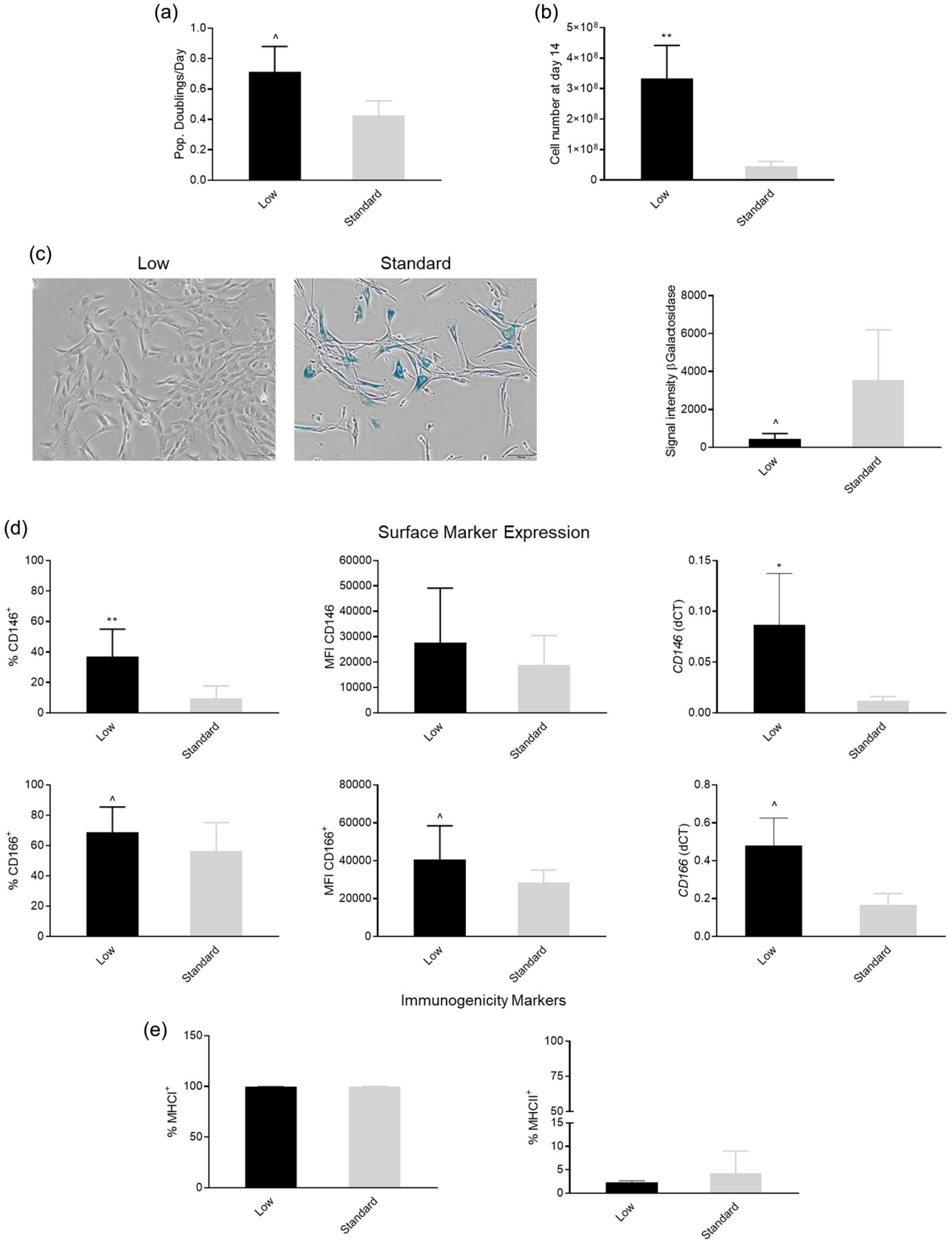

FIGURE 2 (See caption on next page).

comparison with the standard-density cultured counterpart. On the contrary, tissue inhibitor of metalloprotease (TIMP)2, a marker of cartilage remodelling, was significantly downregulated in the cells cultured at low density. A slight downregulation of other markers involved in cartilage remodelling such as TIMP1, matrix metalloproteinase (MMP)3 and MMP13 was also observed in low-density cultured cells.

Cell morphology and matrix deposition after pellet culture

After chondrogenic induction in pellet culture, the low-density cultured cells displayed the typical chondrogenic round morphology in comparison with the more elongated standard-density cultured cells. Moreover, the low-density cultured cells showed a higher matrix deposition appraised as higher cell distance, as well as the presence of a denser matrix compared to pellets from cells cultured at standard density, which often disintegrated when cut with a microtome. Concerning the quality of matrix, alcian blue staining revealed a higher amount of glycosaminoglycans in pellets from low-density cells. The Bern score was higher in pellets obtained from low-density cultured cells (Figure 4a). The immunohistochemical analysis revealed a superior presence of type I and type II collagen (Figure 4b) in pellets from cells cultured at low density that, together with glycosaminoglycans, are fundamental constituents of cartilage matrix. Type II collagen appeared more homogeneously distributed in low-density condition.

Immunomodulatory ability

The expression of macrophage surface markers after co-culture with cartilage cells is reported in Figure 5. The treatment of macrophages with dexamethasone-induced an increase in CD206 and CD163 expression, as compared to untreated macrophages. An increase of CD163 expression on the surface of M0 macrophages was more induced by the co-culture with low-density-cultured cells, than with standard density-cultured cells. Furthermore, akin to dexamethasone treatment, the cells cultured both at low and standard density induced an upregulation in the expression of the M2a marker CD206 in M0 macrophages.

DISCUSSION

In the current study, an innovative approach that involves the combination of low-density culture with the use of hPL to enrich a population of cartilage cells with CPCs is presented. This method aims to harness these cells for enhancing cell therapy-based approach for cartilage repair, with a specific focus on addressing diffuse chondral lesions in the early stages of OA.

The findings presented in this study demonstrate that low-density culture allows obtaining about 7.5-fold more cells than standard-density culture albeit starting from the same amount of cartilage. In addition, cells expanded in low-density condition in hPL do not age even after many passages, which makes them suitable for use in treating diffuse lesions where a large number of cells is needed. Noteworthy, their immunogenic profile suggests a potential allogenic use. In this view, as demonstrated by this study where cartilage cells were isolated from the hip joints of patients who had undergone hip replacement, waste cartilage tissue of OA patients could be seen as an incredible reservoir of precious cells. Envisioning the development of a therapeutic product, our findings suggest that CD146 and CD166 may serve as quality control markers, as they are increased in low-density cultured cells. Finally, from a functional point of view, the reduced expression of hypertrophic markers, the increased deposition of cartilage matrix and a promising immunomodulatory potential of the low-density cultured cells suggest the possibility of using these cells in an OA context.

The identification of novel therapeutic approaches for early OA patients suffering of diffuse chondral lesions is key to reduce and control the socioeconomic burden of this condition. Knee OA patients present a broad variation of response to therapies and the identification of effective interventions is challenging. Injective treatments involving hyaluronic acid, blood-derived products, such as platelet-rich plasma and platelet-rich growth factor, aim to modulate joint homeostasis. Similarly, injections of mesenchymal stem cell-based products, such as bone marrow-derived (i.e., bone marrow aspirate concentrate) or

FIGURE 2 Cell proliferation rate. (a) Data of population doubling/day and (b) cell number at day 14 of cartilage cells expanded at low and standard-density in human platelet lysate (hPL) at first passage (P1) ($n = 5$ donors); senescence analysis. (c) Representative images of β-galactosidase expression in cartilage cells cultured at low and standard density in hPL for 42 days and quantitative analysis of β-galactosidase signal intensity ($n = 4$ donors). ^$p \leq 0.1$. (d) CD146 and CD166 surface expression in cartilage cells expanded at low and standard-density in hPL at third passage (P3). Data are showed as mean and standard deviation (SD) of percentage of positive cells and as mean florescent intensity (MFI) ($n = 9$ donors) and as relative gene expression (dCT) ($n = 4$ donors). Histograms show the dCT of the surface marker genes. Fold change values between 1.5 and 2 for upregulation and between 0.5 and 0.8 for downregulation are showed ($n = 4$ donors). (e) MHC-I and MHC-II surface expression in cartilage cells expanded at low and standard density in hPL at third passage (P3). Data are showed as mean and SD of percentage of positive cells ($n = 5$ donors). ^$p \leq 0.1$, *$p \leq 0.05$, **$p \leq 0.01$.

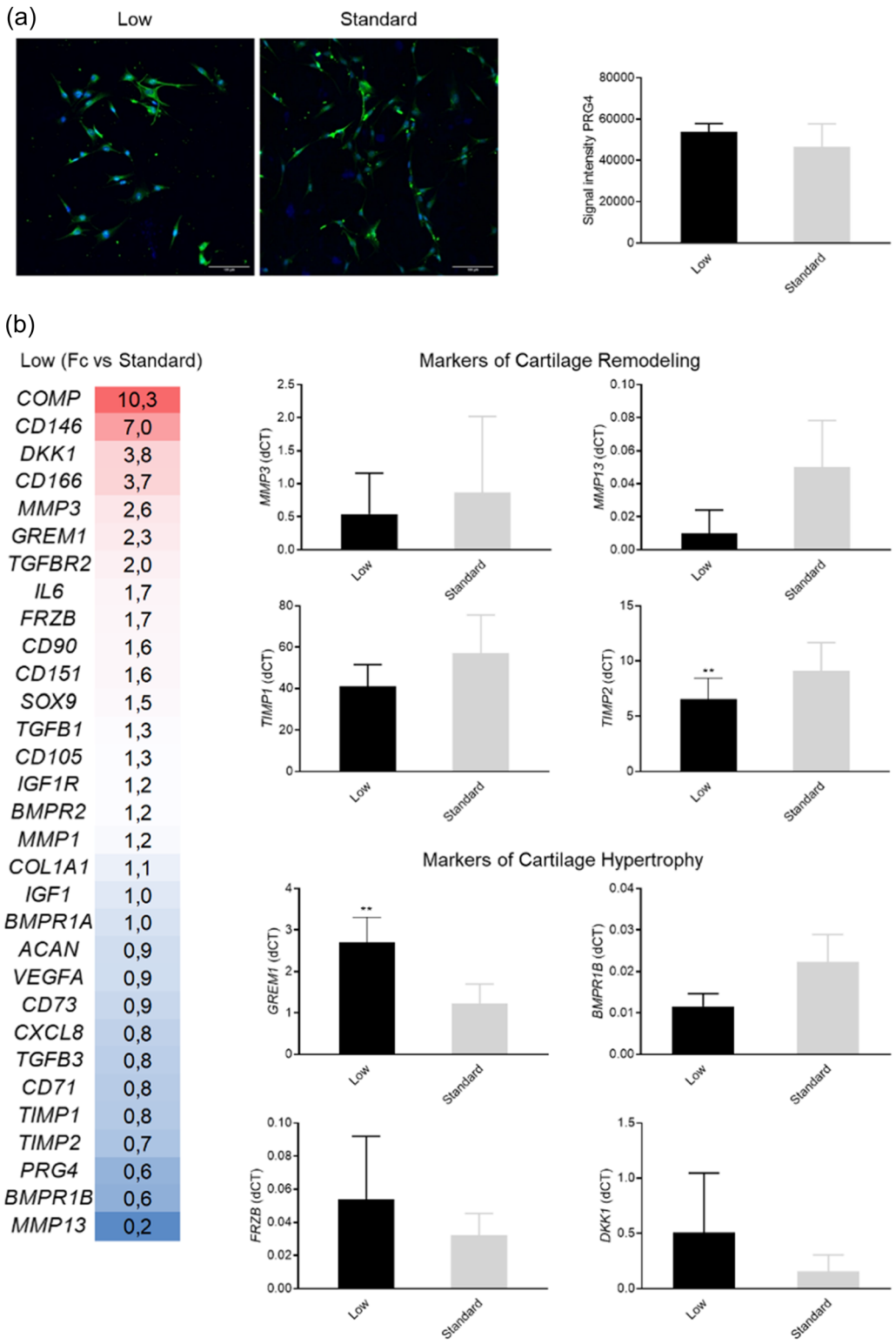

FIGURE 3 Expression of proteoglycan 4 (PRG4). (a) Representative confocal images of cells stained for PRG4 (green) and DAPI (blue), bars = 100 μm and quantitative analysis of signal intensity ($n = 4$ donors). Gene array of a panel of markers. (b) Data are reported in a heat map showing the fold change of the gene expression of cells cultured at low (L) in comparison with standard density (S) in human platelet lysate. Histograms show the dCT of the relevant genes involved in cartilage remodelling and hypertrophy. Relative gene expression (dCT) of genes with fold change values between 1.5 and 2 for upregulation and between 0.5 and 0.8 for downregulation are showed ($n = 4$ donors). $^{**}p \leq 0.01$.

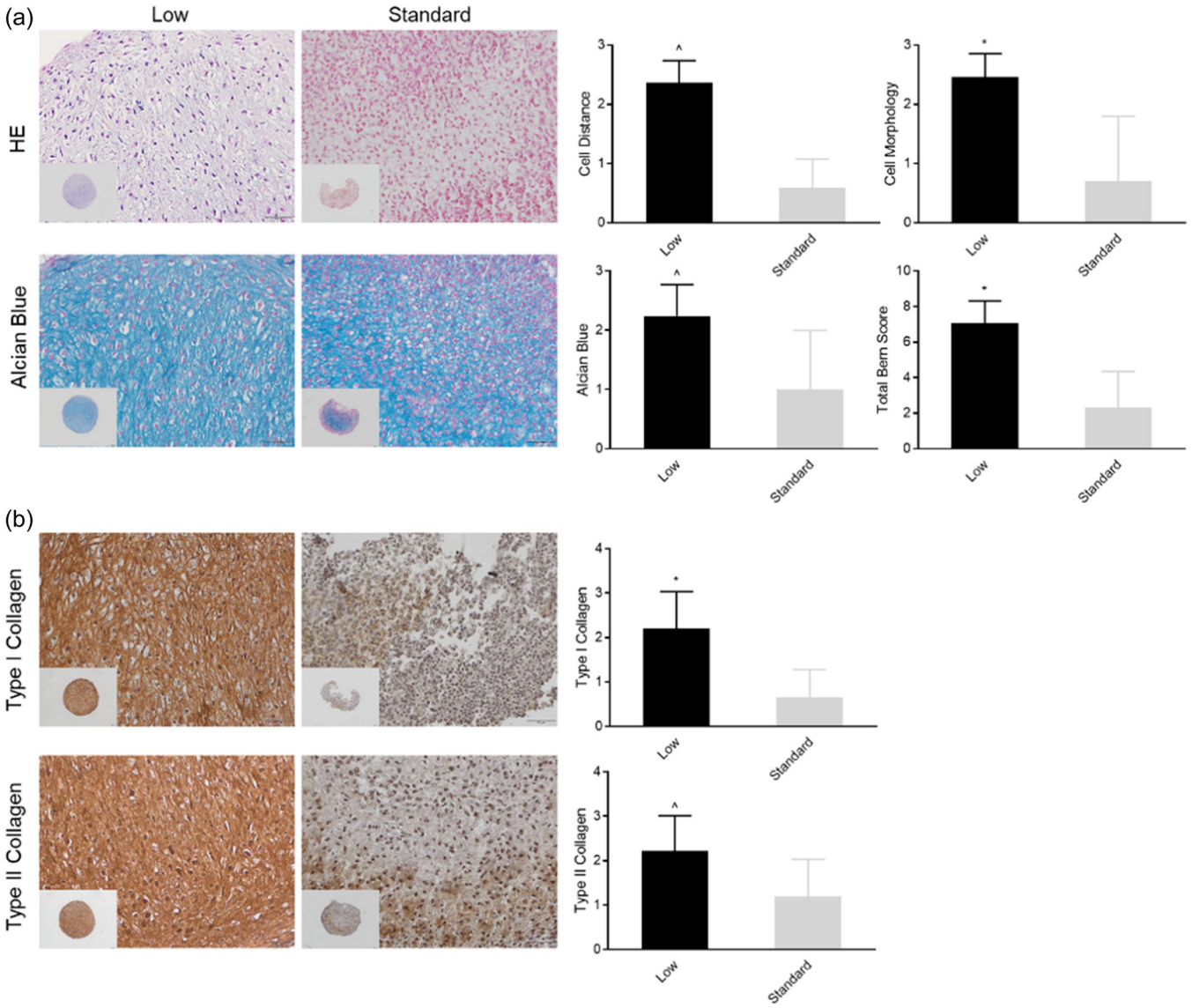

FIGURE 4 Extracellular matrix deposition. (a) Representative histological images and scores of cell morphology stained with hematoxylin and eosin (HE) and glycosaminoglycan content (Alcian Blue staining) of low or standard density cartilage cells in pellet cultured in chondrogenic medium. (b) Representative immunohistochemical images and scores of matrix quality stained for type I and II collagen in low density (L) or standard density (S) cartilage cells in pellet culture exposed to chondrogenic medium. bars of small pictures = 100 μm; bars of large pictures = 200 μm ($n = 5$ donors). ^$p \leq 0.1$, *$p \leq 0.05$.

adipose tissue-derived (i.e., stromal vascular fraction, microfragmented adipose tissue), primarily act on the inflamed environment. However, all these approaches fall short in promoting cartilage regeneration. On the other hand, ACI, designed for focal lesions, promotes cartilage regeneration but has limitations in addressing diffuse lesions in OA patients. In fact, the need for a high cell number and the lack of anti-inflammatory function reduce its application in these patients.

In this scenario, the first advantage of our protocol is that the cells expanded at low density show a superior proliferative ability in comparison with cells at standard density. This result is in line with the findings of a recent study where the features of human bone marrow mesenchymal stem cells (MSCs) cultured at 50 and at 4000 cells/cm^2 were compared [21, 29]. In our study, the higher proliferation rate observed in cells cultured at low density likely depends on the clonal isolation of cells, which allows the selection of CPCs based on their clonogenic activity. Additionally, the plethora of growth factors contained in hPL [4] is likely to synergistically contribute to enhance the proliferation rate. Indeed, platelet products have demonstrated a robust mitogenic response in both cartilage cells and CPCs released from cartilage chips. Specifically, it has been observed that CPCs effectively respond to the potent proliferative stimulus triggered by hPL [5, 20], as previously reported for primary human articular

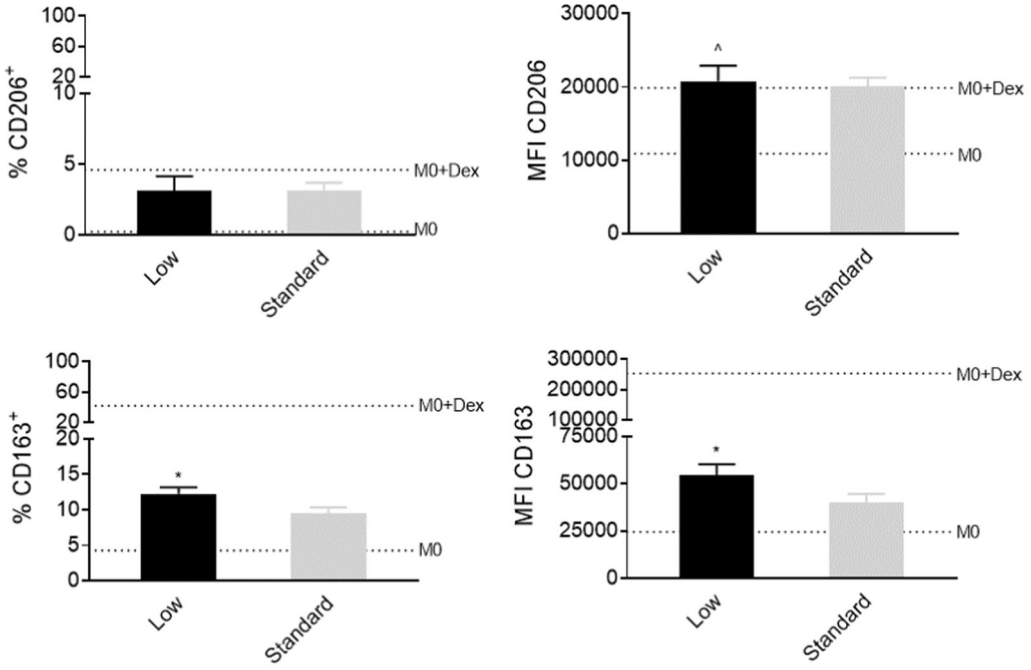

FIGURE 5 Macrophage surface marker expression. flow cytometry analysis for CD206 and CD163 performed on macrophages co-cultured with cells from five donors cultured at low (L) and standard density (S) in human platelet lysate. Data are reported as mean florescent intensity (MFI) ($n = 5$ donors). ^$p \leq 0.1$, *$p \leq 0.05$.

chondrocytes [32, 38]. The increase in CPC proliferation in hPL was reported as concomitant with senescence attenuation [5]. In our study, low-density cultured cells displayed inferior signs of senescence compared to cells cultured at standard density, over an extended expansion time frame. This result can be certainly ascribed to the positive effect of culturing cells at very low density, as recently reported by a study focusing on MSCs, which demonstrated that late passage senescence and MSC life span can be controlled by cell density [21]. However, also in this case, it can be hypothesised that low-density culture and hPL act synergistically in preserving cell phenotype. Indeed, platelet-derived products have been shown to induce senescence recovery and reproliferation in aged stem cells [48] and may very likely exert a similar effect on CPCs. This finding holds significant relevance as senescent cartilage cells are recognised contributors to OA development. Consequently, implanting cells already exhibiting signs of senescence is deemed unsuitable for fostering a pro-regenerative environment and generating high-quality cartilage tissue.

We investigated surface and matrix remodelling markers to further characterise the cells obtained with the devised expansion protocol. In our experiments, CD146, a traditional MSC surface marker, was found to be more expressed both at gene and protein level in cells expanded at low density as compared to standard-density cultured cells. This result may indicate that the low-density culture promotes an enrichment in CPCs, which are characterised by 'stem-like' features. Since a previous study has reported that this surface marker is expressed at higher levels on CPCs cultured in hPL in comparison with the same cells maintained in FBS [20], we can hypothesise that culturing cells in hPL contributes to enrich the cartilage cells population in CPCs. Of note, this marker is not only a typical MSC marker, but it is also associated with enhanced chondrogenesis [13, 45]. For this reason, although CD146 cannot be considered as an exclusive marker of CPCs, being expressed also by mature chondrocytes, we can envision to use the percentage of $CD146^+$ cells to establish a cut-off value for the release of high-quality cell batches for clinical use. Beyond CD146, we also found that CD166 is increased both at gene and protein level in low-density cultured cells compared to standard-density cultured cells. The association of CD146 and CD166 is of particular interest since these markers, together with $PDGFR\beta^+$, have been shown to characterise a population of CPCs with high chondrogenic potential [13]. Akin to CD146, also CD166 has already been identified as expressed at higher levels on articular chondrocytes cultured in hPL in comparison with the same cells maintained in FBS [5], suggesting that low-density culture and hPL may act synergistically in increasing the expression of this marker. Building on these considerations, CD166 should be considered as a marker worthy of attention whose expression should be assessed in a higher number of donors in cells to

confirm these preliminary observations. If the increase in CD166 will be confirmed as significant by increasing the sample size, we could then consider combining the expression of CD146 with that of CD166 as release test to identify the most promising cell batches for clinical use in cartilage repair.

Considering the clinical application of the cells selected with the developed protocol, we analysed their immunogenic profile demonstrating that low-density cultured cells display a profile that closely mirrors that of MSCs in terms of MHC-I and MHC-II expression. This finding suggests the potential application of these cells for allogenic use. Literature reports encouraging data regarding innovative trials involving one-step procedures utilising allogenic MSCs combined with autologous chondrons for the treatment of chondral lesions [40]. Notably, these trials demonstrated the absence of a foreign body response and of serious adverse reactions. Another study confirmed the safety and efficacy of the use of allogenic cartilage cells to treat knee cartilage lesions [36]. These studies indicate that the reasons dissuading clinicians and patients from the use of allogeneic cartilage cells are mainly cultural [16]. Indeed, the advantages far exceed the limitations, stemming from the possibility of carrying out a single procedure instead of the two procedures required by ACI, of being able to select 'super donors' to treat multiple patients and of obtaining a ready-to-use product.

The main limitation of this study lies in the lack of preclinical data regarding the therapeutic efficacy and the safety of the cells obtained with our protocol. However, the assessment of functional markers related to matrix production and remodelling provides encouraging insights. We found that PRG4 was expressed at the same levels in low-density and standard-density cultured cells, suggesting that PRG4 is not a relevant marker to identify the enrichment of CPCs in low-density culture conditions. However, the presence of this marker in low-density cultured cells is crucial since PRG4 is a key factor in joint lubrication, and its expression was found to be higher in CPCs than in MSCs both in two- and three-dimensional conditions [25, 42]. The comparison of the transcriptional profile of donor-matched cells expanded at low and standard density showed a higher expression of GREM1 along with a slight increase in FRZB and DKK1 in low-density cultured cells. Altogether these findings highlight the ability of low-density culture to promote an anti-hypertrophic phenotype. In fact, the BMP antagonist GREM1 and the Wnt signalling antagonists FRZB and DKK1 have previously been defined as natural brakes in hypertrophic differentiation of articular chondrocytes [7, 51]. Interestingly, in vivo, the expression of these markers has been inversely correlated to OA onset [24]. Moreover, a downregulation of such hypertrophy brakes has been obtained by mechanically challenging a cartilage-on-chip model with hyperphysiological compression, which finally resulted in induction of OA features [34]. Finally, BMPR1B was slightly downregulated in low-density cultured cells in comparison with standard-density cultured cells. This is one of the type 1 BMP receptors exploited to inhibit BMP-SMAD1 pathway involved in various physiological functions beyond chondrogenesis, as a potential strategy for mitigating or reversing OA traits [26, 35].

Even from a functional point of view, the cells expanded at low density in hPL present promising features in terms of regenerative potential and immunomodulatory activity. Indeed, cells obtained from low-density culture in hPL exhibit the capacity to produce a greater quantity of a higher-quality matrix. This matrix is notably enriched in glycosaminoglycans and collagens in comparison to that produced by cells cultured at standard density. In accordance with our results, hPL has already shown the ability to promote chondrogenic potential of CPCs both in vitro and in vivo [5, 38]. In particular, the ectopic cartilage formed in nude mice after implantation of CPCs, obtained from migration by cartilage discs and expanded in hPL, was type II collagen positive and type X collagen negative and thus ascribable to a hyaline-like cartilage without hypertrophy signs [5], in accordance with the results obtained in the present study. Moreover, low-density cultured cells showed the ability to switch macrophages towards anti-inflammatory and remodelling activities.

CONCLUSIONS

In summary, our study suggests that the expansion protocol reported in this manuscript efficiently generates a high number of cells with distinct features that make them suitable for treating diffuse cartilage lesions. These cells produce high-quality cartilage ECM and maintain a physiological chondrogenic phenotype in absence of senescence. Moreover, their immunomodulatory function is crucial for addressing cartilage lesions in the context of inflamed joint environment such as in early OA. Additionally, their low immunogenicity allows for allogeneic applications, enhancing cost-effectiveness and logistical efficiency, critical for a broader adoption of cell-based therapies.

AUTHOR CONTRIBUTIONS

Alessandra Colombini: Conceptualisation; methodology; formal analysis; investigation; data curation; writing—original draft preparation; supervision; project administration; funding acquisition. **Silvia Lopa**: Conceptualisation; methodology; formal analysis; investigation; data curation; writing—review and editing. **Francesca Libonati**: Methodology; investigation; data curation; writing—review and editing. **Giuseppe Talò**: Formal analysis; data curation; writing—review and

editing. **Katia Mareschi**: Methodology; resources; writing—review and editing. **Elena Marini**: Methodology; resources; writing—review and editing. **Laura Mangiavini**: Methodology; resources; writing—review and editing. **Vincenzo Raffo**: Methodology; investigation. **Matteo Moretti**: Writing—review and editing; supervision; funding acquisition. **Laura de Girolamo**: Writing—review and editing; supervision; funding acquisition. All authors have read and agreed to the published version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

the datasets presented in this study are stored in a data repository at the following link https://osf.io/wjre6/?view_only=0cf785e5d1304fd7847fdea9330ff1c1.

ETHICS STATEMENT

The study was approved by the local Institutional Review Board (clinical protocol approved by the San Raffaele Hospital Ethics Committee on the date 16 December 2020, registered under number 214/int/2020, for the use of waste biological material). Informed consent was obtained from all subjects involved in the study.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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