RESEARCH ARTICLE

# The impact of cryopreservation in signature markers and immunomodulatory profile of tendon and ligament derived cells

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## Abstract

Tendon and ligament (T/L) engineering strategies towards clinical practice have been challenged by a paucity of understanding in the identification and still poorly described characterization of cellular niches. Prospecting how resident cell populations behave in vitro, and how cryopreservation may influence T/L-promoting factors, can provide insights into T/L-cellular profiles for novel regenerative solutions. Therefore, we studied human T/L-derived cells isolated from patellar tendons and cruciate ligaments as suitable cellular models to anticipate tendon and ligament niches responses for advanced strategies with predictive tenogenic and ligamentogenic value. Our results show that the crude populations isolated from tendon and ligament tissues hold a stem cell subset and share a similar behavior in terms of tenogenic/ligamentogenic commitment. Both T/L-derived cells successfully undergo cryopreservation/thawing maintaining the tenogenic/ligamentogenic profiles. The major differences between cryopreserved and fresh populations were observed at the gene expression of *MKX*, *SCX*, and *TNMD* as well as at the protein levels of collagen type I and III, in which cells from tendon origin (hTDCs) evidence increased values in comparison to the ones from ligament (hLDCs, $p < 0.05$). In addition, low-temperature storage was shown to potentiate an immunomodulatory profile of cells, especially in hTDCs leading to an increase in the gene expression of the anti-inflammatory factors *IL-4* and *IL-10* ($p < 0.05$), as well as in the protein secretion of IL-10 ($p < 0.01$) and IL-4 ($p < 0.001$). Overall, the outcomes highlight the relevance of the cryopreserved T/L-derived cells and their promising immunomodulatory cues as in vitro models for investigating cell-mediated mechanisms driving tissue healing and regeneration.

**KEYWORDS**
cryopreservation, immunomodulatory cues/properties, ligament, tendon

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# 1 | INTRODUCTION

Tendons and ligaments share intricate and complementary relationships despite their different functional roles in the human body. Nevertheless, knowledge of the biology of tendon and ligament cell populations is limited as well as the understanding of their regenerative potential, both with relevant inputs to stimulate the tenogenic and ligamentogenic features of local stem cells and their role in immunomodulation (Lui, 2015b; G. Yang et al., 2013). Hence, there is a strategic interest in the study of tissue-specific cells and their niche interplay in the pathophysiology of T/L tissues and in the onset and progression of T/L disorders.

The native residing cells have a natural role in the maintenance and renewal of tissue composition and architecture, being also involved in the endogenous mechanisms of healing. Considering the epigenetic commitment to respond to tendon and ligament-specific requirements, resident cell populations could provide a more tissue oriented in vitro model for engineering strategies. Bi and coworkers (Bi et al., 2007) investigated tendon-derived cells from human hamstring tendon and mouse patellar tendons. A tendon stem/progenitor cells (TSPCs) population was identified in tendons of both species that possesses clonogenicity, self-renewal, and multipotency and a high expression of tendon associated markers, namely scleraxis (SCX), tenomodulin (TNMD), Comp and Tenascin C (TNC) (Bi et al., 2007). Ni et al. also demonstrated that tendon-derived stem cells used to produce a scaffold-free tendon could promote tendon repair in a rat patellar tendon injury (Ni et al., 2013). In another approach, Komatsu et al., implanted cell sheets made of tendon stem/progenitor cells in an Achilles tendon defect model. After 4 weeks the histological properties and collagen content were significantly improved, indicating that TSPCs sheets promote remodeling in the early stages of tendon healing (Komatsu et al., 2016).

A progenitor cell population was also found in ligament tissues (Lui, 2015a; Steinert et al., 2011). This subset of cells displayed mesenchymal stem cell (MSC) characteristics evidencing self-renewal capacity and multilineage differentiation in vitro, and a positive expression of CD73, CD90, CD105, CD106, CD146 and STRO-1 (Cheng et al., 2010; Steinert et al., 2011). Tendon-related markers, namely TNC, SCX and TNMD, have also been identified in ligament-derived cells (Lee et al., 2017). Similarly to tendon resident cells, ligament cells isolated from ACL tissues have been used in the fabrication of cell sheets. Ligament cell sheets implanted into a rat ruptured ACL resulted in an increased angiogenesis and collagen production demonstrating the potential of LDSCs in treatment strategies for ligament repair (Takayama et al., 2015). However, it is not clear whether resident cell populations respond specifically to fulfill local functional requirements or whether the close functional and structural relationship of tendon and ligament tissues could provide insights both on cellular signatures and shared phenotypic features. Therefore, we propose to study human T/L-derived cells (hLDCs and hTDCs) isolated from patellar tendons (PT) and cruciate ligaments (CLs) and compare their metabolic activity, morphology as well as their clonogenic and tenogenic/ligamentogenic potential.

Most of the cell-based studies use freshly isolated or expanded cells despite the fact that therapeutic approaches cannot fully depend on the immediate availability of cells. Cryopreservation stands as a clinically relevant procedure to enable the storage of living cells in extremely low temperatures for mid to long periods of time. One of the major challenges of cryopreservation methods is to ensure that cell functions and phenotype are maintained after thawing. The effect of cryopreservation has been investigated in cell recovery after thawing and in functional responses with controversial outcomes. Although cryopreservation of periodontal ligament stem cells in cell sheet constructions did not alter their viability, proliferative capacities, and multi-lineage differentiation (Li et al., 2017), other studies demonstrate that cryopreservation may compromise cell function, even if the loss of potency may be recovered with time (Hernandez-Tapia et al., 2020).

Another important aspect for healing and regeneration is the preservation of the natural immunomodulatory cues of implanted cells for a favorable interplay with the immune system. Cryopreservation revealed efficacy in the modulation of the inflammatory profile of adipose-derived stem cells (Othmani et al., 2019) with increased production of pro-inflammatory interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α), and decreased levels of anti-inflammatory interleukin 10 (IL-10). Nevertheless, in a work by Antebi et al. (2019) a significant inhibition of interleukin-1β (IL-1β), TNF-α, and interferon-γ (INF-γ) was observed in cryopreserved MSCs from the bone-marrow origin.

In this study, human resident cells of tendon and ligament tissues were isolated following mechanical dissociation and enzymatic methods, and characterized in terms of morphology, stemness potential, along with the expression of tendon-associated markers for up to 28 days in culture. As the mid- to long-term storage influence the recapitulation in vitro of the characteristics of natural tissues, we also investigated the behavior of cryopreserved T/L-derived cells after expansion in monolayer cultures, and their immunomodulatory profile at the gene and protein levels.

# 2 | MATERIALS AND METHODS

## 2.1 | Isolation of human tendon and ligament (T/L-) derived cells

T/L-derived cells (hLDCs and hTDCs, respectively) were isolated from surplus tissues of knee surgeries, obtained under previously established protocols with Hospital da Prelada (Porto, Portugal, P.I. No. 005/2019), and with informed consent of the patients. The written informed consent and the surgical related procedures were reviewed and approved by the Hospital Ethics Committee. A minimum of three independent donors per each processed anatomical area were considered: cruciate ligaments (CL) and patellar tendons (PT). Cells were used at passage 2 or 3.

After surgical collection, T/L samples were transported in a sterile solution of phosphate buffer saline (PBS; Sigma-Aldrich) with

2% antibiotic/antimycotic solution (A/A, Alfagene). Afterward, T/L samples were carefully washed in PBS and dissected. Each and every T/L sample was divided into three portions with similar dimensions, from which cells were isolated using gravitational or enzymatic procedures.

## 2.2 | Gravitational isolation

One of the portions was placed in a 75 cm$^2$ culture flask (Falcon) containing basic culture medium composed of α-MEM (α-MEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Alfagene) and 1% A/A solution. Cells were allowed to migrate from the explanted tissues into the adherent bottom of the culture flasks by gravity. After 4 days at 37°C in a 5% $CO_2$, cells were washed in PBS and medium exchanged. Cellular detection and morphology were assessed under an inverted microscope (Axiovert 40 CFL; Zeiss).

## 2.3 | Enzymatic isolation

The other two portions of the T/L samples were minced using a sterile scalpel. PBS drops were added in a continuous basis to keep a moist environment and reduce cell damage by mechanical forces. The excess of PBS was removed using a filtration system for 50 ml tubes (Falcon). Minced samples were collected into a 50 ml tube (Falcon), already containing an enzymatic solution of collagenase (C6885; Sigma-Aldrich) with $CaCl_2$ (1:1000; VWR) and BSA (1% w/v; Sigma-Aldrich). A ratio of 1:1 of minced tissue to the enzymatic solution was considered. Two concentrations of collagenase were used: (i) 0.01% collagenase solution overnight, and (ii) 0.1% collagenase solution for 1 h. To stabilize the temperature inside the tube and to assure the temperature was constant at 37°C, samples were incubated in a water bath under constant agitation (1200 rpm). After incubation, digested samples were filtered and centrifuged three times (1200 rpm) for 5 min. Immediately after the first centrifugation, samples were vigorously agitated for 2–5 s and centrifuged again.

After isolation procedures, hTDCs and hLDCs were cultured in basic medium composed of α-MEM, supplemented with 10% FBS, and 1% A/A solution.

## 2.4 | Expansion and cryopreservation of hTDCs and hLDCs

The cells were expanded in a basic medium and the influence of the cryopreservation process was investigated. Tendon and ligament-derived cells were cryopreserved in dimethyl sulphoxide (DMSO, N182-5, VWR) in FBS (1:9). Cell suspension was cooled down to −20°C for 4 h and then to −80°C for 24 h. Afterward, cells were moved into a liquid nitrogen tank (390-Vessel number SC004272-4, Biosystem 24) at −196°C for one month (Neves et al., 2017; Popa et al., 2013). Cryopreserved hTDCs and hLDCs were herein termed

"cryo," and the freshly isolated cells "fresh." Cryo and fresh cells were seeded at a density of $1 \times 10^5$ in six-well tissue culture plates (Falcon), cultured in basic medium and weekly evaluated. Cell cultures were investigated for morphology, gene and protein expression for up to 28 days before and after cryopreservation.

## 2.5 | Morphological analysis of isolated cells

hTDCs and hLDCs were daily monitored using a phase-contrast microscope (Axiovert 40; Zeiss). Representative sections were captured at ×20 magnification by a digital camera (PowerShot G11; Canon).

## 2.6 | Multilineage potential of hTDCs and hLDCs

For the assessment of the multilineage potential, hTDCs and hLDCs were cultured in osteogenic, chondrogenic or adipogenic differentiation media and the lineage differentiation was characterized by Alizarin Red (osteogenic), Safranin O and Toluidine B (chondrogenic) and Oil Red (adipogenic) stains. The protocols are detailed as Supporting Information (S1).

## 2.7 | Metabolic activity

The metabolic activity of hTDCs and hLDCs was weekly evaluated by MTS assay (Cell Titer 96® Aqueous Solution Cell Proliferation Assay; Promega) for 28 days. Cells were washed in PBS before a 3-h incubation in a mixture of FBS-free and phenol red-free medium (D2902; Sigma-Aldrich) and MTS solution (5:1 ratio) at 37°C and 5% $CO_2$. The absorbance was read at 490 nm (Synergy HT; Bio-TeK Instruments). Triplicates were made of each and every sample and a blank reading was performed.

## 2.8 | Flow cytometry analysis

To investigate stemness markers, cell adhesion molecules, and tendon-associated markers, hTDCs and hLDCs were detached using TrypLE Express (12605-028; Alfagene), centrifuged, and resuspended in fresh PBS. Cells were incubated with fluorochrome-conjugated antibodies for (i) stemness assessment: CD105FITC (1807; Bio-Rad), CD73PE (9010996; BD Biosciences), and CD90APC (9352389; BD Biosciences), and for (ii) adhesion molecules detection: CD29PE (42615; BD Pharmingen), CD44PE (9199202; BD Biosciences), and CD49fFITC (4319153; Thermo Fisher Scientific) for 20 min at RT protected from light.

Tenogenic/ligamentogenic-associated markers were investigated using nonconjugated antibodies for Scleraxis (ab58655), Tenascin-C (ab6393), Tenomodulin (ab81328), Collagen I (ab9039), and Collagen III (ab7778) all purchased to Abcam. Cells were incubated with primary antibodies for 30 min before incubation with Alexa Fluor 488

(2072687; Alfagene) for 30 min at RT protected from light. Finally, cells were resuspended in acquisition buffer (PBS with 1% neutral buffered formalin [INOPAT/05-k01009]), and data were acquired in a flow cytometer FACS Calibur (BD Biosciences). A minimum of 10,000 cells were acquired and analysis was performed using the Cell Quest software.

## 2.9 | mRNA extraction and real-time RT-PCR

Total RNA was isolated using the TRI Reagent® RNA Isolation Reagent (T9424; Sigma) following the manufacturer's instructions, and quantified spectrophotometrically (Nanodrop® ND-1000; Thermo Fisher Scientific). RNA was reverse-transcribed to complementary DNA via a qScript™ cDNA Synthesis Kit (Quanta Biosciences) using an Eppendorf Mastercycler® ep realplex gradient. The expression of tenascin C (*TNC*), collagen type I (*COL1A1*), collagen type III (*COL3A1*), decorin (*DCN*), tenomodulin *(TNMD)*, mohawk *(MKX)*, scleraxis *(SCX)*, cycloogenase-2 *(COX-2)*, tumor necrosis factor *(TNF-α)*, *IL-4*, *IL-6*, and *IL-10*, was assessed by real-time RT-PCR. The transcript expression of target genes was analyzed and normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). The $2^{-\Delta\Delta Ct}$ method was used and the results presented as relative gene expression. The primer sequences were obtained from Primer 3 software, and synthesized by MWG Biotech (Table S1).

## 2.10 | Immunolocation of T/L-related markers

After overnight fixation in 10% neutral buffered formalin (INOPAT), "fresh" and "cryo" cells were kept in PBS at 4°C until performing the immune location assays.

Cells were permeabilized with 0.025% Triton-X100 (Sigma-Aldrich) in PBS and then blocked with RTU Normal Horse Serum (RTU Vectastain Kit, PK-7200; Vector) to avoid unspecific reactions. Then, cells were incubated with the primary antibodies overnight at 4°C. Antibodies for collagen I (1:500; ab9039), collagen III (1:100; ab7778), Tenomodulin (1:100; ab81328), and Scleraxis (1:100; ab58655) were purchased to Abcam. Then, samples were incubated with a fluorescent secondary antibody (donkey anti-rabbit Alexa Fluor 488, 1:200, 2072687; Alfagene) for 1 h at RT before staining with 4,6-diamidino-2-phenyindole dilactate (DAPI, 5 μg/μl, D9564; Sigma-Aldrich) for 10 min and with Phalloidin-Tetramethylrhodamine B isothiocyanate (1:200, P1951; Sigma-Aldrich) for 20 min. Samples were observed under a microscope (Imager Z1m; Zeiss) and images acquired using a digital camera (AxioCam MRm5).

## 2.11 | Collagen and non-collagenous proteins quantification

The amount of collagen and non-collagenous proteins deposited by hTDCs and hLDCs, both in "fresh" and "cryo" conditions, was determined by Sirius Red/Fast Green Collagen Staining Kit (9046; Chondrex). After fixation in 10% buffered formalin (Bio Optica), cells were incubated for 30 min in the dye solution. After adding a dye extraction buffer, the OD values were read (Synergy HT; Biotek Instruments) at 540 and 605 nm.

## 2.12 | Release of soluble inflammatory factors

Inflammatory mediators released into the culture media were analyzed using an enzyme immunoassay kit for human IL-6 (900-K16; Peprotech), TNF-α (EK0525; Tebu-Bio), COX-2 (KA0323; Abnova), IL-4 (RAB0298; Sigma-Aldrich), and IL-10 (900-K21; Peprotech), according to the manufacturers' instructions.

## 2.13 | Statistical analysis

All quantitative results are expressed as the mean ± standard error of the mean (*SEM*). The statistical analysis was performed using GraphPad Prism software version 6. Two-way ANOVA was used, unless specified, followed by Bonferroni post hoc test for multiple comparison tests. Data were obtained from three independent experiments (*n* = 3) analyzed in triplicate. Statistically, differences between hTDCs and hLDCs are significant for *p* < 0.05. Different degrees of confidence were assessed and represented with the symbols *, γ, $, Ω for *p* < .05; **, α, θ, β, η for *p* < .01; ***, #, δ, ɜ, φ for *p* < .001; and ****, ****, ψ, &, ε, μ, ∞, κ, ρ, χ, €, π, τ for *p* < .0001, respectively.

## 3 | RESULTS

### 3.1 | Influence of isolation protocols on hTDCs and hLDCs phenotypic profile

The protocols for isolating a crude population of hTDCs and hLDCs aimed to select the method with the highest yield of cells. We defined cell density, that is, the number of adherent cells per tissue portion, as a parameter to determine the yield of tissue cultured adherent cells 6 days after isolation The highest yield of cells was observed after isolation with 0.1% collagenase solution, followed by gravity, and then by 0.01% collagenase solution (Figure 1a). Overall, cells isolated from CL tissues showed an increased number of adherent cells compared to cells obtained from PT samples (Figure 1a). The multi-lineage potential of T/L-derived cell populations was confirmed with Alizarin Red, Safranin O/Toluidine Blue, and Oil Red stains (Figure 1b).

### 3.2 | Impact of cryopreservation in hTDCs and hLDCs

The metabolic activity of both freshly isolated (fresh) and cryopreserved (cryo) cells increased with time in culture (Figure 2a). At each
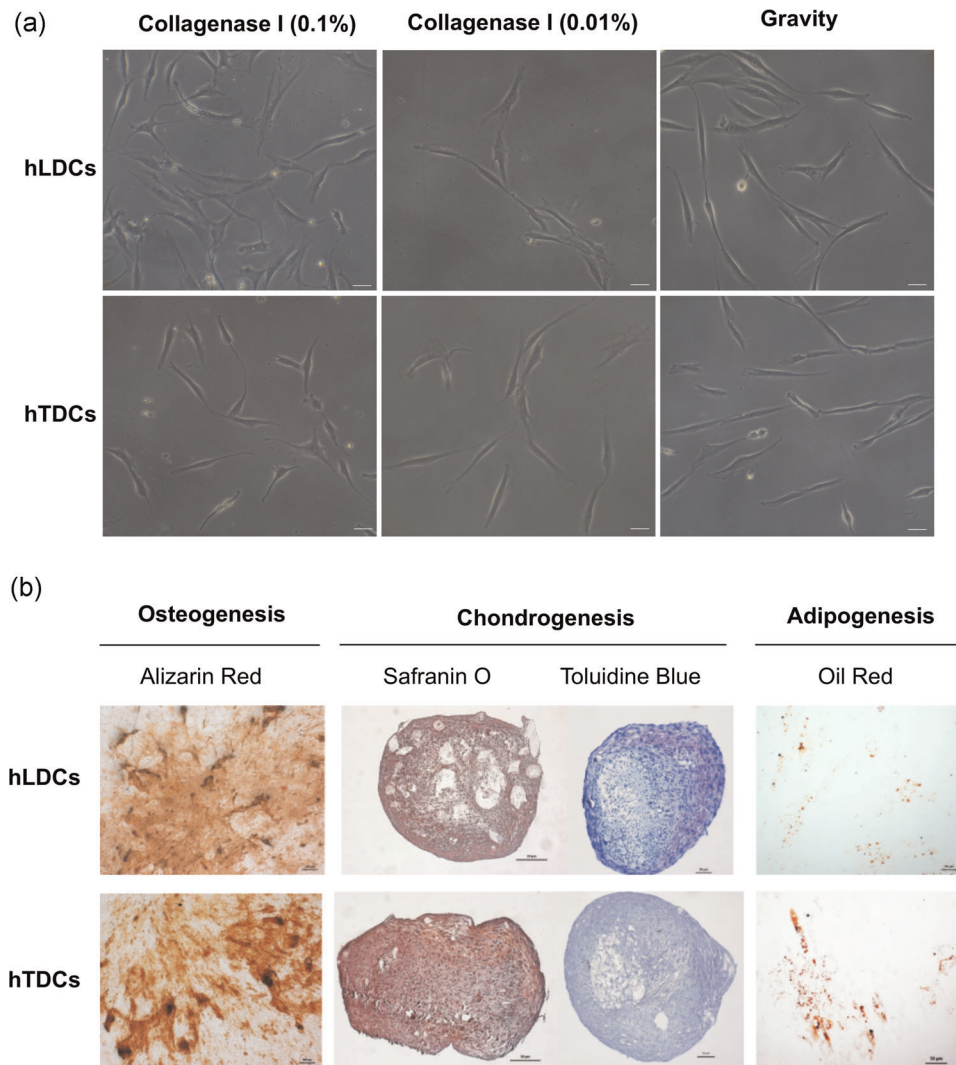
**FIGURE 1** Methods for isolating hLDCs and hTDCs and assessment of cell multilineage potential. (a) Morphological assessment of hLDCs and hTDCs 6 days after enzymatic digestion (collagenase solution) or tissue deposition (gravity). Magnification: ×20. (b) Microscopy photographs of Alizarin Red, Safranin O, Toluidine Blue, and Oil Red stains 21 days after the induction of hLDCs and hTDCs towards an osteogenic, chondrogenic, and adipogenic phenotype. hLDC, human ligament-derived cell; hTDC, human tendon-derived cell

time point, cryo cells have increased activity in comparison to freshly isolated cells ($p < 0.05$), independently of their origin. Additionally, hTDCs are more metabolically active than hLDCs after cryopreservation, which is particularly evident at Day 7 ($p < 0.0001$) and Day 21 ($p < 0.01$). Furthermore, hTDCs and hLDCs proliferated with the time in culture and tended to align in function of time (Figure 2b). After 14 days in culture, both cell populations naturally form lines of cells, that later become parallelly distributed (at Days 21 and 28) in freshly and cryopreserved conditions.

The stemness potential was investigated through the classical stemness markers CD73, CD90, and CD105 (Figure 2ci). The percentage of freshly expanded hTDCs expressing these markers (Figure 2ci) is inferior when compared to cryopreserved hTDCs. The opposite is observed for hLDCs, with a higher variation in the number of cryopreserved cells expressing these molecules.

Furthermore, T/L-derived cells were investigated for the cell–cell and cell–matrix mediating molecules, CD29, CD44, and CD49f (Figure 2cii). Nearly 100% of hTDCs and hLDCs, in both cryo and fresh conditions, express CD29. Despite some variation in freshly isolated hTDCs, more than 90% of hTDCs and hLDCs express CD44. With the exception of fresh hLDCs, which fully express CD49f, there is a wide variation in this marker in hTDCs (fresh and cryo) and hLDCs (cryo) populations.

Independently of the tissue source or culture conditions, over 80% of cells express SCX and TNMD, early and late (mature) tendon associated markers (Figure 2ciii), respectively. Conversely, the percentage of cells that express TNC, COL I, and COL III varied significantly ($p > 0.05$), and may relate to the fact that most of these molecules are extracellularly released to enable extracellular matrix (ECM) formation.
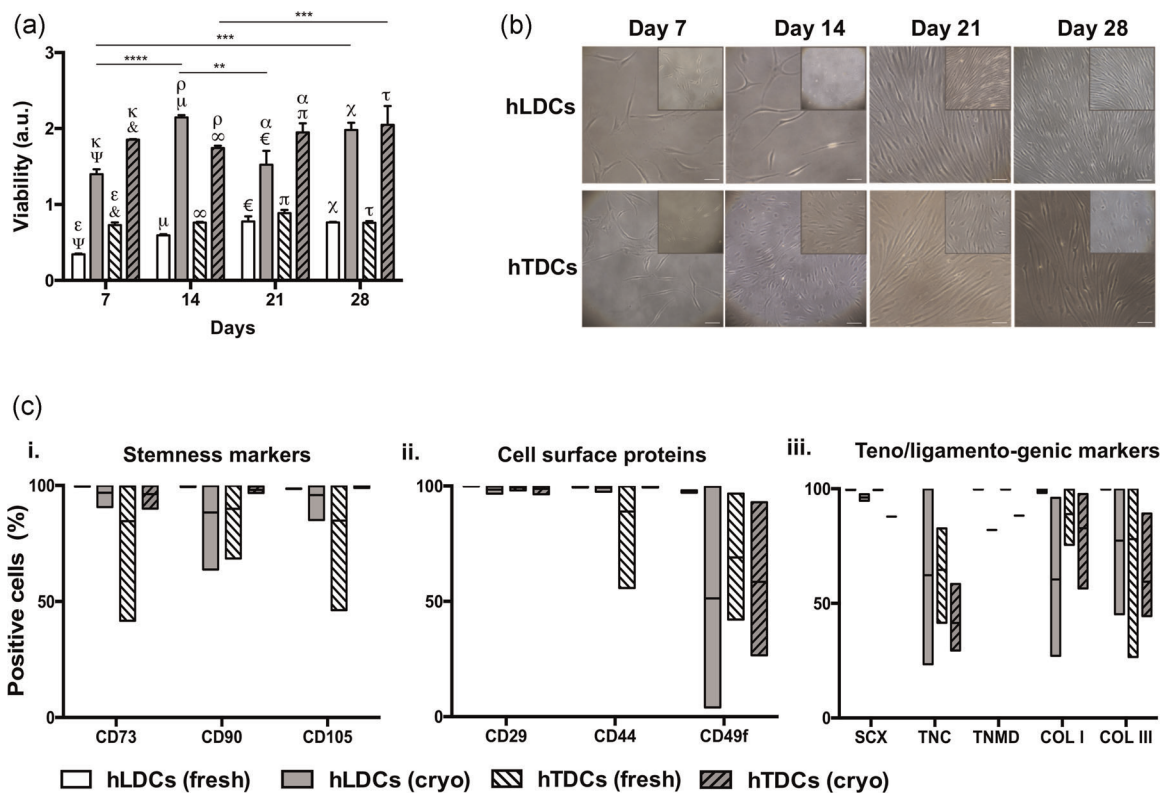
**FIGURE 2** Assessment of viability, morphology, and surface characterization of freshly isolated (fresh) and cryopreserved (cryo) cells up to 28 days in culture. (a) Cell viability analysis by MTS assay of ligament (hLDCs) and tendon (hTDCs) cells. Symbols denote statistical differences for $p < 0.05$; **, $\alpha$ for $p < 0.01$; *** for $p < 0.001$; and ****, $\psi$, &, $\varepsilon$, $\mu$, $\infty$, $\kappa$, $\rho$, $\chi$, €, $\pi$, $\tau$ for $p < 0.0001$, respectively. (b) Weekly morphological assessment of cryopreserved hLDCs and hTDCs expanded up to 28 days. Inset images represent freshly cells. Magnification: ×20; (c) Percentage of positive hLDCs and hTDCs expressing different surface markers. The percentage of positive cells is represented by mean, minimum, and maximum values

The expression of tenogenic/ligamentogenic genes tended to increase after cryopreservation/thawing when compared to freshly expanded cells (Figure 3a). SCX and TNMD increase in cryopreserved hTDCs, mainly at Days 14 and 28 (TNMD: $p < 0.05$ for Day 14 and $p < 0.01$ for Day 28; SCX: $p < 0.01$ for Day 14 and $p < 0.05$ for Day 28) (Figure 3a).

The relative expression of MKX, DCN and TNC is enhanced in hTDCs cryo when compared to hLDCs ($p < 0.05$) (Figure 3a). In particular MKX and DCN levels increased from Days 7 to 28 (MKX, $p < 0.0001$; DCN, $p < 0.001$), while TNC increased from Days 7 to 21 ($p < 0.05$). Overall, the gene expression associated to tendon/ligament markers was enhanced in both types of cells after cryopreservation, however, with higher values in hTDCs ($p<0.05$ in comparison to hLDCs cryo).

The matrix produced by the cells during culture was also investigated. The deposition of SCX and TNMD was also analyzed by immunofluorescence (Figure 3b). Consistent production of SCX and TNMD proteins was observed over time in both fresh and cryo cells.

The synthesis of a collagen-rich matrix by hTDCs and hLDCs was confirmed by collagen gene expression and collagen synthesis. COL1A1 and COL3A1 increased in hTDCs ($p<0.05$) after cryopreservation with the exception of COL3A1 at day 14 (Figure 4ai,aiii).

Despite the significant increment in COL1A1 of hTDCs cryo ($p < 0.05$) in comparison to hLDCs cryo, the immune-detection of COL I and COL III was similar during the time in culture (Figure 4aii,aiv). To infer the quality of ECM synthesized by the cells, both collagen, and non-collagenous proteins were quantified (Figure 4b), and showed to increase in hTDCs cryo ($p < 0.01$) with time of culture. Moreover, cryopreserved hTDCs synthetized higher quantities of both classes of proteins ($p < 0.05$ in comparison to fresh hTDCs, and to hLDCs).

## 3.3 | Immunomodulatory capacity of T/L-derived cells after cryopreservation/thawing

After confirming the post cryopreservation recovery of relevant functional cues, we investigated the immunomodulatory potential of hTDCs and hLDCs assessing relative gene expression and secreted forms of inflammatory mediators (Figure 5a,b). With a few exceptions at intermediary time points, the expression of COX-2, IL-6, and TNF-$\alpha$ diminished to levels below the baseline in hTDCs cryo (IL6: $p < 0.01$ at Days 7 and 21; TNF-$\alpha$: $p < 0.01$ at Day 14 and $p < 0.05$ at Days 21 and 28, both in comparison to hTDCs fresh) (Figure 5ai). In hLDCs cryo, the decrease in the expression of inflammatory genes
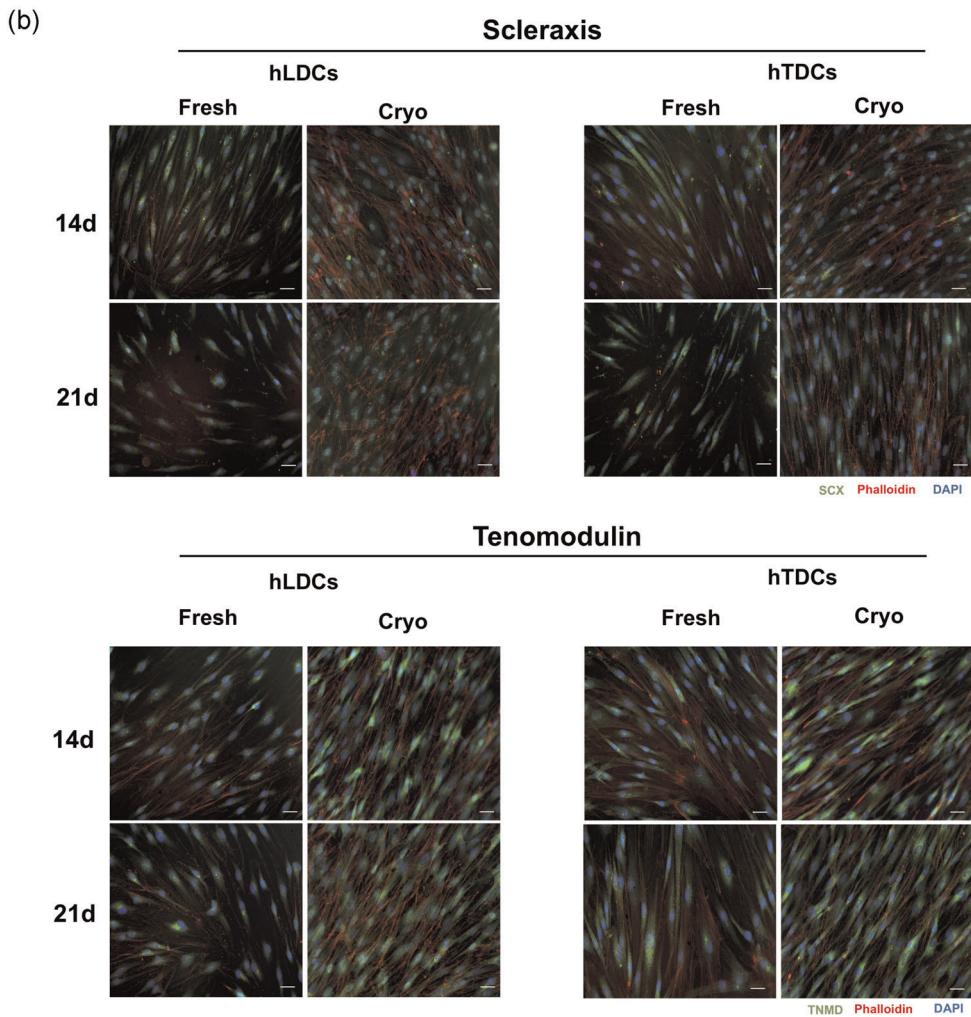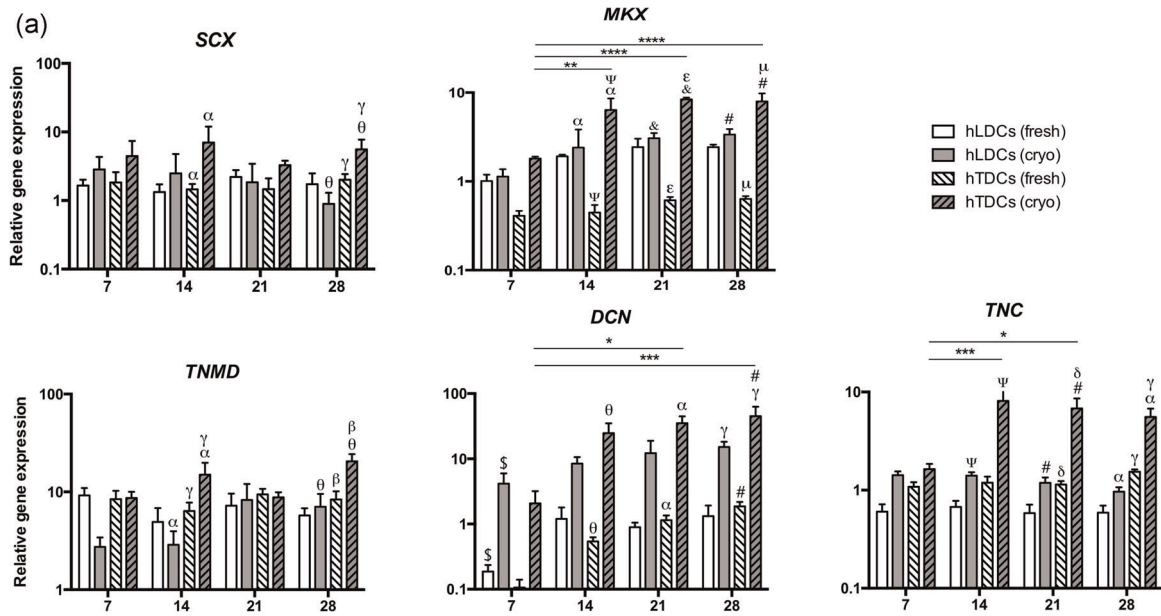
(a)



(b)



**FIGURE 3** (See caption on next page)

was only significant for *COX-2* ($p < 0.01$ at Days 7 and 21; $p < .05$ at Day 28 in comparison to hLDCs fresh) (Figure 5ai). The decrease in the pro-inflammatory gene expression is accompanied by an upregulation of the anti-inflammatory *IL-4*, especially in hTDCs, from Day 7 to Day 28 ($p < 0.0001$) and by a consistent expression of *IL-10* over time, with significantly increased levels in comparison to hLDCs ($p < 0.05$ at Day 14 and $p < 0.01$ at Day 28) (Figure 5aii).

We further investigated the translation of inflammatory genes into soluble molecules with paracrine action. The release of COX-2, TNF-α, IL-4, IL-6, and IL-10 was assessed 3 and 7 days after thawing, considering the timeline of acute inflammatory responses (Figure 5bi,bii). Three days after cryopreservation, hTDCs show a decrease in IL-6 ($p < 0.001$) and an increase in both IL-4 ($p < 0.001$) and IL-10 ($p < 0.01$), in comparison to hLDCs. Also, the release of COX-2, IL-6 and TNF-α was significantly reduced in hTDCs cryo ($p < 0.05$) by Day 7.

Overall, the results suggest that cryopreserved T/L-derived cells evidence an improved immunomodulatory response, resulting in higher expression and synthesis of anti-inflammatory factors, with a more pronounced effect in hTDCs.

# 4 | DISCUSSION

In this study, we hypothesized that resident cell populations from tendon and ligament tissues would be suitable for advanced bioengineering strategies, whose tenogenic/ligamentogenic responses and regenerative potential could be influenced by the cell source (PT or CL tissues), phenotypic commitment towards tenogenic/ligamentogenic lineages, and standard cryopreservation procedures.

Firstly we isolated hTDCs and hLDCs using different methods. The enzymatic digestion of the tissues with a 0.1% collagenase solution was the most efficient and favorable method to obtain the highest yield of resident cells. Cells isolated from CL tissues were in a higher number in comparison to PT cells, which is in agreement with studies reporting that the PT is more hypocellular than ligaments (Amiel et al., 1984).

Although the cells isolated by enzymatic digestion are typically heterogenic in nature, in this study we did not aim to separate the stem cells or other cell populations (e.g. mature tenocyte or ligamentocytes) from hTDCs or hLDCs. In fact, our goal was to explore resident cell populations as a crude population, which is expected to better mimic tendon/ligament niches responses and to provide more suitable cell models aiming at tendon and ligament regeneration.

The resident cell populations from both PT and CL evidenced a cellular subset that meets the stemness criteria of MSCs. The identification of a stem cell population in these tissues is expected and supported by the literature (Bi et al., 2007; Mos et al., 2007; Salingcarnboriboon et al., 2003; Tempfer et al., 2009; J. Zhang & Wang, 2010), and may assist the reparative mechanisms in living tissues.

The ability for cell machinery revival after cryopreservation has been investigated as a long-term storage procedure for cells and tissues (Antebi et al., 2019; Hernandez-Tapia et al., 2020). However, the impact of cryopreservation/thawing in the maintenance of stemness, tissue-specific functionalities, and immunomodulatory cues of hTDCs and hLDCs has not been reported.

The highly expression of stem cell markers observed in thawed hTDCs and hLDCs is likely due to a stem cell subset that is viable through the process cryopreservation/thawing. The presence of the adhesion molecules, CD29 and CD44, which mediate cell–cell and cell–matrix interactions as well as cellular migration capacity (Pittenger et al., 1999) also support this outcome, since these molecules are described to be expressed by MSCs (Suto et al., 2017), tendon progenitor stem cells (Lui, 2015a; C. Zhang et al., 2019), and ligament progenitor cells (Steinert et al., 2011).

Resident T/L-derived cell populations also show a high variation in the CD49f expression with cell sourcing and in both fresh/cryopreserved conditions. CD49f holds multiple roles including the enhancement of multipotency and stemness maintenance in MSCs (Kim et al., 2020). It has also been reported to participate in cell sensing of inflammatory cues (Z. Yang et al., 2015).

To support the identification of a T/L-phenotype, the gene expression of tenogenic/ligamentogenic-associated markers was investigated in freshly isolated and cryopreserved cells. After cryopreservation/thawing, the expression of *SCX*, *TNMD*, *MKX*, *DCN*, and *TNC* was enhanced, especially in hTDCs. Interestingly, in a previous study in the rat PT, no significant differences were detected in *SCX*, *COL I*, and *TNMD* between control- and cryo- groups (Dai et al., 2017). Apart from species, we hypothesize that the pool of stem cells available in hTDCs and hLDCs may be more active upon cryopreservation in response to the environmental stresses induced by the temperature changes and to the regain of cellular activities (Bissoyi et al., 2016; Cabrera et al., 2020).

After cryopreservation/thawing, the expression of *COL1A1* and *COL3A1* is induced in hTDCs, which is consistent with the higher collagen production in tendon tissues (Amiel et al., 1984; Gabriel, 2002; Little et al., 2014). We also observed an increase in

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**FIGURE 3** Gene expression and immunolocalization of tendon and ligament markers in freshly isolated (fresh) and cryopreserved (cryo) cells. (a) Real-time RT-PCR analysis of *SCX*, *MKX*, *TNMD*, *DCN*, and *TNC* for gene expression of hTDCs and hLDCs expanded up to 28 days. (b) Fluorescence microscopy images for the cellular localization of the tenogenic markers (green), scleraxis, and tenomodulin after 14 and 21 days in culture (fluorescence microscopy ×20, scale bar = 50 μm). Nuclei and cytoskeleton were counterstained with DAPI (blue) and phalloidin (red), respectively. Symbols *, γ, $ denote statistical differences for $p < 0.05$; **, α, θ for $p < 0.01$; ***, #, δ for $p < 0.001$; and ****, ψ, &, ε, μ for $p < 0.0001$, respectively. DAPI, 4,6-diamidino-2-phenyindole dilactate; *DCN*, decorin; hLDC, human ligament-derived cell; hTDC, human tendon-derived cell; *MKX*, mohawk; RT-PCR, reverse-transcription polymerase chain reaction; *SCX*, scleraxis; *TNC*, tenascin C; *TNMD*, tenomodulin
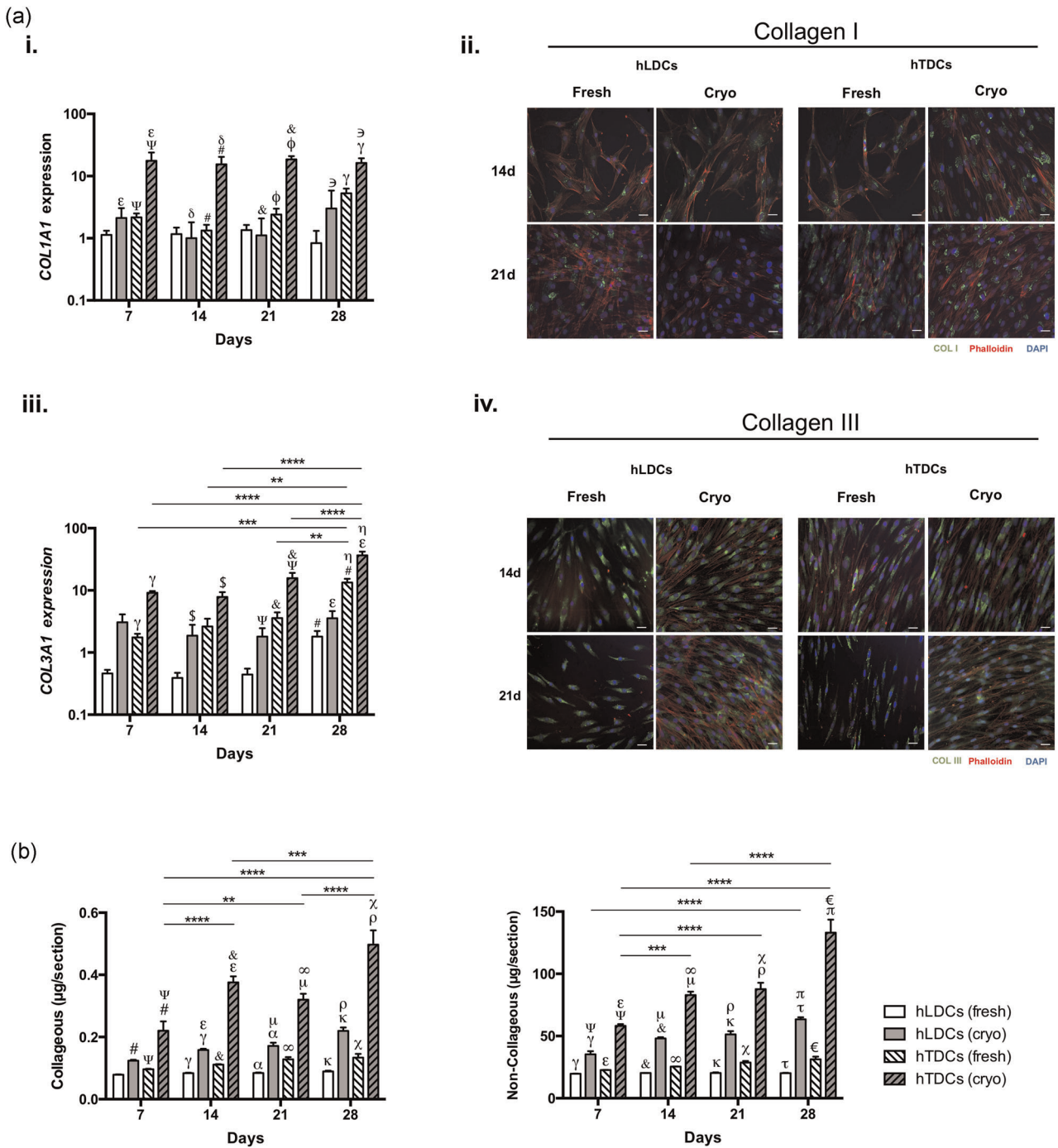
(a)

i.



ii.



Collagen I

iii.



iv.



Collagen III

(b)





**FIGURE 4** Evaluation of tendon extracellular matrix production of freshly isolated (fresh) and cryopreserved (cryo) cells. (ai, aiii) Real-time RT-PCR analysis of *COL1A1* and *COL3A1* for gene expression of hLDCs and hTDCs expanded up to 28 days. (aii, aiv) Immunolocation of collagen type I and III (green) after 14 and 21 days in culture (fluorescence microscopy x20, scale bar = 50 μm). Nuclei and cytoskeleton were counterstained with DAPI (blue) and phalloidin (red), respectively. (b) Quantification of collagen and non-collagenous proteins secreted by hLDCs and hTDCs using a Sirius Red/Fast Green Collagen Staining Kit. Symbols γ, $, denote statistical differences for $p < 0.05$; **, α, η for $p < 0.01$; ***, #, δ, ə, φ for $p < 0.001$; and ****, ψ, &, ε, μ, ∞, κ, ρ, χ, €, π, τ for $p < 0.0001$, respectively. COL1A1, Collagen type I; COL3A1, Collagen type III; DAPI, 4,6-Diamidino-2-phenyindole dilactate; hLDC, human ligament-derived cell; hTDC, human tendon-derived cell; RT-PCR, reverse-transcription polymerase chain reaction
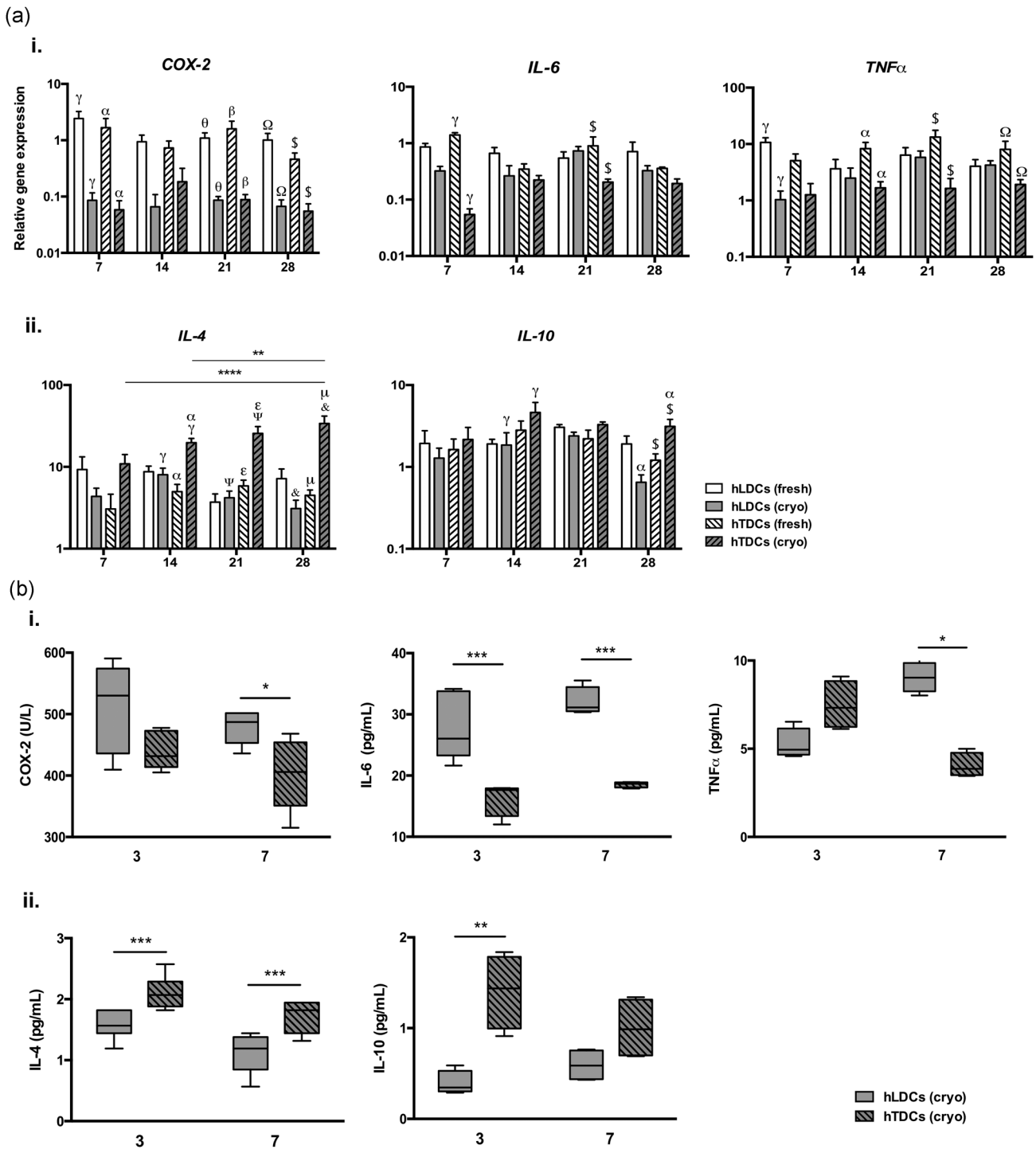
(a)

i.



ii.



(b)

i.



ii.



**FIGURE 5** Immunomodulatory profile of freshly isolated (fresh) and cryopreserved (cryo) hLDCs and hTDCs. Real-time RT-PCR analysis of (ai) pro-inflammatory factors, *COX-2*, *IL-6*, *TNF-α*, and (aii) anti-inflammatory factors, *IL-4*, *IL-10* for the gene expression of hLDCs and hTDCs expanded up to 28 days. Quantification of (bi) COX-2, IL-6, TNF-α, and (bii) IL-4 and IL-10, released to the culture medium after 3 and 7 days in culture. Statistically significant differences between freshly isolated and cryopreserved cells were only investigated for gene assessment. Symbols *, γ, $, Ω denote statistical differences for $p < 0.05$; **, α, θ, β for $p < 0.01$; *** for $p < 0.001$; and ****, ψ, ε, &, μ for $p < 0.0001$, respectively. *COX-2*, cycloogenase-2; hLDC, human ligament-derived cell; hTDC, human tendon-derived cell; *IL-4*, interleukin-4; RT-PCR, reverse-transcription polymerase chain reaction; *TNF-α*, tumor necrosis factor-α

the concentration of both collagen and non-collagenous proteins, the latter being necessary for the proper assembly of collagen molecules into a functional matrix (Screen et al., 2015).

Overall, the panel of markers assessed in cells isolated from T/L-tissues identified few cell-source divergences. These outcomes are in part expected due to the similarities in physiological and constituent features shared by tendons and ligaments, but may also be restrained by the limited knowledge of distinctive and exclusive biomarkers for tendon and ligament tissues.

Furthermore, we explored the effect of the cryopreservation/ thawing process in the expression and synthesis of inflammatory mediators as an enabling tool to study the contributions of resident tissue cells' for improved tissue healing. The interplay between implanted cells and host tissues influences the successful outcomes of cell-based strategies to stimulate repair and regeneration at the implantation site. Cryopreserved hTDCs evidenced a more promising response regarding immunomodulatory cues in relation to hLDCs, with higher expression and release of anti-inflammatory IL-4 and IL-10. These results are in accordance to other studies that demonstrated the influence of cryopreservation to the inhibition of TNF-$\alpha$ and IL-1$\beta$, and to an increase in IL-10 in human MSCs assessed 24 h post-thawing (Antebi et al., 2019).

Overall, the present work contributed with new perspectives to the characterization of T/L-derived cell populations, and on the impact of cryopreservation/thawing on desirable immunomodulatory communication, offering new possibilities to explore in vitro the intercellular networks between resident cell populations and immune cells driving healing events.

## 5 | CONCLUSIONS

The influence of the cryopreservation/thawing process in the expression and synthesis of inflammatory mediators reinforces the benefits of using cryopreserved T/L-derived cells to study the resident cells' endowment for in situ repair and for advancing regenerative strategies. The exploitation of the cryopreservation effect on cellular phenotype is pertinent in light of the global challenge of bench-to-bedside translational therapies. Cryopreserved cells can act as off-the-shelf living agents to assist therapeutic approaches, including immunotherapies. Furthermore, it is also important to assure the maintenance of signature markers that preserve cellular identity and epigenetic memory driving tendon and ligament healing.

In sum, this study provides relevant information on the predictive behavior of hTDCs and hLDCs as suitable in vitro models with immunomodulatory competencies to successfully engineer optimal therapeutic solutions towards T/L functional regeneration.

## CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

## AUTHOR CONTRIBUTIONS

Ana I. Gonçalves, Adriana Vinhas, and Márcia T. Rodrigues performed the experiments. Adriana Vinhas, Ana I. Gonçalves, and Márcia T. Rodrigues analyzed the data. Ana I. Gonçalves, Márcia T. Rodrigues, and Manuela E. Gomes provided guidance and/or senior supervision. Adriana Vinhas, Ana I. Gonçalves, and Márcia T. Rodrigues wrote the manuscript. Adriana Vinhas, Ana I. Gonçalves, and Márcia T. Rodrigues prepared the figures. All authors appraised the manuscript and figures.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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