Pulsed Electromagnetic Field Modulates Tendon Cells Response in IL-1β-Conditioned Environment

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ABSTRACT: Strategies aiming at controlling and modulating inflammatory cues may offer therapeutic solutions for improving tendon regeneration. This study aims to investigate the modulatory effect of pulsed electromagnetic field (PEMF) on the inflammatory profile of human tendon-derived cells (hTDCs) after supplementation with interleukin-1β (IL-1β). IL-1β was used to artificially induce inflammatory cues associated with injured tendon environments. The PEMF effect was investigated varying the frequency (5 or 17 Hz), intensity (1.5, 4, or 5 mT), and duty-cycle (10% or 50%) parameters to which IL-1β-treated hTDCs were exposed to. A PEMF actuation with 4 mT, 5 Hz and a 50% duty cycle decreased the production of IL-6 and tumor necrosis factor-α (TNF-α), as well as the expression of *TNFα*, *IL-6*, *IL-8*, *COX-2*, *MMP-1*, *MMP-2*, and *MMP-3*, while *IL-4*, *IL-10*, and *TIMP-1* expression increased. These results suggest that PEMF stimulation can modulate hTDCs response in an inflammatory environment holding therapeutic potential for tendon regenerative strategies. © 2019 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 38:160–172, 2020

Keywords: human tendon-derived cells; inflammation; IL-1β; cytokines; pulsed electromagnetic field

Tendons are musculoskeletal tissues with limited healing and regenerative capacity. The inadequate capacity to heal toward mechano-competent efficiency and the involvement of inflammation mediators in tendon injuries in general and tendinopathic conditions in particular demands for new approaches to improve healing and to encourage regeneration.[1–3]

Interleukin-1β (IL-1β) is a crucial pro-inflammatory mediator, secreted by immune cells and cells of some connective tissues at the inflammation site[4] with a pivotal role in both homeostatic and pathological mechanisms. Increased levels of IL-1β have been detected in tendons after prolonged mechanical loading (overuse), a known risk factor for tendinopathy.[4] The exogenous supplementation of IL-1β increases the expression of IL-6, COX-2, and MMPs[5–7] in tendon cells[8] and reduces the expression of tendon-associated genes such as scleraxis and tenomodulin and collagen 1 and 3 in injured tendon-derived progenitor cells (TPCs).[9] Blocking IL-1β has been also reported to resolve inflammation in several inflammatory diseases.[10] The influence of IL-1β in stimulating inflammatory cues that are known to be present in tendon pathologies has motivated its use as an in vitro model to study biological responses to inflammation-conditioned niches. The strategies for successful tendon treatments may rely in the creation of innovative approaches to control and guide inflammation toward proper healing envisioning contributions for tendon regeneration.

Magnetic platforms have emerged as a technological and versatile field of research to improve cell-based therapies and tissue engineering and regenerative medicine approaches[11–14] enabling the design of non-invasive and remote-actuated systems to trigger and modulate physiological processes, stimulating healing, and regeneration. The clinical potential of magnetic stimulation is enormous. Studies in the literature reported a positive effect of magnetic actuation to improve healing of non-unions and delayed unions for treatment of bone fractures and cervical spine fusion surgery.[15,16]

In recent years, pulsed electromagnetic field (PEMF) actuation, which works by emitting a pulsating, varying intensity, and frequency electromagnetic field, was reported to modulate the cell processes. PEMF was shown to decrease pro-inflammatory cytokines such as IL-1β and tumor necrosis factor-α (TNF-α) and to increase anti-inflammatory cytokines as IL-10 by tendon cells in vitro,[17–19] inhibited the synthesis of PGE2 and promoted anabolic activity of the chondrocytes.[17,19,20] In addition, PEMF induced a downregulation of TNFα and factor nuclear factor-κB (NF-κB) on macrophages, evidencing the potential of PEMF-actuated approaches for modulating inflammatory responses. Despite the promising outcomes and the remote actuation over inflicted tissues, the cellular and molecular mechanisms involved are still unveiled. Thus, we hypothesized that IL-1β can stimulate inflammatory cues in human tendon cells (hTDCs) and be used as a model for inflammatory-compromised tendon niches. We also hypothesize that the inflammatory response of IL-1β-conditioned hTDCs can be modulated toward a non-inflammatory/repair response exposing hTDCs to PEMF actuation.

In this study, hTDCs were stimulated with different concentrations of IL-1β and exposed to six combinations of PEMF parameters, namely magnetic field intensity (1.5, 4, or 5 mT), frequency (5 or 17 Hz), and duty cycle (10% or 50%), some of which referred to hold potential in tendon inflammation-related studies.[17–19] As

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inflammation mediators have been associated to tendinopathic conditions, recreating the inflammatory cues of pathological environments combined with the potential of PEMF to modulate the inflammatory profile in hTDCs anticipates new opportunities approaching tendon regeneration.

MATERIALS AND METHODS

Cells: Isolation and Expansion

The hTDCs were isolated from surplus tissue samples of the knee tendons and ligaments, namely patellar tendon, collected from four adult patients undergoing orthopedic reconstructive surgeries under protocols previously established with Hospital da Prelada (Porto, Portugal) and with informed consent of the patients. The healthy nature of the tissues was confirmed at the surgery room by the orthopedic surgeon. The hTDCs were isolated by enzymatic digestion of tendon samples with a solution of collagenase I from *Clostridium histolyticum*.[21–23] In brief, harvested samples were immersed in a sterile solution of phosphate-buffered saline (PBS; Sigma-Aldrich, Saint Louis, MO) with 2% antibiotic/antimicotic solution (A/A; Alfagene, Life Technologies Limited, Paisley, UK) before being minced and digested in collagenase (C6885; Sigma-Aldrich, Saint Louis, MO) with 2 M $CaCl_2$ (VWR, Darmstadt, Germany) (1:1,000) and 1% bovine serum albumin (BSA) (Sigma-Aldrich, Saint Louis, MO) for 1 h at 37°C under agitation. The digested samples were filtered, centrifuged three times at 1,200 rpm for 5 min, and the supernatant discarded. The hTDCs were expanded in α-MEM (A-MEM; Alfagene, Life Technologies Limited, Paisley, UK) supplemented with 10% fetal bovine serum (FBS) (Alfagene, Life Technologies Limited, Paisley, UK) and 1% A/A solution.

The multilineage differentiation potential and the cell markers panel for stemness profile had been determined and confirmed in previous studies using the described isolation protocol.[22]

Inducing Inflammatory Cues in hTDCs With IL-1β

The hTDCs, from passage 2-4, were incubated for 16 h with serum-deprived αMEM medium and plated at 10,000 cells/cm^2. Afterward, the hTDCs were treated with exogenous IL-1β (Alfagene, Life Technologies Limited, Paisley, UK) (0.01–1 ng/ml) and cultured for 1, 2, 3, or 7 days. The cells were assessed for cell viability, proliferation, and messenger RNA (mRNA) transcriptional levels of metalloproteinases. The media was also collected and screened for inflammatory cytokines.

Assessing the PEMF effect on hTDCs response to IL-1β stimulation

After pre-selecting the best concentration of IL-1β (1 ng/ml), hTDCs were IL-1β treated for 24 h before 1 h exposure to different PEMF conditions using a Magnetotherapy device (Magnum XL Pro; Globus Corporation, Italy) (Table 1). Afterward, the cells were cultured for 1, 2, 3, and 7 days and assessed for cell viability, proliferation, mRNA transcriptional levels of inflammatory-associated markers, and protein deposition. A non-PEMF group (static control) was kept in identical conditions but without PEMF stimulation.

hTDCs Characterization

Metabolic Activity and Proliferation

The metabolic activity and proliferation of hTDCs were evaluated by MTS assay (CellTiter96® AQueous One Solution; Promega Corporation, Madison) and Quant-It PicoGreen dsDNA assay kit (Thermo Fisher Scientific, Molecular Probes, Eugene, OR), respectively. For the MTS assay, the samples were washed with PBS and incubated with a mixture of serum-free medium without phenol red and MTS solution (5:1 ratio) for 3 h at 37°C and 5% CO_2, protected from light. The supernatant was then transferred to a 96-well plate and the absorbance read at 490 nm (Synergy™HT; BIO-TEK Instruments, Winooski, Vermont). Samples were prepared in triplicate and a blank reading (no cells) was performed.

For the dsDNA quantification assay, the samples were washed with PBS and then transferred to a microtube containing 1 ml of sterile ultrapure water and kept at −80°C until analysis. The samples were then thawed, sonicated, and analyzed at an excitation/emission wavelength of 485/528 nm. The samples and standards were made in triplicate.

Table 1. Experimental Scheme of PEMF Parameters on IL-1β-Treated hTDCs. The cells were exposed to one of the following conditions for 1 h

Frequency (Hz)	Intensity (mT)	Duty Cycle (%)
5 or 17	1.5	10
		50
	4	10
		50
	5	10
		50

Live and Dead Staining

The influence of PEMF on cell viability was also determined by live/dead assay. The cells were incubated for 20 min with calcein-AM (2 μg/ml), and propidium iodide (3 μg/ml), both from Life Technologies Limited, Paisley, UK. Afterward, the cells were washed with PBS and visualized under a fluorescence inverted microscope (Axio Observer; ZEISS, Göttingen, Germany).

Cytokine Analysis

The release of pro-inflammatory cytokines, IL-6 and TNF-α by hTDCs was determined at day 1 and 3 after treatment with IL-1β and PEMF stimulation in culture medium and stored at −80°C until analysis. The IL-6 (900-K16; Peprotech, Rocky Hill, NJ) and TNFα (EK0525; Tebu-Bio, Fremont, CA) concentrations were quantified using commercial enzyme human immunoassay kits, following the manufacturer's instructions.

RNA Isolation and Gene Expression Analysis

The total RNA was extracted using TRI reagent® RNA Isolation Reagent (T9424; Sigma-Aldrich, Saint Louis, MO) following the manufacturer's instructions. RNA was quantified using a Nanodrop® ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) at 260 nm. The first-strand complementary DNA was synthesized from 1 μg of RNA of each sample (qScript™ cDNA Synthesis Kit; Quanta Biosciences, Gaithersburg, MD) in a 20 μl reaction using a Mastercycler® ep realplex gradient S machine (Eppendorf, Hamburg, Germany).

The quantification of the transcripts was carried out by quantitative polymerase chain reaction (qPCR) using the PerfeCTA SYBR Green FastMix kit (Quanta Biosciences, Gaithersburg, MD) following the manufacturer's protocol, in a Real-Time Mastercycler ep realplex thermocycler (Eppendorf,

Hamburg, Germany). The primers were designed with Primer3 software (Supplementary Table S1) and synthesized by MWG Biotech. The $2^{-\Delta\Delta C_t}$ method was used to evaluate the relative expression for each target gene.[24]

The transcript expression of target genes was analyzed and normalized to the expression of endogenous housekeeping gene *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) and then to the samples collected at day 0 ($n = 3$).

Immunolocation Analysis

The cells were washed three times with warm PBS, before and after fixation with 10% (v/v) neutral buffered formalin overnight and kept in PBS at 4°C until usage. Subsequently, the cells were incubated with 0.025% Triton-X100 in PBS solution (Sigma-Aldrich, Saint Louis, MO) and the blocking step was performed using RTU Normal Horse Serum (RTU Vectastain Kit, PK-7200; Vector, Burlingame, California). The hTDCs cells were incubated overnight with connexin 43/GJA1, 1:100 (ab11370; Abcam, Cambridge, UK) and phospho-p44/42 MAPK, 1:200 (Cell Signaling Technology, Danvers, MA), diluted in antibody diluent with background reducing components (Dako, Santa Clara, CA) at 4°C, followed by 1 h incubation at room temperature with the secondary antibody (donkey anti-rabbit Alexa Fluor 488, 1:200; Alfagene, Life Technologies Limited, Paisley, UK). The samples were washed three times with PBS and stained with 4′,6-diamidino-2-phenyindole, dilactate (DAPI, 5 μg/μl, D9564; Sigma-Aldrich, Saint Louis, MO) for 10 min and with phalloidin-tetramethylrhodamine B isothiocyanate (Phalloidin), prepared according to the manufacturer's instructions (P1951, 1:200; Sigma-Aldrich, Saint Louis, MO).

All samples were observed under a microscope (Imager Z1m; Zeiss, Göttingen, Germany) and images acquired using a digital camera (AxioCam MRm5). A minimum of two wells per sample, condition and endpoint were analyzed. Three independent experiments were investigated for protein detection by immunofluorescence.

Western Blot Analysis

The total cell lysates were prepared using RIPA buffer (Sigma-Aldrich, Saint Louis, MO) supplemented with protease inhibitor cocktail (Sigma-Aldrich, Saint Louis, MO). The samples were centrifuged for 15 min at 14,000 rpm at 4°C and the supernatants collected. The protein content was evaluated using the BCA protein assay kit (Alfagene, Life Technologies Limited, Paisley, UK) according to the manufacturer's instructions.

The protein extracts of 15 μg were resolved in 10% sodium dodecyl sulfate-polyacrylamide gels, followed by semi-dry transfer to Amersham™ Protran® Western blotting membranes (nitrocellulose; Sigma-Aldrich, Saint Louis, MO), using a Pierce Power Station (Thermo Fisher Scientific). The membranes were blocked with 5% BSA in Tris-buffered saline with Tween 20 (Sigma-Aldrich, Saint Louis, MO) (TBS-T) and incubated with rabbit anti-phospho-p44/42 MAPK (Erk1/2) (1:1,000) (9102; Cell signaling Technology, Danvers, MA) and anti-α smooth muscle actin (1:2,000) (ab32575; Abcam, Cambridge, UK) antibodies for 1 h at room temperature under mild agitation. The membranes were washed three times with TBS-T and then incubated with a secondary antibody (1:2,000) (Anti-Rabbit IgG Alkaline Phosphatase antibody, A9919, Sigma-Aldrich, Saint Louis, MO) for 1 h at RT. A colorimetric AP substrate reagent kit (1706432; BioRad, Hercules CA) was used for color development.

Statistical Analysis

The results are expressed as mean ± standard error of the mean (SEM). The statistical analysis was performed using GraphPad Prism6 software. Data were obtained from three independent experiments ($n = 3$) analyzed in triplicate, and evaluated by two-way analysis of variance followed by multiple comparison tests. A difference was considered significant with a confidence interval of 95% for different degrees of confidence, $p < 0.05$, $p < 0.01$, $p < 0.001$, and $p < 0.0001$. A multifactorial analysis was also performed to investigate the impact of the PEMF, IL-1β treatment and times, as well as their potential interactions (PEMF × IL-1β, PEMF × time, IL-1β × time) in the expression of individual genes, using SPSS software (vs23) with a degree of confidence of $p < 0.05$.

RESULTS

Assessment of Human Tendon-Derived Cells Response to an IL-1β-Induced Environment

The metabolic activity and proliferation of hTDCs treated with different concentrations of IL-1β (0.01, 0.1, and 1 ng/ml) increased between day 1 and 7, and tended to increase with increasing concentrations of IL-1β (Fig. 1A).

The MTS values showed an increase 3 and 7 days after treatment with 1 ng/ml of IL-1β in comparison with all other concentrations ($p < 0.05$) and to non-treated hTDCs (control without IL-1β, $p < 0.05$). Also, dsDNA increased beyond 3 days in hTDCs treated with 1 ng/ml of IL-1β (in comparison with non-treated hTDCs, $p < 0.001$).

In Figure 1B, secreted IL-6 and TNF-α increased in IL-1β treated hTDCs (1 ng/ml of IL-1β, $p < 0.0001$) in an IL-1β concentration-dependent manner. Moreover, a significant increase in metabolic activity, gene expression, and release of cytokines was observed upon hTDCs stimulation with 1 ng/ml of IL-1β ($p < 0.05$), (Fig. 1C and D). The expression of *MMP-1*, *MMP-2*, and *MMP-3* increased for 1 ng/ml of IL-1β $p < 0.05$ (Fig. 1C).

Altogether, the 1 ng/ml of IL-1β stimulated more effectively inflammatory cues in hTDCs, being selected as the most promising condition to be investigated in the following PEMF-actuated experiments.

Assessment of PEMF Parameters on IL-1β-Treated hTDCs

After the establishment of the optimal concentration of IL-1β (1 ng/ml) to stimulate inflammatory cues on hTDCs, we investigated PEMF actuation to modulate the response of IL-1β treated hTDCs. Thus, the effect of magnetic field parameters namely strength, frequency, and duty cycle were investigated on IL-1β treated hTDCs.

Effect of PEMF Actuation in Metabolic Activity and Cell Content of IL-1β Treated hTDCs

Having in consideration previous studies,[18–20] different PEMF combinations of strength (1.5, 4, and 5 mT), frequency (5 and 17 Hz), and duty cycle (10% and 50%) were assessed (Table 1) on IL-1β treated hTDCs. The metabolic activity of IL-1β treated hTDCs tended to increase with time in culture for PEMF associated with both frequencies studied, 5 and 17 Hz (Figs. 2A and 3A). However, a decrease in the metabolic activity

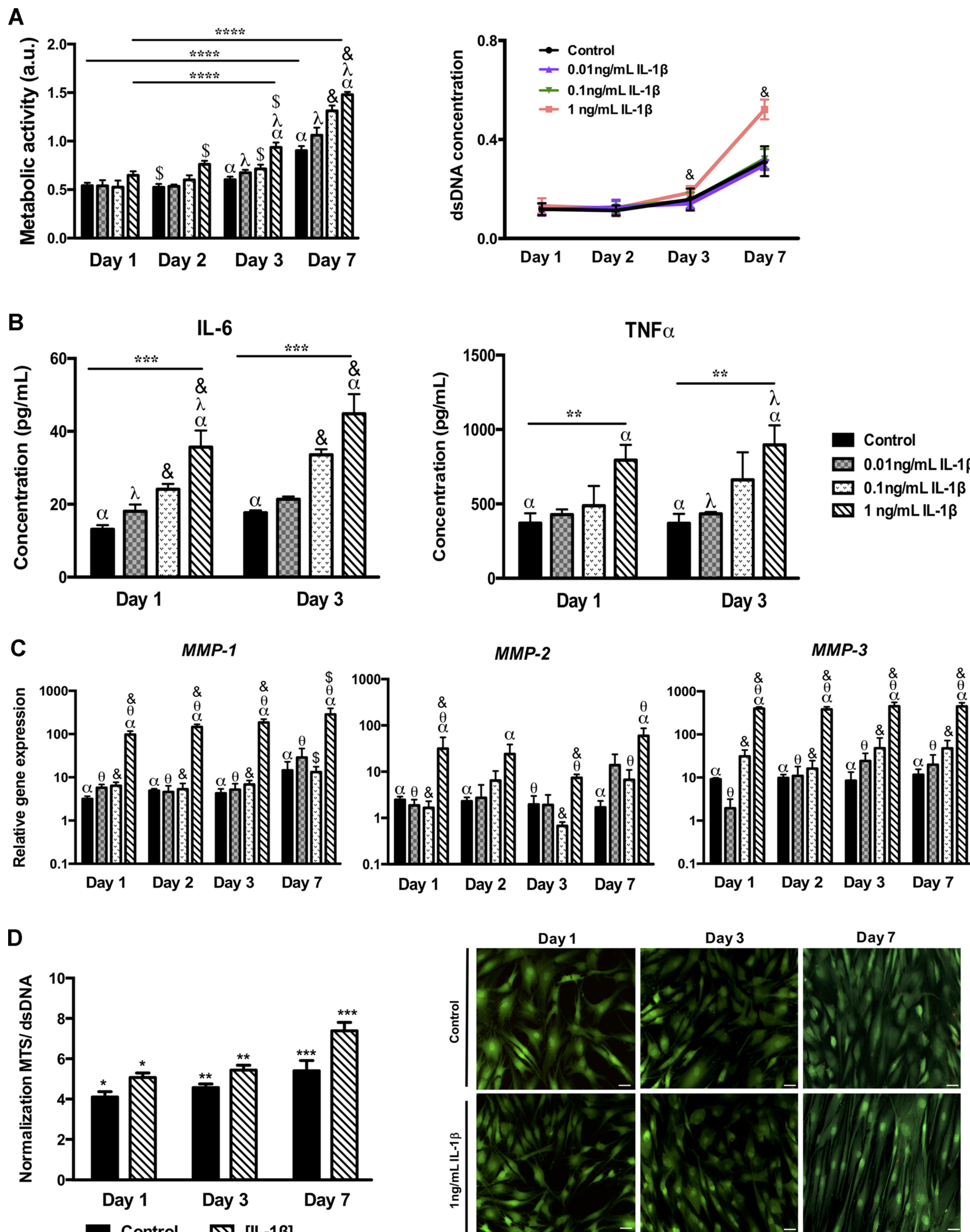

Figure 1. Effect of interleukin-1β (IL-1β) concentration (0.01, 0.1, and 1 ng/ml) on human tendon-derived cells (hTDCs) for up to 7 days in culture. (A) The metabolic activity was determined by the MTS assay and the cell content was determined by the PicoGreen assay. (B) hTDCs relative expression of genes involved in extracellular matrix (ECM) remodeling: *MMP-1*, matrix metalloproteinase 1; *MMP-2*, *MMP-3*. (C) Profile of IL-6 and tumor necrosis factor-α (TNF-α) cytokines secreted by hTDCs treated with different concentrations of IL-1β after 1 and 3 days in culture. (D) Cell metabolic activity, viability, and morphology of hTDCs cultured in the presence of 1 ng/ml of IL-1β. Ratio of metabolic activity and dsDNA content of hTDCs after 1, 3, and 7 days in culture. The morphology and viability of hTDCs treated with 1 ng/ml of IL-1β was also assessed 1, 3, and 7 days after pulsed electromagnetic field (PEMF) actuation by live and dead staining. Viable and dead cells are represented in *green* and *red*, respectively (fluorescence microscopy ×20, scale bar 50 μm, merged images). Results are presented as mean ± standard error of the mean (SEM) ($n = 3$, experimental replicates from three biological replicates) using two-way analysis of variance (ANOVA) followed by multiple comparisons tests. Control condition refers to hTDCs without IL-1β treatment. Symbols and letters denote statistically significant differences with different degrees of confidence. Symbols *, $ denote statistical differences for $p < 0.05$; **, λ for $p < 0.01$; ***, &, θ for $p < 0.001$; and ****, α for $p < 0.0001$. [Color figure can be viewed at wileyonlinelibrary.com]

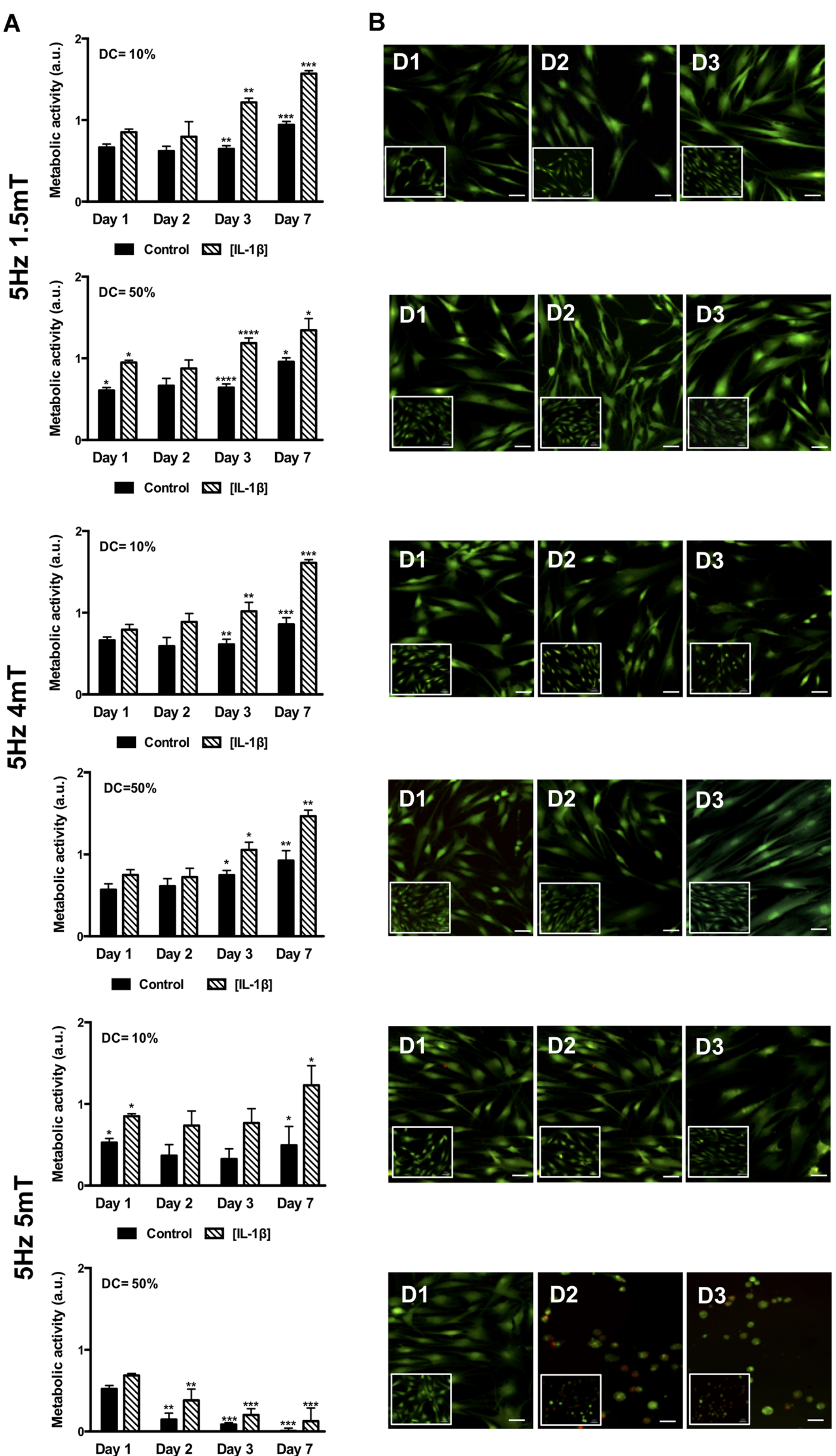

Figure 2. Effect of 5 Hz pulsed electromagnetic field (PEMF) in human tendon-derived cells (hTDCs) treated with interleukin-1β (IL-1β) up to 7 days in the culture. (A) Metabolic activity of hTDCs, with a variation of intensity (1.5–5 mT) and duty cycle (10–50%, DC,%). (B) Live and dead staining for viable (*green*) and dead cells (*red*) in hTDCs cells 1, 3, and 7 days (D1, D3, and D7), respectively, after PEMF actuation (fluorescence microscopy ×20, scale bar 50 μm, merged images). Insets are representative images of hTDCs without IL-1β treatment (control). Results are presented as mean ± standard error of the mean (SEM) ($n = 3$), three experimental replicates from three biological replicates) and data analyzed using two-way analysis of variance (ANOVA) followed by multiple comparisons tests (GraphPad Prism). Control condition refers to hTDCs without IL-1β treatment. Statistically, significant differences are shown with different degrees of confidence: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$ in comparison with controls. [Color figure can be viewed at wileyonlinelibrary.com]

(Figs. 2A and 3A) and viability (Figs. 2B and 3B) of hTDCs was observed after exposure to a 5 mT PEMF with a 50% duty cycle, independently of the frequency applied.

Considering the detrimental exposure of hTDCs to 5 mT strength PEMF combined with a 50% duty cycle, this condition was eliminated from the PEMF assessment of IL-1β treated hTDCs.

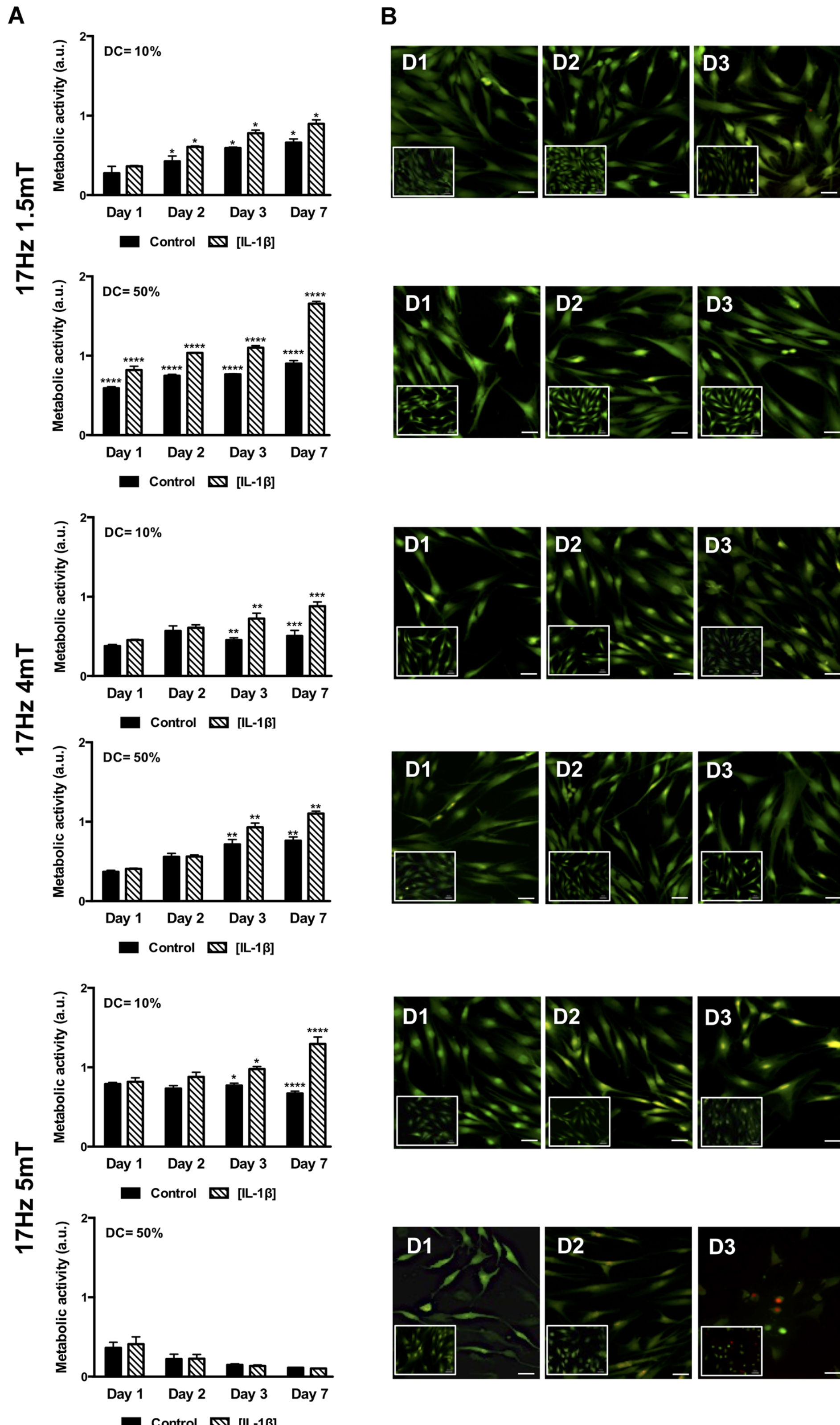

Figure 3. Effect of 17 Hz pulsed electromagnetic field (PEMF) in human tendon-derived cells (hTDCs) treated with interleukin-1β (IL-1β) up to 7 days in the culture. (A) Metabolic activity of hTDCs in function of intensity (1.5–5 mT) and duty cycle (10–50%, DC,%) of PEMF. (B) Live and dead staining for viable (*green*) and dead cells (*red*) in hTDCs cells 1, 3, and 7 days after actuation (fluorescence microscopy × 20, scale bar 50 μm, merged images). Insets are representative images of hTDCs without IL-1β treatment (control). Results are presented as mean ± standard error of the mean (SEM) ($n = 3$, three experimental replicates from three biological replicates) and data analyzed using two-way analysis of variance followed by multiple comparisons tests (GraphPad Prism). Statistically significant differences are shown with different degrees of confidence $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$, $^{****}p < 0.0001$ in comparison with controls. [Color figure can be viewed at wileyonlinelibrary.com]

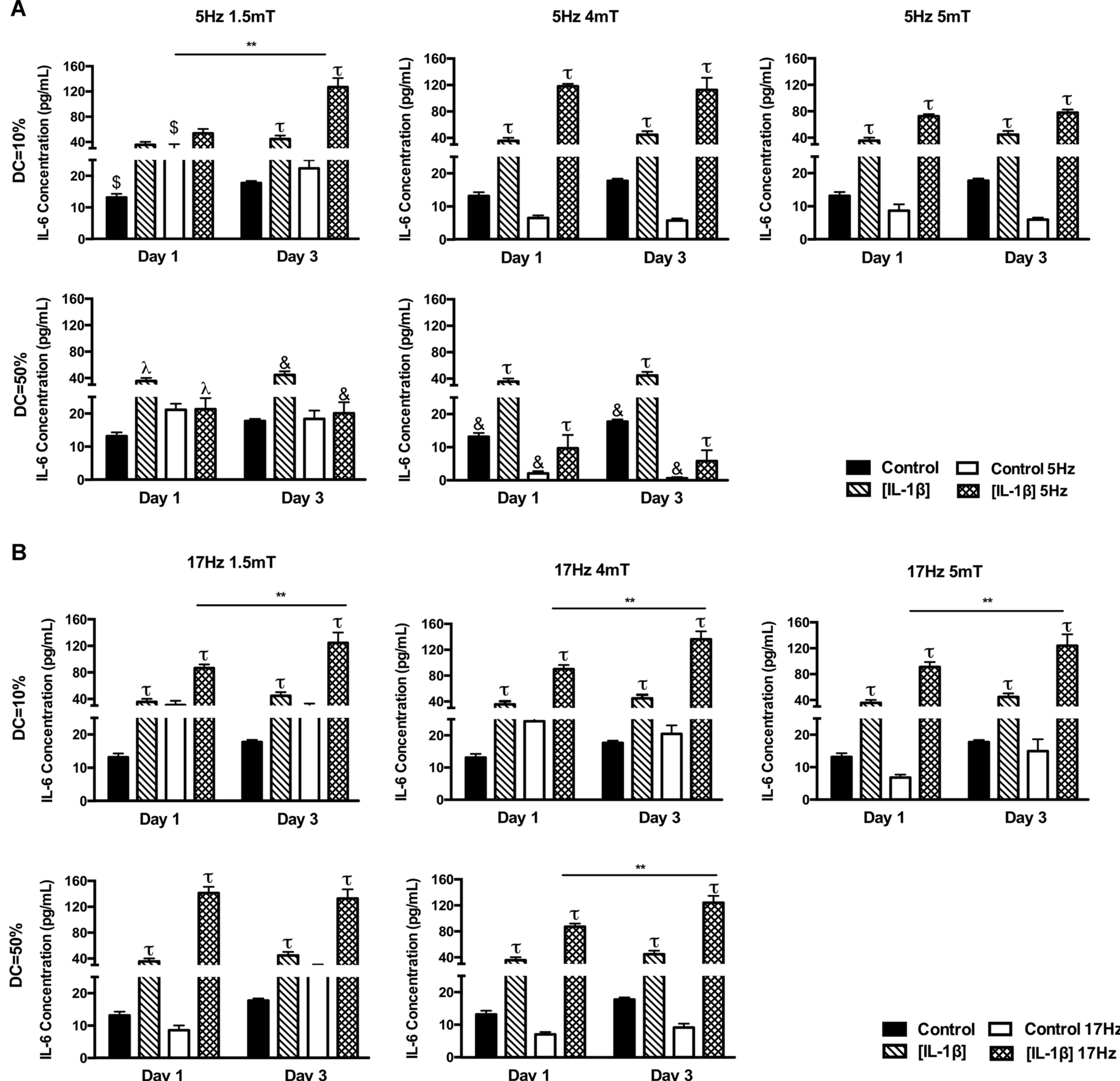

Figure 4. Release of interleukin-1β (IL-6) after human tendon-derived cells (hTDCs) treatment with IL-1β and pulsed electromagnetic field (PEMF) stimulation. (A) The effect of 5 Hz PEMF in IL-6 levels secreted by hTDCs with a variation of intensity (1.5–5 mT) and duty cycle (10–50% DC,%); (B) The effect of 17 Hz PEMF in IL-6 levels secreted by hTDCs, with a variation of intensity (1.5–5 mT) and duty cycle (10–50 DC,%). IL-6 was quantified in the culture medium 1 and 3 days after IL-1β and PEMF stimulation. Results are presented as mean ± standard error of the mean (SEM) ($n = 4$, experimental replicates from three biological replicates). Data were analyzed using a two-way analysis of variance (ANOVA) followed by multiple comparisons tests (GraphPad Prism). Statistically significant differences are shown with different degrees of confidence: $ for $p < 0.05$; ** and λ for $^{**}p < 0.01$; & for $p < 0.001$, and τ for $p < 0.0001$.

Influence of PEMF in the Cytokine Release Profile of IL-1β Treated hTDCs

The effect of PEMF parameters in the release of pro-inflammatory cytokines, IL-6 and TNF-α, by IL-1β treated hTDCs are presented in Figures 4 and 5, respectively.

In non-PEMF conditions, IL-6 and TNF-α concentrations tended to increase in IL-1β treated hTDCs.

When a PEMF is applied, IL-6 concentration increased with 5 Hz PEMF combined with a 10% duty cycle for a strength of 1.5, 4, or 5 mT (Fig. 4A) but not with a 5 Hz, 4 mT PEMF 50% duty cycle, in which IL-6 decreases in comparison with controls ($p < 0.001$) (Fig. 4A). The application of 17 Hz PEMF resulted in a strongly increased IL-6 release in all conditions in comparison with IL-1β treated hTDCs without PEMF actuation ($p < 0.0001$) (Fig. 4B).

The release of TNF-α tended to increase after PEMF exposure (Fig. 5A and B), with the exception for the

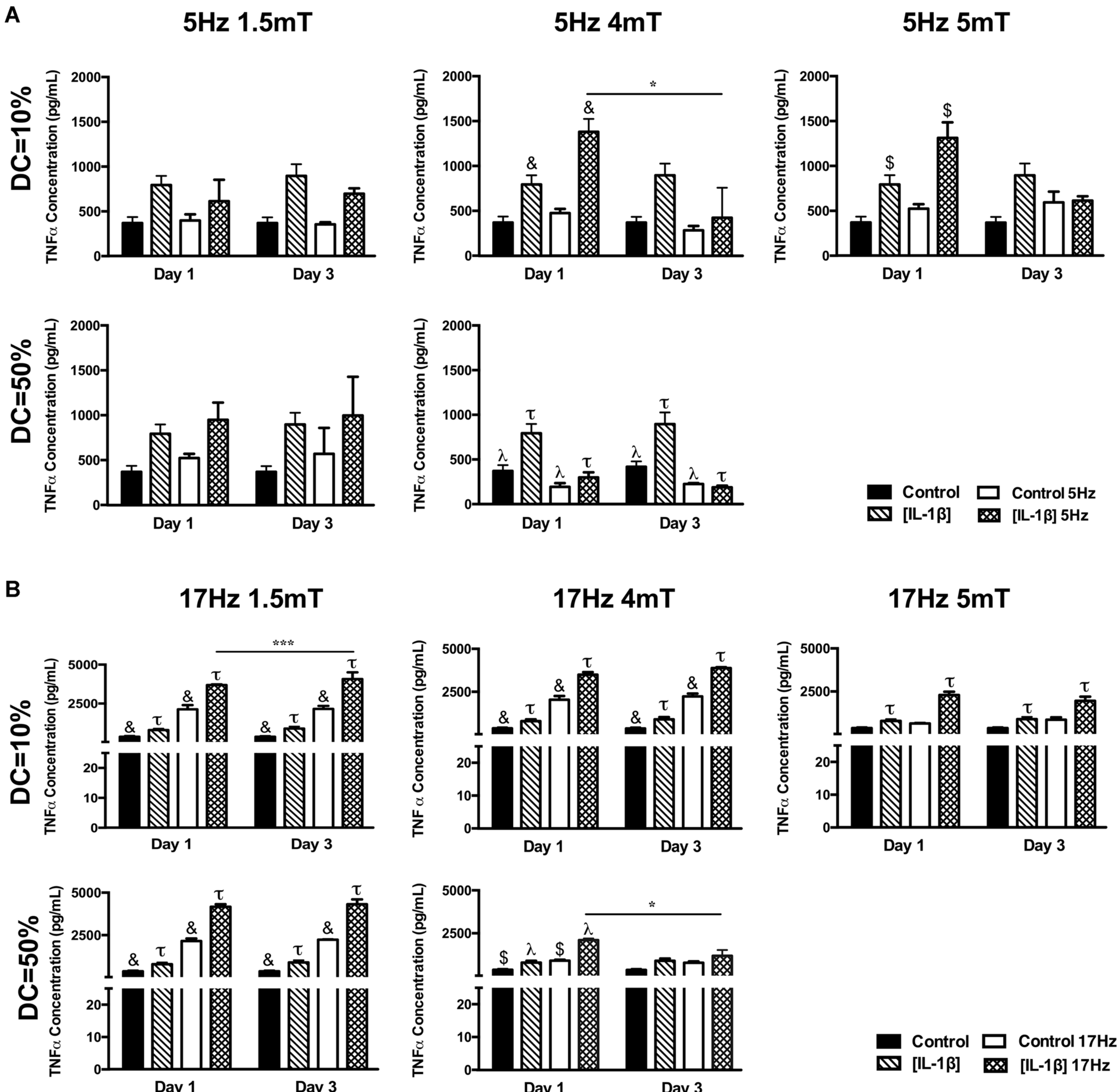

Figure 5. Tumor necrosis factor-α (TNF-α) secreted after human tendon-derived cells (hTDCs) treatment with interleukin 1β (IL-1β) and pulsed electromagnetic field (PEMF) stimulation. (A) The effect of 5 Hz PEMF in TNF-α levels secreted by hTDCs with a variation of intensity (1.5–5 mT) and duty cycle (10–50% DC,%); (B) The effect of 17 Hz PEMF in IL-6 levels secreted by hTDCs, with a variation of intensity (1.5–5 mT) and duty cycle (10–50 DC,%), TNF-α was quantified in the culture medium 1 and 3 days after IL-1β and PEMF stimulation. Results are presented as mean ± standard error of the mean (SEM) ($n = 4$, experimental replicates from three biological replicates). Data were analyzed using a two-way analysis of variance (ANOVA) followed by multiple comparisons tests (GraphPad Prism). Statistically significant differences are shown with different degrees of confidence: *, $ for $p < 0.05$; λ for $p < 0.01$; ***, & for $p < 0.001$, and τ for $p < 0.0001$.

5 Hz and 4 mT PEMF with a 50% duty cycle. As for the IL-6 release, the concentration of TNF-α increased in 17 Hz PEMF conditions (Fig. 5B).

In summary, hTDCs showed a reduced cytokine release after 1 h stimulation with a 5 Hz and 4 mT of intensity PEMF combined with 50% of duty cycle, suggesting a modulatory effect on pro-inflammatory cytokines in both physiological and inflammation-conditioned niches. Thus, this PEMF condition was further investigated in the expression of inflammation-related genes of IL-1β treated hTDCs.

PEMF Effect on the Genetic Profile of IL-1β Treated hTDCs

The gene expression of pro-inflammatory factors (*TNFα*, *IL-6*, *IL-8*, *IL-1β*, and *COX-2*) was increased in IL-1β treated hTDCs in comparison with control

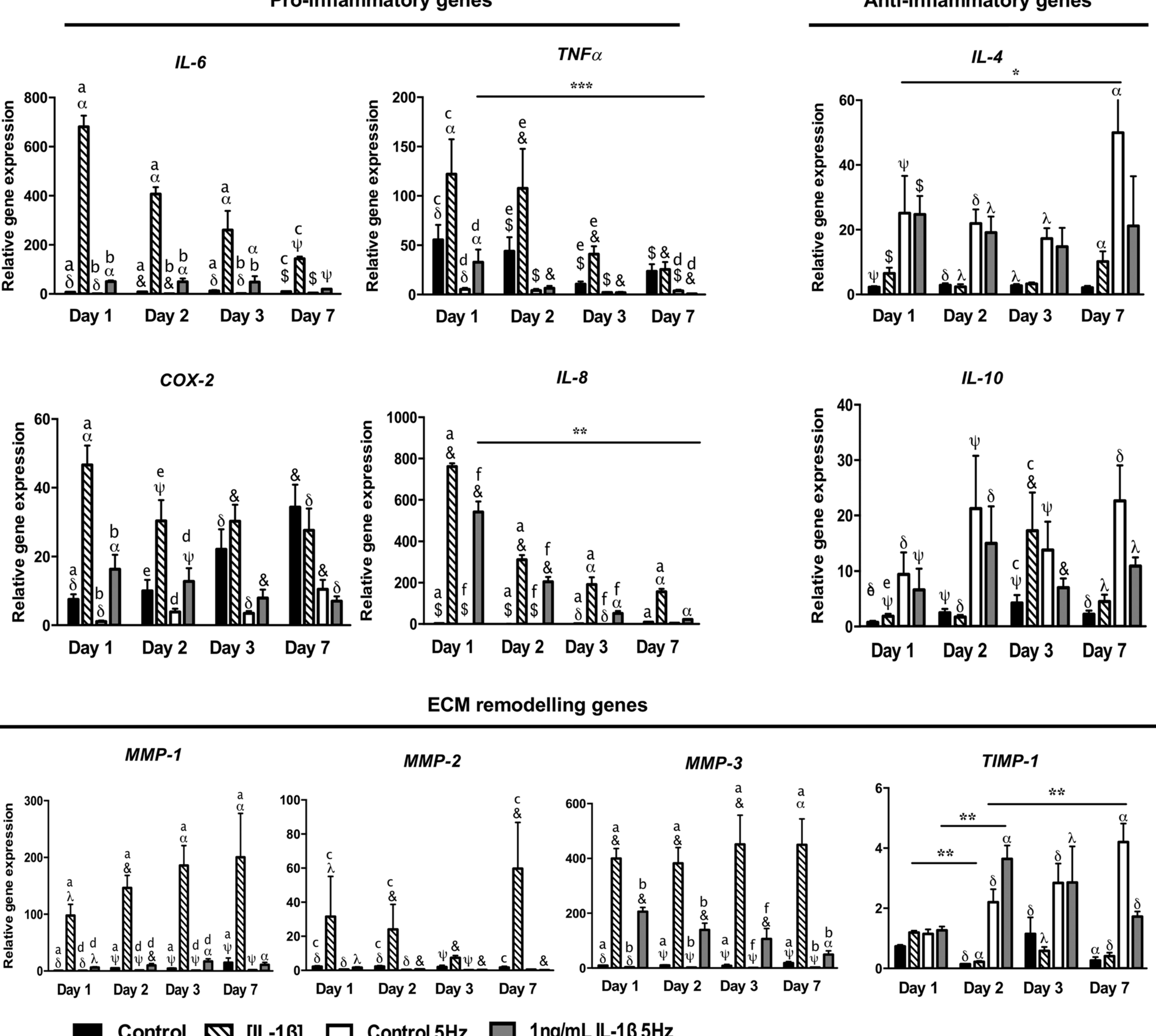

Figure 6. Relative expression of genes related to inflammation and extracellular matrix (ECM) remodeling in human tendon-derived cells (hTDCs) treated with interleukin 1β (IL-1β) and stimulated with pulsed electromagnetic field (PEMF) by reverse-transcription polymerase chain reaction (RT-PCR) analysis. (A) Relative expression of pro-inflammatory genes: *IL-6*, *TNFα*, *IL-8*, and *COX-2*. (B) Relative expression of anti-inflammatory genes: *IL-4* and *IL-10*. (C) Relative expression of ECM-associated remodeling genes: *MMP-1*, *MMP-2*, *MMP-3*, and *TIMP-1*. Results are presented as mean ± standard error of the mean (SEM) ($n = 3$). Data were analyzed using a two-way analysis of variance (ANOVA) followed by multiple comparisons tests (GraphPad Prism). Control condition refers to hTDCs without IL-1β treatment. Statistically significant differences are shown with different degrees of confidence: *,Ψ, $, d, e for $p < 0.05$; **, λ, δ, c for $p < 0.01$; ***, &, a, b for $p < 0.001$ and α, f for $p < 0.0001$.

($p < 0.01$) (Fig. 6). Furthermore, 5 Hz and 4 mT PEMF combined with 50% of duty cycle reduced the genetic expression of *IL-6*, *TNFα*, *COX-2*, and *IL-8* in both non-treated and IL-1β treated hTDCs ($p < 0.05$). In the particular case of *TNFα* and *COX-2*, the values found for IL-1β treated cells stimulated with PEMF are lower than non-PEMF stimulated hTDCs ($p < 0.05$). Conversely, PEMF stimulated hTDCs showed an increase in *IL-4* and *IL-10* in both IL-1β treated and non-treated cells ($p < 0.05$) (Fig. 6). Overall, PEMF stimulated cells showed increased anti-inflammatory gene expression over non-stimulated cells, with IL-1β treated hTDCs and non-treated hTDCs expressing similar values.

The ECM remodeling and maturation during healing depend on the balance of catabolic MMPs and anabolic TIMPs. Thus, *MMP-1*, *-2*, -3, and *TIMP-1* levels were also evaluated (Fig. 6). *MMP-1*, *-2*, and *-3* were upregulated in IL-1β treated hTDCs in comparison with non-treated cells 1, 2, and 7 days ($p < 0.05$) after IL-1β supplementation (Fig. 6). After PEMF actuation, MMPs expression was considerably decreased

in both IL-1β treated and non-treated hTDCs ($p < 0.05$). However, *TIMP-1* levels increased in non-treated hTDCs and decreased in IL-1β treated hTDCs 7 days after treatment ($p < 0.01$).

The multifactorial analysis performed showed that *TNFα* expression is significantly affected by all the parameters assessed: PEMF ($p = 0.002$), IL-1β ($p = 0.026$), and time ($p = 0.042$) while *COX-2* and *TIMP-1* are influenced by the interaction of PEMF and time ($p = 0.002$ and $p = 0.004$, respectively). *IL-4* is both influenced by time ($p = 0.037$) and by the interaction of IL-1β with PEMF ($p = 0.025$).

ECM-related genes, namely *MMP-1*, is strongly influenced by time ($p = 0.011$) and by the interaction effects of time with PEMF ($p = 0.021$) and of time with IL-1β ($p = 0.041$). Interestingly, PEMF particularly influences the *MMP-2* expression ($p = 0.014$) while *MMP-3* is more influenced by the PEMF and time interactions ($p = 0.028$).

In summary, the cytokine gene profile was changed upon exposure of hTDCs to a PEMF with 5 Hz, 4 mT and an applied 50% duty cycle, evidencing the PEMF impact in the expression of inflammation-related genes. Moreover, the interactions between PEMF and time of culture parameters also seem to play a role in the expression of inflammatory cytokine genes and ECM-related genes.

PEMF Effect on the Cell–Cell Communication of IL-1β Treated hTDCs

Cx43 expression associated to the cell–cell dissemination of inflammatory cues is lower in IL-1β treated hTDCs after exposure to PEMF (Fig. 7A).

The influence of IL-1β treatment and PEMF stimulation in endogenous MAPK(Erk1/2) is not as clear as for Cx43. MAPK(Erk1/2) immunolocation shows a decrease in the total protein after PEMF stimulation (Fig. 7B). Western blot outcomes indicate a differential detection in p42 and p44 bands of phospho-MAPK (Erk1/2). By day 1 it is increased in static control conditions (day 1) and in IL-1β treated hTDCs exposed to PEMF (day 1). With the exception of static control conditions, it tends to decrease by day 3 (Fig. 8).

DISCUSSION

The role of inflammation cues in healing and regeneration depends on coordinated events with the release of bioactive molecules and precise cell-to-cell communication.

Previous works have demonstrated IL-1β as an inflammation model in vitro,[25,26] being able to stimulate inflammatory mediators including COX-2, MMPs, TNF-α, and IL-6.[5]

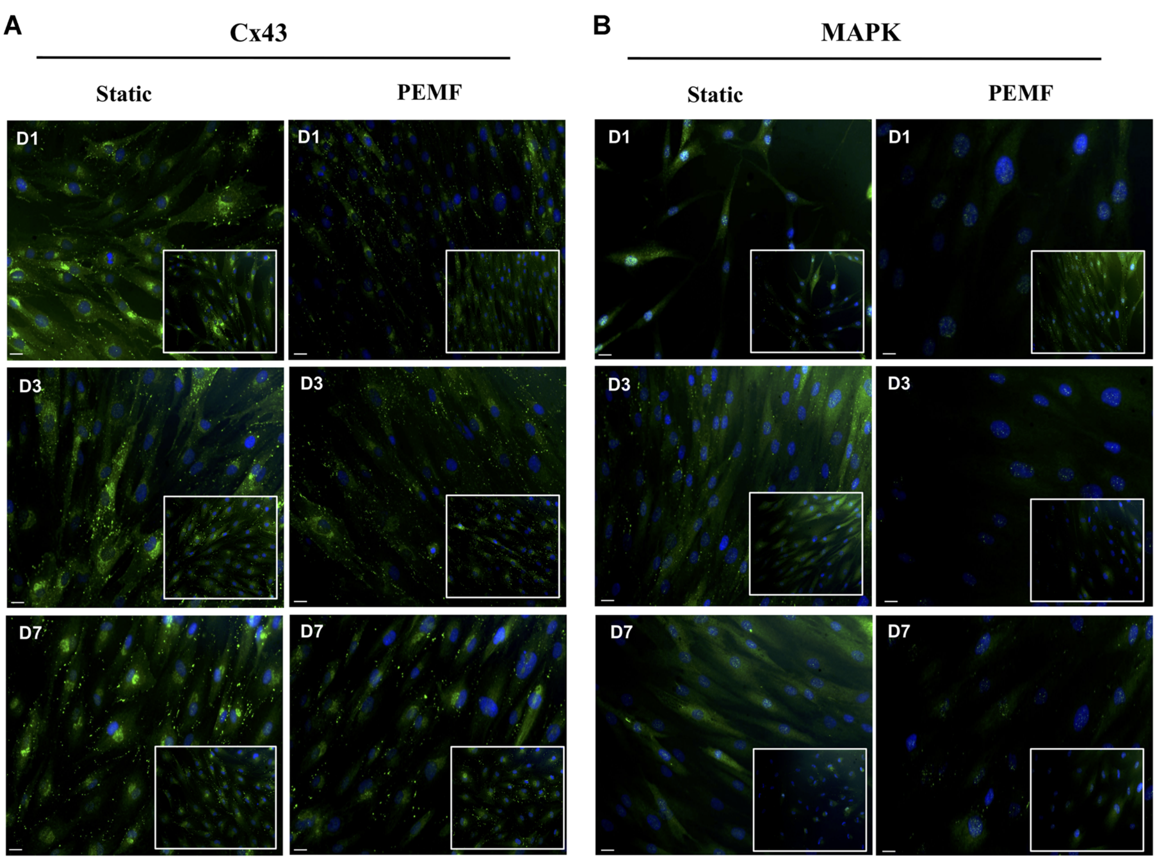

Figure 7. Representative images of Cx43 and MAPK expression in human tendon-derived cells (hTDCs) treated with interleukin-1β (IL-1β) and stimulated with pulsed electromagnetic field (PEMF). Fluorescence images of (A) Cx43 (green), nucleous (blue) and (B) MAPK (green), nucleous (blue) immunolocation in hTDCs cells after 1, 3, and 7 days in the culture, D1, D3, and D7, respectively (fluorescence microscopy ×20, scale bar 50 μm). Insets are representative images of hTDCs without IL-1β treatment (control, bottom right) and to the negative control (top right). Results are presented as mean ± SEM ($n = 3$, three experimental replicates from three biological replicates). [Color figure can be viewed at wileyonlinelibrary.com]

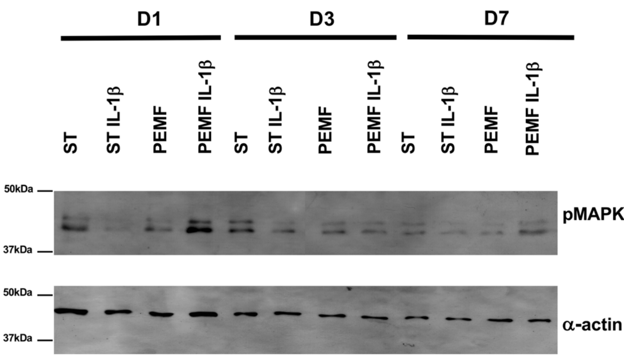

Figure 8. Western blot assay for the detection of phosphorylated MAPK (pMAPK (Erk1/2). Whole cell protein lysates were analyzed by probing for phospho-MAPK(Erk1/2) and α-smooth muscle actin (control) in human tendon-derived cells (hTDCs) cells treated with interleukin 1β (IL-1β) for 1, 3, and 7 days in the culture (D1, D3, and D7) after pulsed electromagnetic field (PEMF) stimulation (two experimental replicates from two biological replicates).

In our study, the concentrations of IL-1β investigated increased cell proliferation beyond 3 days post-treatment ($p < 0.05$), which may relate to the IL-1β influence in cell proliferation. Moreover, the significant increment in *MMPs* (-1, -2, and -3) around 100-fold suggests that hTDCs respond to IL-1β treatment. MMPs are responsible for the degradation of collagens and proteoglycans and have been linked to the degenerative changes in chronic tendinopathy.[27]

The release of pro-inflammatory cytokines IL-6 and TNF-α tended to increase in IL-1β concentration-dependent manner, which also suggests the role of IL-1β as an inflammatory inducer for hTDCs. Although these outcomes were expected and complied with other published works,[28] they were necessary to establish the optimal IL-1β concentration (1 ng/ml) to induce inflammatory cues on hTDCs without causing detrimental or cytotoxic effect to the cells and to set IL-1β model for future tendon-related studies.

In recent years, magnetic-actuated technologies propose to offer remote control and non-invasive tools to trigger and control biological processes, therefore, the promise of therapeutic value for the management and treatment of tissue pathologies. The tendon cells exposed to a 75 Hz, 1.5 mT magnetic field for 8 h showed an increment on IL-10 expression without affecting the production of pro-inflammatory cytokines,[19,29] while rat Achilles tendons showed a reduction of inflammation signals after a 17 Hz PEMF exposure for 15 min, five sessions a week.[20] However, studies on PEMF actuation require further insights for the identification of the main parameters or a combination of these holding a therapeutic action to potentiate proper healing at the cellular and tissue levels. Thus, we investigated sets of PEMF parameters, namely frequency, intensity, and duty cycle in modulating hTDCs cytokine profile.

An increase in cell metabolic activity, as well as an elongated hTDCs shape, was observed after PEMF exposure suggesting that the PEMF conditions investigated did not exert a cytotoxic effect. An exception was observed in hTDCs exposed to 5 mT PEMF and 50% duty cycle indicating that this condition is not favorable for cellular processes. Also, these results suggest the combination of 5 mT and 50% duty cycle has more impact in cell viability than any of the frequencies applied. Overall, the PEMF parameters studied showed the potential to modulate inflammatory cues of tendon cells enabling external control in non-invasive applications to assist regeneration, whose parameters can be adjusted if desirable, along the temporal healing timeline to promote the most suitable therapeutic action.

In the present study, IL-1β induces an increment in IL-6 and TNF-α secretion, antagonized by PEMF actuation with 5 Hz, 4 mT and 50% of duty cycle. This effect is also verified in non-treated cells, suggesting that PEMF may also influence cytokine profile in physiological conditions. In terms of gene expression, the PEMF application shows a modulatory effect in the genetic profile of IL-1β treated hTDCs. As mRNA levels of inflammatory cytokines are remarkably upregulated in injured tendons,[9] the inhibition of IL-1β via PEMF actuation could be beneficial for tendon-healing strategies. These outcomes are consistent with those of other studies applying magnetic forces combined with 5, 17, or 75 Hz frequencies.[17,19,29] Accordingly, the strong IL-1β-induction of *MMP-1* and *MMP-3* expression has been demonstrated in rabbit Achilles tendon cells exposed to cyclic strain and inflammatory cytokines.[30] In our work, PEMF stimulation not only decreases *MMPs* expression but favors *TIMP-1* increment, suggesting a tentative action of hTDCs toward an anabolic ECM turnover.

Connective tissues as tendons are excellent coupled cell networks that can facilitate/stop the spread of inflammatory cues.[31] The cell–cell communication established via gap junction channels as connexin 43 is of extreme importance to prevent or perpetuate inflammatory signals between cells. IL-1β was reported to stimulate an increase in Cx43 hemichannels[32] via activation of MAPK, iNOS, COX2, and PGE2. IL-1β was also described to increment the production of pro-inflammatory factors (IL-1, IL-17, IL-8, TGF-β, and TNF-α) in inflammation-associated diseases via MAPK pathway.[33] Further, the upregulation of Cx43 by synovial fibroblasts has been associated with the production of pro-inflammatory factors,[34] proposing a functional connection between IL-1β, Cx43, and the propagation of inflammation signals to neighboring cells.

In our study, the expression of Cx43 in IL-1β treated hTDCs was decreased after exposure to PEMF, which can limit the IL-1β-mediated signaling cascade. Besides the validation of IL-1β to induce inflammatory cues on the hTDCs, enabling studies on inflammation-conditioned niches, our outcomes disclose the potential role of PEMF as an antagonist of hTDCs response to the presence of pro-inflammatory signals. The actuation of 5 Hz 4 mT PEMF combined with 50% duty cycle led to a decrease in the expression of pro-inflammatory and MMP genes and to a diminished expression of Cx43.

MAPK signaling has been described to mediate multiple cell responses[35,36] and could be involved in the response of IL-1β-conditioned tendon cells stimulated with PEMF. The tendon cells expressed pMAPK(Erk1/2) in all conditions studied and immunochemistry studies pointed that it was initially localized within the nucleus. Although phosphorylation of MAPK (Erk1/2) is typically associated with a translocation into the nucleus, cytoplasmic pMAPK(Erk1/2) also enables control over cell fate,[37] whose expression is increased in static conditions. Moreover, in this study, p42 and p44 bands of pMAPK(Erk1/2) were detected in all conditions by western blotting. Interestingly though is the fact that thicker bands were identified in static control and IL-1β treated hTDCs exposed to PEMF. The increment in pMAPK(Erk1/2) of hTDCs exposed to PEMF treated with IL-1β did not correlate with the gene downregulation of pro-inflammatory markers TNF-α and COX-2 (in comparison with PEMF or IL-1β alone). These results suggest that complex signaling networks are likely involved in triggering inflammatory cues in the tendon cells and that these cues can be modulated by magnetic actuation.

CONCLUSIONS

The findings of this work show that PEMF parameters of 5 Hz, 4 mT, and 50% duty cycle can modulate the response of tendon cells to induced inflammatory stresses, influencing intracellular mechanisms at the gene and protein levels, envisioning the applicability of magnetic-actuated therapies for tendon-healing approaches.

AUTHORS' CONTRIBUTION

A.V. and A.G. performed the experiments, M.R. assisted with lab techniques. A.V. and M.R. analyzed the data. A.G., M.R., R.R., and M.E. provided guidance and/or senior supervision. A.V., A.G., and M.R. wrote the manuscript. A.V. and M.R. prepared the figures. All authors appraised the manuscript and figures.

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SUPPORTING INFORMATION

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