INTERLEUKIN-1β MODULATES ENDOCHONDRAL OSSIFICATION BY HUMAN ADULT BONE MARROW STROMAL CELLS

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Abstract

Inflammatory cytokines present in the milieu of the fracture site are important modulators of bone healing. Here we investigated the effects of interleukin-1β (IL-1β) on the main events of endochondral bone formation by human bone marrow mesenchymal stromal cells (BM-MSC), namely cell proliferation, differentiation and maturation/remodelling of the resulting hypertrophic cartilage. Low doses of IL-1β (50 pg/mL) enhanced colony-forming units-fibroblastic (CFU-f) and -osteoblastic (CFU-o) number (up to 1.5-fold) and size (1.2-fold) in the absence of further supplements and glycosaminoglycan accumulation (1.4-fold) upon BM-MSC chondrogenic induction. In osteogenically cultured BM-MSC, IL-1β enhanced calcium deposition (62.2-fold) and BMP-2 mRNA expression by differential activation of NF-κB and ERK signalling. IL-1β-treatment of BM-MSC generated cartilage resulted in higher production of MMP-13 (14.0-fold) *in vitro*, mirrored by an increased accumulation of the cryptic cleaved fragment of aggrecan, and more efficient cartilage remodelling/resorption after 5 weeks *in vivo* (i.e., more TRAP positive cells and bone marrow, less cartilaginous areas), resulting in the formation of mature bone and bone marrow after 12 weeks. In conclusion, IL-1β finely modulates early and late events of the endochondral bone formation by BM-MSC. Controlling the inflammatory environment could enhance the success of therapeutic approaches for the treatment of fractures by resident MSC and as well as improve the engineering of implantable tissues.

Keywords: Mesenchymal stem cells; tissue engineering; chondrogenesis; osteogenesis; endochondral ossification.

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Introduction

Fracture healing is a finely orchestrated process which recapitulates bone development and typically results in functional tissue regeneration (Gerstenfeld *et al.*, 2003; Behonick *et al.*, 2007; Marsell and Einhorn, 2011). Inflammation plays a crucial role in promoting and directing several aspects of bone regeneration (e.g., vascularisation, cell recruitment, cartilaginous callus production) and involves secretion of tumour necrosis factor-α (TNF-α); interleukin-1 (IL-1), IL-6, IL-11 and IL-18 (Gerstenfeld *et al.*, 2003). Importantly, the typical expression pattern of TNF-α and IL-1, the two master regulators of inflammation in fracture healing, is bimodal, with a peak within the first 24 hours which ends after 7 days, and a second peak after 4 weeks, with some variability, depending of the age of the patient (Cho *et al.*, 2002; Gerstenfeld *et al.*, 2003; Lange *et al.*, 2010). Briefly, the purpose of the first peak is to promote cell recruitment into the haematoma and vascularisation, while the second peak regulates cartilaginous callus remodelling (Cho *et al.*, 2002; Gerstenfeld *et al.*, 2003).

Even though the literature is controversial, numerous clinical studies indicate that the use of nonsteroidal anti-inflammatory drugs (NSAID), frequently used for postoperative pain control, may increase the risk of delayed fracture healing (Giannoudis *et al.*, 2000; Burd *et al.*, 2003; Bhattacharyya *et al.*, 2005). Moreover, several studies in different animal models indicated that a long-acting NSAID therapy is more deleterious than a short-acting NSAID treatment (Krishak *et al.*, 2007; O'Conner *et al.*, 2009; Ochi *et al.*, 2011). Taken together, these results highlight a key role of inflammation in influencing the processes of fracture healing.

Most fractures heal by indirect fracture healing, which consists of a combination of endochondral ossification, leading to formation of a cartilaginous callus, and intramembranous ossification, which results in formation of a periosteal callus. However, the key feature of this process is the remodelling and ossification of the cartilaginous callus (Gerstenfeld *et al.*, 2003). The interaction of a tissue undergoing the endochondral route with inflammatory signals is a crucial process to be investigated. Most *in vitro* studies assessed the effects of inflammatory cytokines on either osteogenic or chondrogenic differentiation capacity of mesenchymal stromal cells (MSC). In particular, IL-1β and TNF-α stimulation has been shown to enhance the extent of mineralisation and expression of osteoblast-related genes during MSC culture in osteogenic medium

(Ding *et al.*, 2009; Hess *et al.*, 2009; Cho *et al.*, 2010), and to inhibit MSC chondrogenesis in a dose-dependent manner (Wehling *et al.*, 2009). However, no study has yet reported on the role of inflammatory cytokines on the different phases of proliferation, differentiation and maturation/remodelling of an endochondral tissue, based on human bone marrow MSC (BM-MSC) *in vitro* or *in vivo*.

With the final goal of identifying specific tissue repair processes regulated by IL-1β during endochondral/perichondral bone formation, we studied the effects of IL-1β on (i) proliferation and commitment of BM-MSC; (ii) osteogenic and chondrogenic differentiation of BM-MSC; (iii) maturation/remodelling of the BM-MSC-generated cartilage tissue during *in vitro* and (iv) *in vivo* endochondral bone formation. Since *in vivo* BM-MSC are typically exposed to a hypoxic environment (Das *et al.*, 2010), the effects of IL-1β during osteogenic and chondrogenic culture were also investigated at different oxygen percentages (19 %, 5 % and 2 %).

Materials and Methods

Cell harvest

Human bone marrow aspirates were harvested from 7 individuals (all male, mean age: 36.7 years, range: 24-49 years) during routine iliac crest bone grafting, in accordance with the rules of the local ethical committee (University Hospital Basel) and after informed consent was obtained.

BM-MSC culture

BM-MSC were isolated from bone marrow aspirates as previously described (Braccini *et al.*, 2005). Nucleated cells were counted after staining with Crystal Violet 0.01 % (Sigma-Aldrich, St. Louis, MO, USA) and seeded in culture dishes at a density of $4.5x10^3$ cells/cm^2 for the clonogenic culture or in culture flasks at a density of $1.7x10^5$ cells/cm^2 for the expansion of BM-MSC.

Clonogenic culture

Cells were cultured for 2 weeks in alpha-MEM (minimal essential medium) (Gibco/Life Technologies, Carlsbad, CA, USA) supplemented with 10 % foetal bovine serum, 1 mM sodium pyruvate, 100 mM HEPES buffer, 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.29 mg/mL L-glutamine (complete medium) with IL-1β (Sigma-Aldrich) (0, 50 or 1000 pg/mL) in the presence or not of 5 ng/mL fibroblast growth factor-2 (FGF-2) in a humidified incubator at 37 °C with 5 % CO_2 with medium changes twice a week.

Expansion of BM-MSC

Cells were cultured for two passages in complete medium supplemented with 5 ng/mL FGF-2 as described earlier (Frank *et al.*, 2002).

Osteogenic differentiation

Osteogenic differentiation was induced in 2D cultures using a defined medium (osteogenic medium) as previously described (Frank *et al.*, 2002). Briefly, BM-MSC were seeded in 6- or 12-well plates at a density of $3x10^3$ cells/cm^2 in alpha-MEM supplemented with 10 % foetal bovine serum (FBS), 10 mM β-glycerophosphate (Sigma-Aldrich), 10 nM dexamethasone (Sigma-Aldrich) and 0.1 mM L-ascorbic acid-2-phosphate (Sigma-Aldrich) and cultured for 2 or 3 weeks, with medium changes twice per week. IL-1β at different concentrations (i.e., 0, 10, 50, 100, 250 and 1000 pg/mL) was added at the beginning of the experiment and at each medium change. Cell layers were cultured for 3 weeks in a humidified incubator at 37 °C with 5 % CO_2 and 19 % oxygen or in a "Sci-tive" Workstation (Ruskinn Technology, Pencoed, South Wales, UK), to maintain constant hypoxic conditions (i.e., 5 % and 2 % oxygen). Medium was changed twice per week.

BM-MSC from three donors were used to study whether inhibition of NF-κB or ERK modulates IL-1β osteogenic responses. BM-MSC were grown for 7 days in 12-well plates with the last three days without FGF-2. Confluent layers were then supplemented with 50 nM pyrrolidine dithiocarbamate (PDTC, NF-κB inhibitor, Sigma-Aldrich) or 10 µM UO126 (MEK/ERK inhibitor, Sigma-Aldrich) (Cho *et al.*, 2010) while in osteogenic medium. After 3 hours IL-1β (50 pg/mL) was added to selected wells and maintained only for the first three days. Cells were then cultured in osteogenic medium (without IL-1β and inhibitors) for an additional 11 days. Control wells were cultured in osteogenic medium without inhibitors and/or IL-1β for the entire 14 days. U0126 was dissolved in dimethyl sulphoxide (DMSO) at a stock concentration of 10 mM. DMSO 1:1000 supplemented to the cells as control was observed not to modulate osteogenic differentiation.

Chondrogenic differentiation

Chondrogenic differentiation was induced in 3D pellet cultures using a defined serum-free medium (chondrogenic medium), as previously described (Jakob *et al.*, 2001). Briefly, BM-MSC were resuspended in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 mg/mL D-glucose, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 100 mM HEPES buffer, 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.29 mg/mL L-glutamine supplemented with ITS+1 (10 µg/mL insulin, 5.5 µg/mL transferrin, 5 ng/mL selenium, 0.5 mg/mL bovine serum albumin, 4.7 µg/mL linoleic acid (Sigma-Aldrich), 0.1 mM ascorbic acid 2-phosphate (Sigma-Aldrich), 10 ng/mL TGFβ 1 (R&D Systems, Minneapolis, MN, USA) and 10^{-7} M dexamethasone (Sigma-Aldrich). Aliquots of $4x10^5$ cells/0.5 mL were centrifuged at 1,200 rpm for 5 min in 1.5 mL polypropylene conical tubes (Sarstedt, Numbrecht, Germany) to form spherical pellets. IL-1β at the concentrations described above was added at the beginning of the experiment and at each medium change. Pellets were cultured under different oxygen percentages as previously described.

Endochondral priming in vitro

BM-MSC were cultured in type I collagen meshes (Ultrafoam®, Davol, Warwick, RI, USA) at a density of $40x10^6$ cells/cm^3 in chondrogenic medium. After 3 weeks, cartilaginous tissues were cultured for an

additional 2 weeks in a serum-free hypertrophic medium without TGF-β 1, supplemented with 50 nM thyroxine, 10 mM β-glycerophosphate, 10^{-8} M dexamethasone, and 0.1 mM L-ascorbic acid-2-phosphate (Scotti *et al.*, 2010). Endochondral primed constructs were then analysed or implanted ectopically in nude mice (as described below).

Endochondral bone formation in vivo

Constructs cultured for 5 weeks *in vitro* were implanted subcutaneously in the back of nude mice (CD-1 nu/nu, athymic, 6- to 8-week old females) (4 samples/mouse), following approval by the local veterinary authorities, and retrieved after 5 or 12 weeks. The *in vivo* experiment was performed with BM-MSC from only one human donor, previously selected out of 7 independent preparations based on the capability to generate hypertrophic cartilage *in vitro*.

Analytical methods

CFU-f and CFU-o

After 2 weeks of clonogenic culture, dishes were rinsed with phosphate-buffered saline (PBS) and stained for alkaline phosphatase (AP) using the 104-LL kit (Sigma Diagnostics, St. Louis, MO, USA). The number of AP positive colonies (with more than 32 cells/colony) was counted by three independent investigators to estimate the fraction of colony forming units osteoblastic (CFU-o). The same dishes were then stained with 1 % methylene blue (MB) and the total number of MB positive colonies (AP positive or negative, with more than 32 cells/colony) were counted to estimate CFU-fibroblastic (CFU-f). The diameter of the MB positive colonies was measured using the UTHSCSA ImageTool 3.0 software.

Histological staining, immunohistochemistry and in situ hybridisation (ISH)

Chondrogenic pellets and constructs were fixed in 4 % paraformaldehyde for 24 h at 4 °C, dehydrated in an ethanol series and embedded in paraffin. Sections (5 µm thick) were stained for safranin-O, alcian blue, haematoxylin and eosin (H&E) (J.T. Baker Chemical, Phillipsburg, N.J., USA), alizarin red and Masson's trichrome. Immunohistochemical analyses were performed using primary antibodies against Osterix (Abcam, Cambridge, UK), Osteocalcin (EMD Millipore, Billerica, MA, USA), MMP13 (Abcam) and aggrecan cryptical epitope-DIPEN (MD Biosciences, St Paul, MN, USA) (Scotti *et al.*, 2010). Upon rehydration in a graded ethanol series, sections were digested according to the manufacturer's instructions. The immunobinding was detected with biotinylated secondary antibodies and by Vectastain ABC (Vector Labs, Burlingame, CA, USA) kit. The red signal was developed with Fast red kit (Dako Cytomation; Dako, Glostrup, Denmark), with haematoxylin counterstaining. Negative controls were performed during each analysis by omitting the primary antibodies. Osteogenically cultured layers were washed twice with PBS, fixed for 10 min in 4 % formalin and stained with alizarin red 2 %. Hydroxyapatite deposits in osteogenically cultured layers were stained using the Osteoimage Mineralisation Assay (Lonza, Walkersville, MD, USA), following the manufacturer's instructions. Quantification of safranin O positive areas (21 slides, 10 samples, total 7.35 cm^2) and bone marrow content in Masson's trichrome staining (20 slides, 10 samples, total 7 cm^2) was performed with ImageJ 1.46d (National Institutes of Health, Bethesda, MD, USA) using thresholding and manual selection. ISH for human Alu repeats was performed as previously published (Scotti *et al.*, 2010).

Immunofluorescence images

Samples after *in vivo* culture were fixed in 4 % paraformaldehyde (Sigma-Aldrich), decalcified with EDTA (Sigma-Aldrich) solution, embedded in OCT and snap frozen in liquid nitrogen. Sections (20 µm thick) were incubated with the primary antibodies anti CD31 (PECAM-1; BD Pharmingen, Franklin Lakes, NJ, USA). When needed, a secondary antibody labelled with Alexa Fluo 546 (Invitrogen/Life Technologies, Carlsbad, CA, USA) was chosen and DAPI was used as nuclear staining. Fluorescence images were acquired using a Zeiss (Oberkochen, Germany) LSM-510 confocal microscope. The percentage of area infiltrated by vessels (CD31) was calculated on cross sections of the implant excluding the outer fibrotic capsule (n = 6 per experimental group) with ImageJ 1.46d (National Institutes of Health) using thresholding and manual selection.

Quantification of glycosaminoglycan (GAG) and DNA contents

Chondrogenic pellets and constructs were digested in proteinase K (1 mg/mL proteinase K in 50 mM Tris with 1 mM EDTA, 1 mM iodoacetamide, and 10 mg/mL pepstatin A) for 16 h at 56 °C. The GAG content of the cartilaginous tissues as well as in the supernatant harvested after each media change was determined spectrophotometrically using dimethylmethylene blue, with chondroitin sulphate as standard (Farndale *et al.*, 1986). The DNA content of constructs and cell layers (lysed with 0.01 % sodium dodecyl sulphate, SDS) was measured using the CyQuant cell proliferation assay kit (Molecular Probes, Eugene, OR, USA) and used to normalise the GAG content.

Quantification of calcium

Osteogenically cultured layers and constructs following hypertrophic culture were lysed with 0.5 N HCl. For quantification of calcium deposition, cell layers were harvested and analysed using the RANDOX (Crumlin, Co. Antrim, UK) CA 590 according to the manufacturer's protocol.

Quantification of VEGF and MMP13

VEGF and MMP13 protein levels were determined according to manufacturer in total protein lysates collected from constructs cultured for 5 weeks (Quantikine, human VEGF and human pro-MMP13, R&D Systems).

Quantitative real-time RT-PCR

Total RNA was extracted from cells using TRIzol (Invitrogen/Life Technologies), treated with DNAse and retrotranscribed into cDNA, as previously described (Frank *et al.*, 2002). Real-time reverse transcriptase-polymerase chain reaction (RT-PCR; 7300 Applied Biosystems/Life

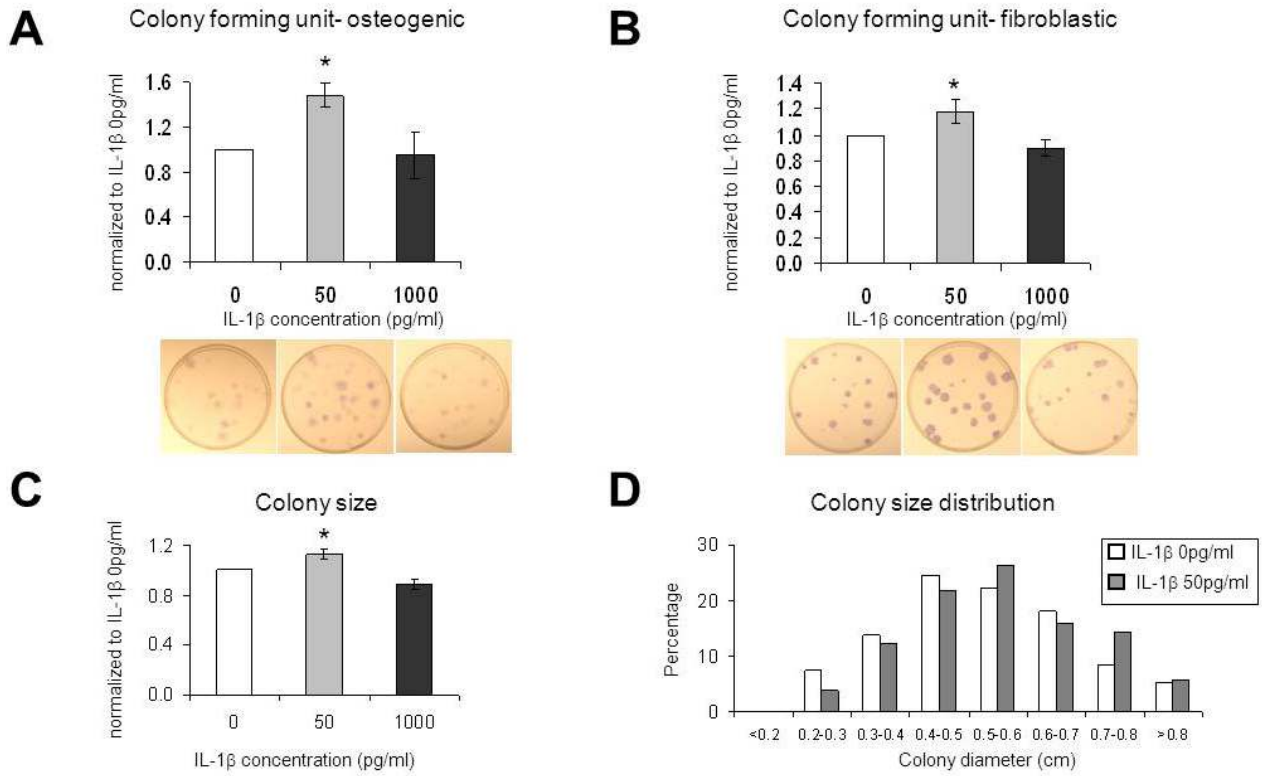

Fig. 1. Effects of IL-1β during clonal culture of human bone marrow stromal cells (BM-MSC). (**A**) Colony forming unit (CFU) -osteoblastic (quantification upper graph, representative colonies stained for alkaline phosphatase, bottom) and (**B**) CFU-fibroblastic (quantification upper graph, representative colonies further stained with methylene blue, bottom). (**C**) Quantification of colony size. $n = 3$ experiments with cells from 3 different donors, 3 plates/donor analysed. Values are mean ± SD, * = $p < 0.05$ from IL-1β 0 pg/mL. (**D**) Colony size distribution of a BM-MSC donor.

Technologies) was performed as previously described (Barbero *et al.*, 2003) to quantify expression levels of mRNA of genes expressed in cartilage (collagen type II), hypertrophic cartilage (collagen type X) in undifferentiated mesenchymal tissues and/or bone (type I collagen, bone sialoprotein, osteocalcin, bone morphogenetic protein (BMP)-2), as well as of genes involved in ECM remodelling, apoptosis and cell proliferation (MMP-13, caspase 3 and Ki-67), using human specific primers and probes. For each sample, the Ct value of each target sequence was subtracted from the Ct value of the reference gene (glyceraldehyde-3-phosphate dehydrogenase, GAPDH), to derive ΔCt. The expression level of each target gene was calculated as $2^{\Delta Ct}$.

Microtomography

Microtomography (μCT) was performed on constructs at different *in vivo* time points, after fixation in formalin and storage in PBS. μCT data were acquired using a SkyScan 1174 table top scanner (SkyScan, Kontich, Belgium) with unfiltered X-rays at an applied voltage of 32 kV and a current of 800 μA. Transmission images were acquired for a 360° scan rotation with an incremental rotation step size of 0.4°. Reconstruction was made with a modified Feldkamp algorithm at an isotropic voxel size of 6.26 μm. Threshold-based segmentation and 3D measurement analyses (bone mineral density and volume) were performed using the CT-Analyser program (SkyScan NV), as previously described (Papadimitropoulos *et al.*, 2007). 3D rendering of the structures was performed using the commercial software VGStudio MAX 1.2.1. (Volume Graphics, Heidelberg, Germany).

Statistical analysis

For each experiment and donor, at least triplicate specimens were assessed and the values presented as mean ± standard deviation of measurements. For the dose response experiments, repeated measures ANOVA was performed using a linear mixed-effects model with a *post-hoc* Dunnett comparison to baseline, corrected for multiple comparisons. This computation was performed with R v. 2.14.2 and the packages "nlme" and "multcomp". Differences between experimental groups were otherwise assessed by two-tailed Wilcoxon tests and considered statistically significant with $p < 0.05$ (Sigma Stat software, SPSS, IBM, Amonk, NY, USA).

Results

Effect of IL-1β on the clonogenicity and proliferation of BM-MSC

We first investigated whether supplementation of IL-1β during the culture of bone marrow cells affected their (i) clonogenicity, (ii) osteogenic commitment in the absence of differentiating factors, and (iii) proliferation. Freshly

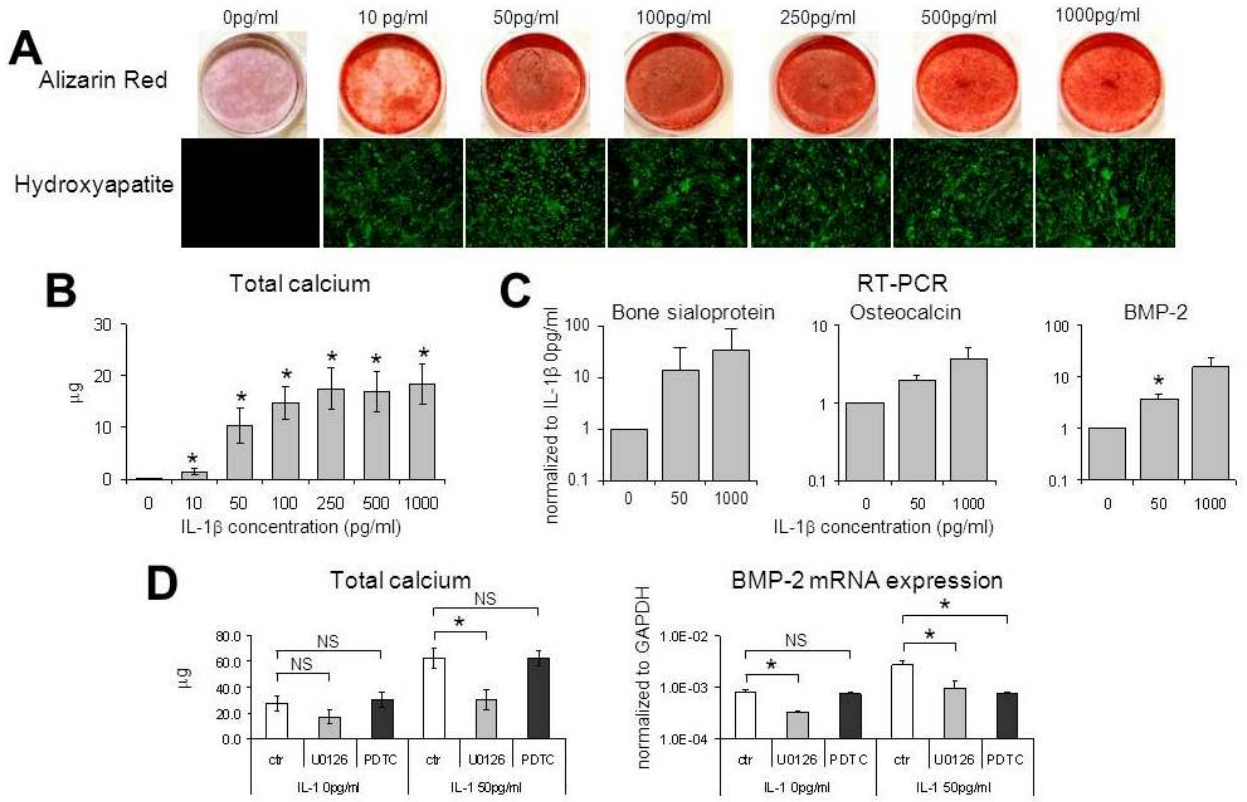

Fig. 2. Effects of IL-1β on the osteogenic differentiation of human bone marrow stromal cells (BM-MSC). (**A**) Representative alizarin red (top) and hydroxyapatite-specific fluorescence (bottom) staining and (**B**) total calcium contents of osteogenically cultured BM-MSC. (**C**) Real time RT-PCR analysis of the expression of bone sialoprotein, osteocalcin and bone morphogenetic protein (BMP)-2, levels are normalised to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and expressed as fold of difference from those measured in cells not stimulated with IL-1β. $n = 5$ experiments with cells from 5 different donors, 10 specimens analysed. (**D**) Effect of ERK and NF-κB inhibition (through 72 h incubation with 10 µm UO126 and 50 nM pyrrolidine dithiocarbamate (PDTC) respectively) on total calcium accumulation and on BMP-2 mRNA expression (see materials and methods for the description of the experimental design). $n = 3$ experiments with cells from 3 different donors, 6 specimens analysed. Data are mean ± SD. * = $p < 0.05$ from IL-1β 0 pg/mL, NS = no significant differences.

isolated bone marrow nucleated cells were then cultured at clonogenic density without IL-1β or in the presence of 50 pg/mL or 1000 pg/mL of IL-1β. IL-1β at the lowest dose significantly enhanced the fraction of CFU-o and CFU-f (1.5- and 1.2-fold respectively, Fig 1A-B). The average colony size was also increased (by 1.2-fold) following exposure to 50 pg/mL IL-1β (Fig. 1C), indicating an enhanced propensity of the cells to proliferate. By grouping the colony diameters in arbitrary size groups we observed a shift in the distribution upon exposure to 50 pg/mL IL-1β (median value from 0.45 cm to 0.55 cm) (Fig. 1D). The presence of FGF-2 during the clonogenic culture of BM-MSC reduced the IL-1β mediated increases of CFU-o, CFU-f and colony size (data not shown).

Effect of IL-1β on the differentiation of BM-MSC

We then investigated whether IL-1β stimulation enhanced the differentiation capacity of BM-MSC when cultured under chondrogenic or osteogenic conditions. Different doses of IL-1β (0 to 1000 pg/mL) were tested.

Osteogenic differentiation

Following culture of BM-MSC in osteogenic medium, the intensity of staining for calcium and hydroxyapatite deposit strongly increased in presence of IL-1β up to 50 pg/mL and remained almost unchanged at higher doses (Fig 2A). Biochemical analyses confirmed the histological trend: calcium contents increased up to 78-fold by IL-1β 50 pg/mL, higher doses of IL-1β induced a further increase in calcium contents that was not statistically significant (Fig. 2B). RT-PCR analyses indicated that IL-1β at both tested concentrations resulted in the up-regulation of the bone sialoprotein, osteocalcin and BMP-2, however statistically significant differences were observed only in the expression of BMP-2 at the lower dose of IL-1β (Fig. 2C). Interestingly, even a short exposure (3 days) of BM-MSC to 50 pg/mL IL-1β induced a significant increase of calcium deposition and expression of BMP-2 mRNA (Fig. 2D). To understand how IL-1β induces enhanced osteogenic differentiation of BM-MSC, cells were pre-treated with U0126 (an ERK inhibitor) or PDTC (an NF-κB inhibitor), exposed or not with 50 pg/mL IL-1β (3 days) and then induced to osteogenic differentiation. We observed that: (i) U0126 specifically inhibited the IL-1β induced calcium deposition and caused a general down-regulation of BMP-2 expression, (ii) PDTC did not affect calcium deposition but specifically inhibited the IL-1β mediated up-regulation of BMP-2 mRNA (Fig. 2D). No significant

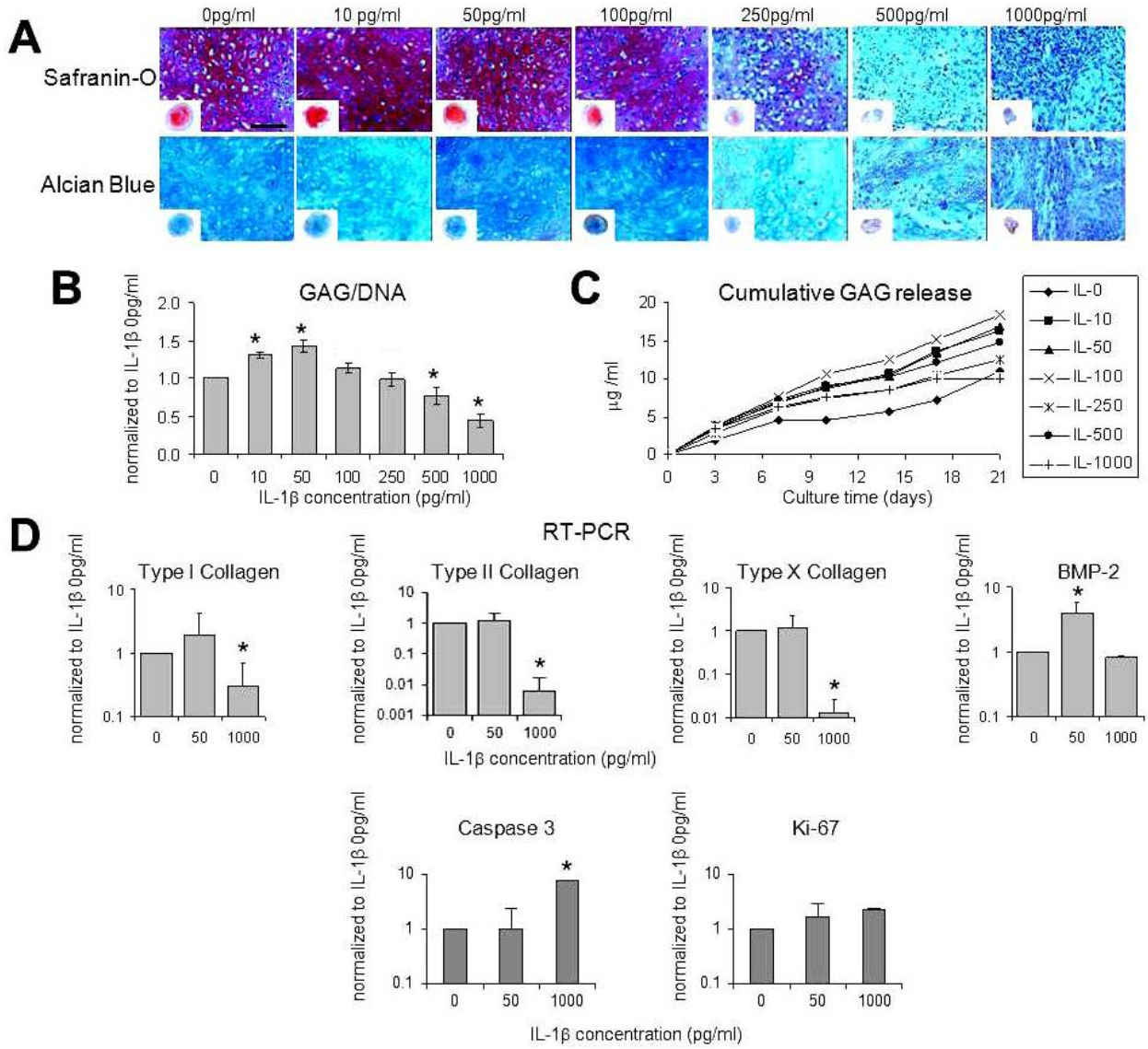

Fig. 3. Effects of IL-1β on the chondrogenic differentiation of human bone marrow stromal cells (BM-MSC). (**A**) Representative safranin-O (top) and Alcian Blue (bottom) staining of chondrogenic pellets. Bar = 50 µm (**B**) Sulphated glycosaminoglycan (GAG) content normalised to the DNA amount of the pellets. Levels are expressed as difference from those measured in cells not stimulated with IL-1β. n = 3 experiments with cells from 3 different donors, 6 specimens analysed. (**C**) GAG released during the chondrogenic culture of a representative BM-MSC donor. (**D**) Real time RT-PCR analysis of the expression of types I, II and X collagen, bone morphogenetic protein (BMP)-2, caspase 3 and Ki67 levels are normalised to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and expressed as fold of difference from those measured in cells not stimulated with IL-1β. n = 3 experiments with cells from 3 different donors, 6 specimens analysed. Data are mean ± SD. * = $p < 0.05$ from IL-1β 0 pg/mL.

differences in the DNA contents were observed between BM-MSC treated or not with U0126 or PDTC (data not shown), suggesting that the inhibitory effects in calcium deposition and BMP-2 expression of these two compounds were not due to a reduction in cell number/survival.

Collectively these results indicate that IL-1β induced an enhanced mineralisation and expression of BMP-2 by BM-MSC more reproducibly when applied at a low dose. Inhibition of ERK but not of NF-κB counteracted both IL-1β mediated mineralisation increase and BMP-2 upregulation.

Chondrogenic differentiation

MSC were also induced to differentiate in pellet in the absence or presence of IL-1β during the entire culture time. Histological analyses indicated that tissues formed by BM-MSC exposed to low doses of IL-1β (10 and 50 pg/mL) were more intensely stained for cartilage specific matrix than tissues formed in absence of IL-1β, while those exposed to high doses (≥ 250 pg/mL) were less intensely stained (Fig. 3A). Biochemical analyses generally confirmed this trend: GAG contents increased up to 1.4-fold (at the IL-1β dose of 50 pg/mL) and decreased up to 2.3-fold at the highest IL-1β dose (Fig. 3B). DNA

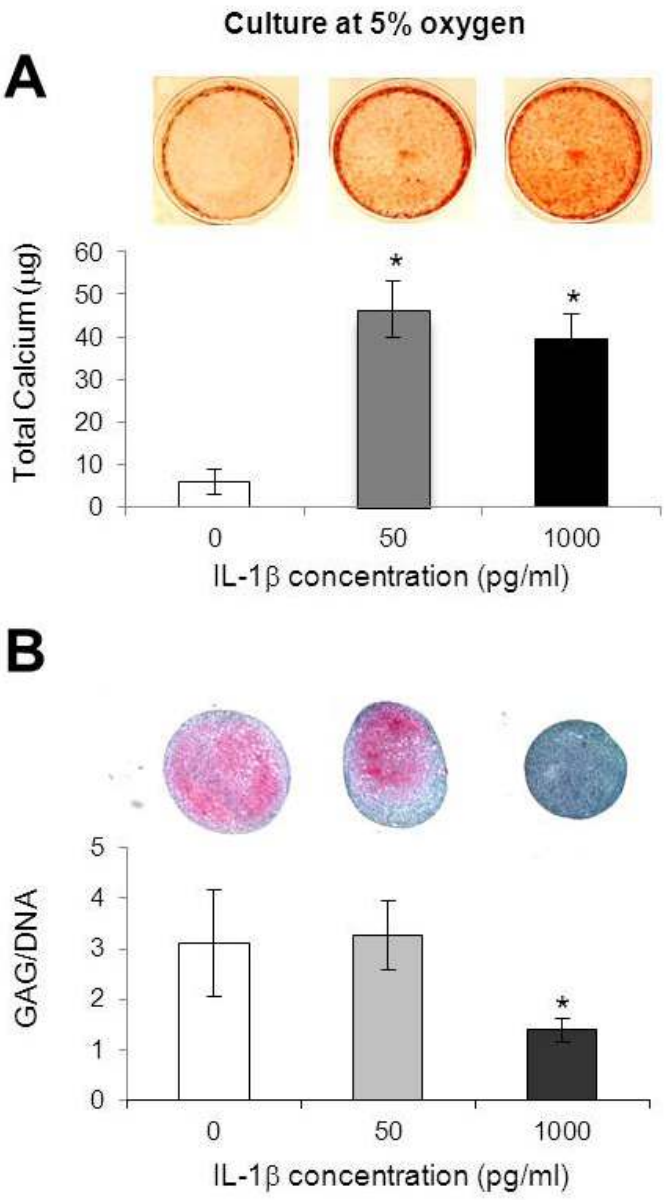

Fig. 4. Effects of IL-1β on the osteogenic and chondrogenic differentiation of human bone marrow stromal cells (BM-MSC) under hypoxic culture. (**A**) Representative alizarin red staining (top) and total calcium contents of osteogenically cultured BM-MSC under 5 % oxygen. (**B**) Representative safranin-O staining (top, scale bar = 500 µm) of and sulphated glycosaminoglycan (GAG) content normalised to the DNA amount of chondrogenic pellets. $n = 3$ experiments with cells from 3 different donors, 6 specimens analysed. Data are mean ± SD. * = $p < 0.05$ from IL-1β 0 pg/mL.

content of pellets gradually decreased with IL-1β, with no statistically significant difference between adjacent groups (data not shown). Biochemical analyses of culture medium harvested at different time of chondrogenic culture indicated that GAG was released to a higher extent by tissues exposed to the lower doses (≥ 100 pg/mL) of IL-1β (Fig. 3C). Cartilaginous tissues generated by BM-MSC not exposed to IL-1β or exposed to low (50 pg/mL) or high (1000 pg/mL) doses of IL-1β were also analysed by RT-PCR. The expression of type I, II and X collagen was not modulated by IL-1β at 50 pg/mL but strongly decreased in samples treated with IL-1β at 1000 pg/mL (3.4-, 170- and 80.1-fold respectively). BMP-2 expression, instead, was enhanced by IL-1β at 50 pg/mL (3.9-fold) but not effected by IL-1β at 1000 pg/mL (Fig. 3D). Interestingly, caspase 3 expression was significantly enhanced by IL-1β at 1000 pg/mL, possibly explaining the loss of DNA and the reduced pellet size, while Ki67 expression was not significantly modulated by IL-1β (Fig. 3D).

Overall these results indicate that low doses of IL-1β during the chondrogenic culture of BM-MSC enhanced the production (accumulation and release) of GAG and the expression of BMP-2 mRNA. Instead high doses of IL-1β reduced extracellular matrix production and chondrogenesis.

Effects of IL-1β under hypoxic culture

BM-MSC were cultured at reduced oxygen percentages (i.e., 5 % and 2 %) under osteogenic and chondrogenic conditions without IL-1β or with 50 pg/mL or 1000 pg/mL IL-1β. At 5 % oxygen, (i) calcium deposition of osteogenically cultured BM-MSC was enhanced following exposure to IL-1β (by 8.0- and 6.8-fold respectively for the concentration 50 pg/mL and 1000 pg/mL) (Fig. 4A), (ii) GAG amounts of pellets was not affected by 50 pg/mL IL-1β but reduced following exposure to 1000 pg/mL IL-1β (by 2.3-fold) (Fig. 4B). At 2 % oxygen, IL-1β (i) enhanced ECM mineralisation (at 50 and 1000 pg/mL) but to lower

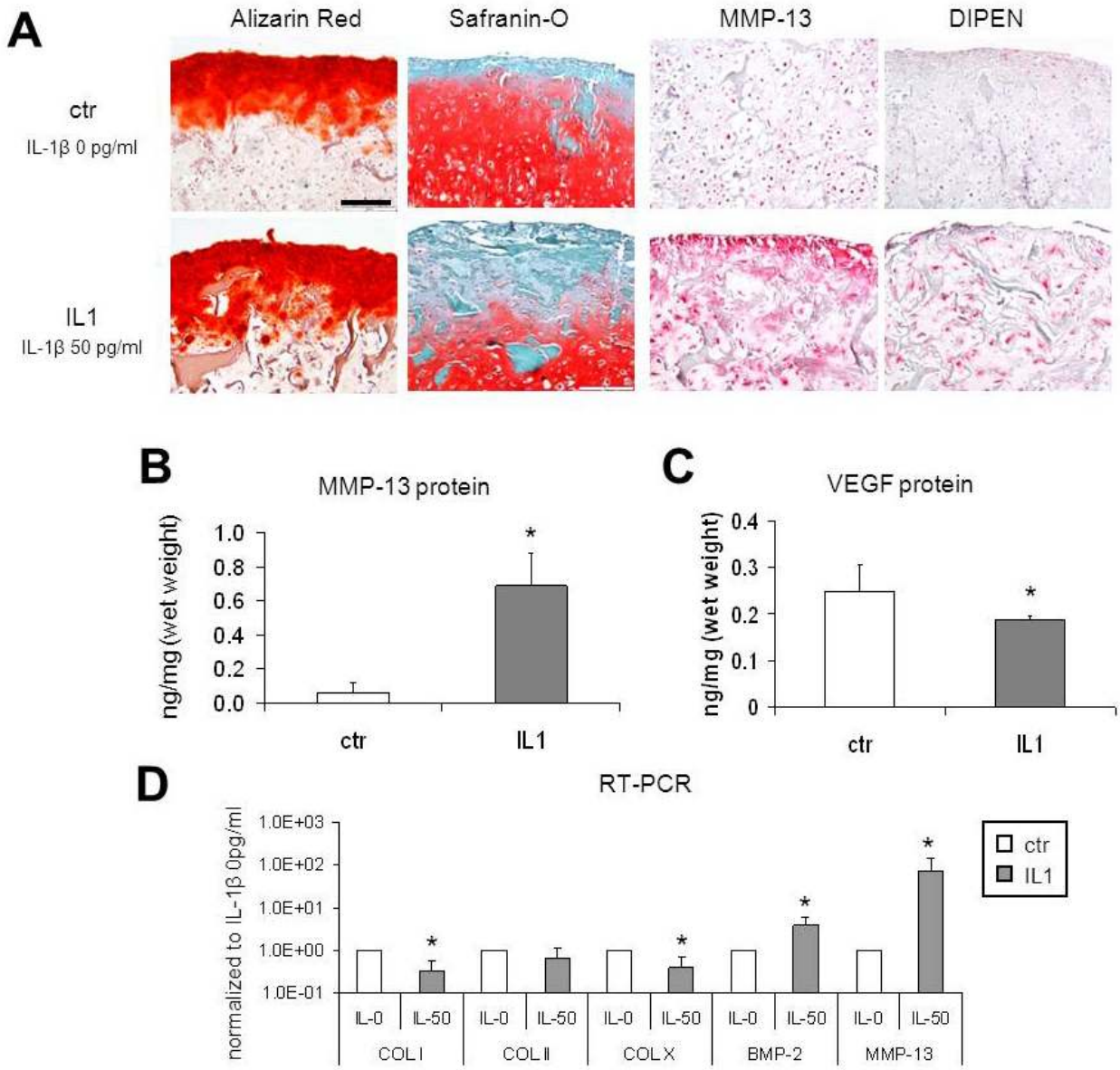

Fig. 5. Effects of IL-1β on *in vitro* formation and remodelling of a newly formed hypertrophic cartilage template by human bone marrow stromal cells (BM-MSC). (**A**) Representative alizarin red, safranin-O, metalloproteinase (MMP)-13 and Aggrecan cryptical epitope (DIPEN) staining of chondrogenic tissues cultured for the last two weeks without or with 50 pg/mL IL-1β. (**B**) MMP-13 protein quantification. (**C**) VEGF protein quantification. (**D**) Real time RT-PCR analysis of the expression of types I, II and X collagens, bone morphogenetic protein (BMP)-2, and MMP-13, levels are normalised to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) level and expressed as fold of difference from those measured in cells not stimulated with IL-1β. Scale bar = 200 μm; $n = 3$ experiments with cells from 3 different donors, 6 specimens analysed. Data are mean ± SD. * = $p < 0.05$ from IL-1β 0 pg/mL.

extents (data not shown), (ii) reduced GAG accumulation by 24.3 % ($p < 0.05$) at 50 pg/mL and to levels close to the limit of detection at 1000 pg/mL.

Effects of IL-1β on the endochondral bone formation

After demonstrating that IL-1β influenced the capacity of BM-MSC to differentiate towards both the chondrogenic and osteogenic lineage, we investigated the effects of this inflammatory chemokine on the maturation/remodelling of hypertrophic cartilage templates and subsequent endochondral bone formation *in vivo*, using our recently published model (Scotti *et al.*, 2010).

***In vitro* hypertrophic cartilage template formation**

Compared to controls, samples exposed to 50 pg/mL IL-1β contained (i) 38 % more calcium (37.95 ±5.47 μg/mg *vs*. 23.42 ±4.02 μg/mg, $p < 0.05$) resulting in a thicker calcified layer (as evidenced by alizarin red, Fig. 5A), (ii) 12 % less GAG (17.72 ±3.98 μg/mg *vs*. 15.59 ±1.6 μg/mg, $p < 0.05$), (iii) 14-fold higher amounts of MMP-13 protein as demonstrated by ELISA. Immunostaining of the extracellular matrix confirmed increased amount of MMP-13 and higher extent of MMP-mediated activity, as assessed by an increased accumulation of the cryptic cleaved fragment of aggrecan (DIPEN) (Fig 5A). MMP-

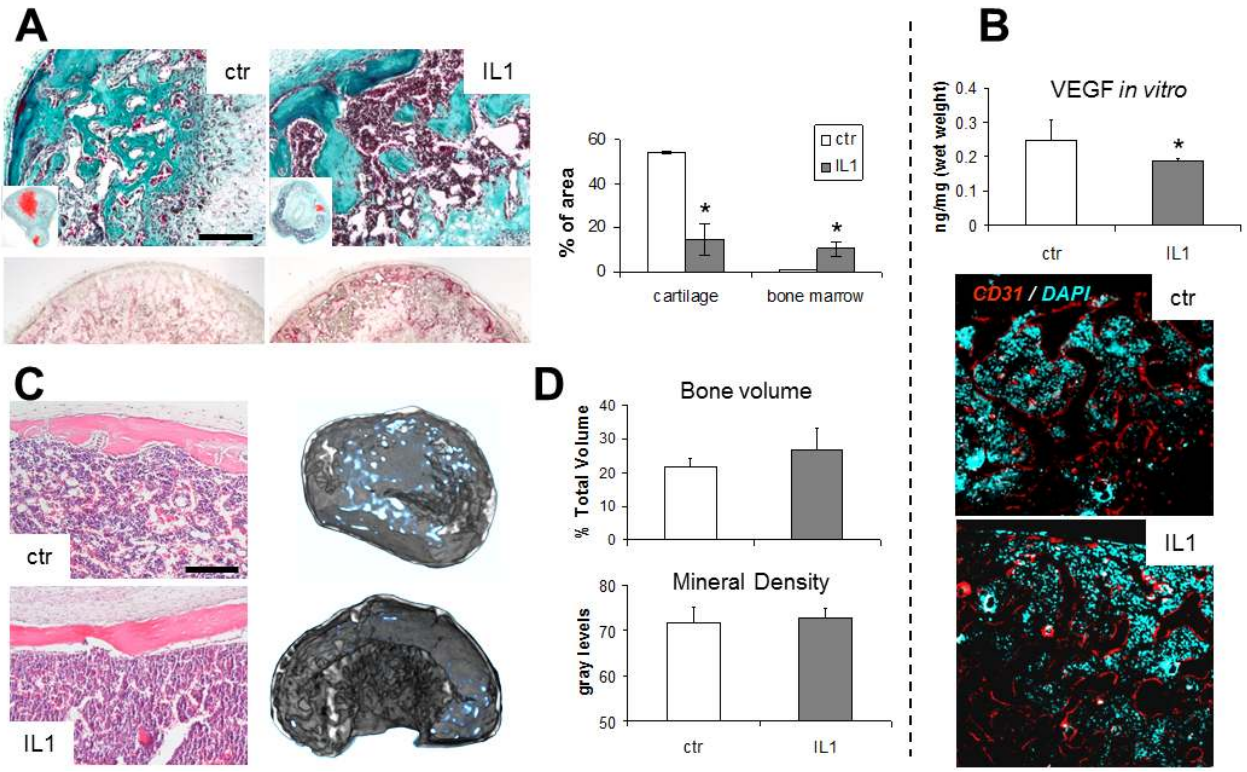

Fig. 6. Effects of IL-1β on the ectopic endochondral bone formation in nude mice of cartilaginous tissues generated with human bone marrow stromal cells (BM-MSC). (**A**) Representative Masson's trichrome and safranin-O (inset) (top) and tartrate-resistant acid phosphatase (TRAP, bottom) stainings and contents of cartilage and bone marrow of tissues harvested after 5 weeks *in vivo*. Scale bar = 200 μm. (**B**) Representative CD31/DAPI staining of constructs after 12 weeks *in vivo*. (**C**) Representative Haematoxylin and Eosin staining (scale bar = 200 μm, left) and 3D reconstructed μCT (right) images of constructs after 12 weeks *in vivo*. (**D**) Quantitative histomorphometric μCT data of constructs after 12 weeks *in vivo*. $n = 2$ experiments with cells from 1 donor, 8 specimens analysed. Data are mean ± SD. * = $p < 0.05$ from IL-1β 0 pg/mL.

13 accumulation within the tissue was also measured and resulted significantly increased in IL-1β treated samples (Fig. 5B). On the contrary, VEGF content was slightly but significantly reduced in IL-1β treated samples (Fig. 5C). Immunostaining for the osteoblastic markers Osterix and osteocalcin increased over time, with no relevant difference between controls and IL-1β treated samples (data not shown). TRAP staining was negative (data not shown). RT-PCR analyses showed that IL-1β treatment did not modify the expression of type II collagen, caused a limited but significant down-regulation of type I collagen (3.2-fold) and type X collagen (2.6-fold), an up-regulation of BMP-2 (3.9-fold) and, in accordance with the biochemical and histological results, a significant and strong up-regulation of MMP-13 mRNA (74.1-fold) (Fig 5D).

Collectively, these results indicated that IL-1β did not enhance the *in vitro* hypertrophic differentiation of BM-MSC but the extent of extracellular matrix calcification and the onset of remodelling of the cartilaginous template at least in part through MMP-13 upregulation.

In vivo tissue development

IL-1β strongly enhanced the remodelling process of the hypertrophic cartilage into bone. In particular, as compared to controls, after 5 weeks *in vivo* IL-1β treated samples showed, (i) reduced safranin-O positive cartilaginous areas (3.6-fold), (ii) larger bone marrow areas (9.1-fold), and (iii) higher density of multinucleated TRAP-positive cells (Fig. 6A). Interestingly, the decrease in VEGF protein within the tissue, measured with ELISA after IL-1β treatment *in vitro*, did not result in an impaired vascularisation *in vivo* (Fig. 6B). As a matter of fact, vessels quantification, performed on CD31-stained sections, showed no difference between controls and IL-1β -treated samples (data not shown).

At the latest *in vivo* time point (12 weeks), both groups could finalise the endochondral process, showing mature bone formation and bone marrow engrafted within the bone trabeculae (Fig. 6C,D). Human cells survived and could be detected within the newly formed bone with ISH for human Alu repeats (data not shown), confirming our previous report (Scotti *et al.*, 2010). Taken together, these data suggest that IL-1β treatment resulted in an accelerated remodelling of the hypertrophic cartilage, ultimately leading to a bone tissue formation similar to that of controls.

Discussion

In this study, we demonstrated that IL-1β modulates the main stages of endochondral/perichondral bone formation of human adult BM-MSC. In particular we reported that

treatment with low dose of IL-1β (50 pg/mL) resulted in: (i) enhanced proliferation and clonogenicity, (ii) enhanced chondrogenic and osteogenic differentiation, (iii) enhanced *in vitro* MMP13-mediated cartilage remodelling and (v) enhanced cartilage resorption (through recruitment of TRAP-positive cells).

In order to achieve an efficient bone fracture healing, tissue repair-competent MSC have first to be recruited to proliferate within an inflammatory milieu. In our clonogenic culture experiment, we have shown that 50 pg/mL IL-1β enhanced the total number of CFU-f and CFU-o as well as the dimension of the resulting colonies. These effects may be partially due to an IL-1β enhanced production of BMP-2. This factor, known to promote mesenchymal progenitor cell proliferation and osteoblastic commitment (Lou *et al.*, 1999; Katagiri *et al.*, 1990) was in fact enhanced in response to inflammatory signals, consistently with previous works (Hess *et al.*, 2009; Cho *et al.*, 2010). In agreement with our findings, Mohanty *et al.* (2010) in their study aimed at assessing changes in the bone marrow during the onset of inflammatory arthritis, observed an increase in CFU-f and CFU-o in the bone marrow of IL1ra-/- *vs*. wild type mice (Mohanty *et al.*, 2010). In contrast, a previous report indicated the inhibitory effects of IL-1β on both the number of BM-MSC derived colonies and colony size (Wang *et al.*, 2002). This discrepancy may be attributed to the modality of IL-1β application (at the time of seeding *vs*. 24 hours after seeding of bone marrow nucleated cells) or the use of different culture medium (DMEM containing 10 % foetal bovine serum *vs*. Iscove's modified Eagle's medium containing 25 % equine serum and hydrocortisone). Indeed, we have also observed in our study that the supplementation of FGF-2 to the culture medium reduced the IL-1β mediated increases of colony numbers and sizes, suggesting the sensitivity of the biological process to accessory signals.

Fracture healing consists of both intramembranous, mainly subperiosteally, and endochondral ossification (Marsell and Einhorn, 2011). We first investigated the effects of IL-1β on the direct osteoblastic differentiation of BM-MSC. Previous studies addressing this issue reported an inhibitory effect of IL-1β as well as of TNF-α on the capacity of *murine* MSC to mineralise the extracellular matrix (Lacey *et al.*, 2009; Lange *et al.*, 2010). The results of our study and of other reports (Hess *et al.*, 2009; Cho *et al.*, 2010), instead, demonstrate that inflammatory cytokines strongly enhanced the mineralisation capacity and the expression of key osteogenic genes by human MSC. Such discrepancy may be due to the inter-species differences in MSC biology (Meisel *et al.*, 2011). Interestingly, we have shown that the IL-1β induced increases of extracellular matrix mineralisation and expression of the osteo-inductive growth factor BMP-2 are caused by the activation of different signalling pathways. While the inhibition of ERK signalling (through the use of U0126) blocked both the IL-1β induced osteogenic responses, the inhibition of NF-κB signalling (through the use of PDTC) blocked significantly only the IL-1β-induced increase of BMP-2 expression. Similarly to our results, Hess *et al.* (2009) showed that a genetic block of the NF-κB pathway inhibits the TNF-α induced increase of BMP-2 expression, but does not block mineralisation of BM-MSC. In order to more extensively elucidate the molecular mechanism of action of IL-1β on this model, further studies on the pathway-associated kinases would be required.

We then investigated the effects of IL-1β on the chondrogenic differentiation of BM-MSC, as the initial stage towards endochondral ossification. In one previous study it was reported that IL-1β decreased proteoglycan synthesis in a dose-dependent manner starting from the lowest dose used (100 pg/mL) (Wehling *et al.*, 2009). Using a broader range of IL-1β concentrations, we instead observed that GAG accumulation by BM-MSC was significantly enhanced at low doses (50 pg/mL), unaffected at intermediate doses (100-250 pg/mL) and significantly reduced at higher doses (≥ 250 pg/mL) of IL-1β. These differences in the IL-1β dose responses can be due to the different type of tissues from which MSC were isolated (bone marrow aspirate from young patients – year range: 24-49 – *vs*. diaphyseal intramedullary reaming of long bone from old patients – year range: 71-78). We recently reported that even a short exposure of 50 pg/mL IL-1β to articular and nasal chondrocytes caused a significant GAG loss (Scotti *et al.*, 2012). However, MSC are less differentiated cells which have osteogenesis as standard differentiation pathway and can more likely respond positively to signals associated with tissue damage, such as inflammation (Caplan and Correa, 2011). Most importantly, this can also be explained by the upregulation of BMP-2, which is crucial for starting the healing process and the formation of the cartilaginous callus (Tsuji *et al.*, 2006). In contrast, chondrocytes are differentiated cells that typically respond to tissue damage with poor regeneration and further damage through MMP-13 upregulation (Goldring *et al.*, 2011).

Since the bone fracture site is a hypoxic environment, we also investigated the effects of IL-1β in BM-MSC cultured at reduced oxygen percentages. We found that IL-1β (50 and 1000 pg/mL) still enhanced ECM mineralisation by BM-MSC osteogenically cultured at oxygen percentages lower than 19 %, a condition known to inhibit osteogenic differentiation of mesenchymal cells (Malladi *et al.*, 2006; D'Ippolito *et al.*, 2006; Hirao *et al.*, 2006; Pattappa *et al.*, 2010; Wang *et al.*, 2011). Instead, IL-1β 50 pg/mL did not alter GAG accumulation at 5 % and slightly reduced it at 2 % oxygen. The latter finding can be due to a more pronounced production of cartilage matrix degradation agents in response to IL-1β under hypoxic conditions. Mathy-Harter *et al.* (2005), in fact, demonstrated that the IL-1β mediated production of nitric oxide by *in vitro* cultured bovine chondrocytes was more pronounced at low (i.e., 1 %) *vs*. atmospheric (i.e., 21 %) oxygen percentages. It is important however to consider that our experimental condition consisting on the culture of BM-MSC under a continuous hypoxic environment does not fully reproduce the variation in oxygen levels during the different stages of fracture healing (Lu *et al.*, 2011).

Endochondral ossification involves differentiation to hypertrophy followed by remodelling of cartilage into bone. We observed that the supplementation of IL-1β during the hypertrophic culture of human BM-

MSC derived chondrogenic tissues did not significantly enhance the expression of type X collagen but markedly increased MMP-13 expression and activity. The absence of an effect on type X collagen is in accordance with a previously published work which compared the effects of IL-1β, TNF-α and macrophage conditioned medium (MCM) on human chondrocytes seeded on a silk scaffold and reported an upregulation of collagen type X only in MCM-treated samples (Sun *et al.*, 2011). It should also be considered that, in our conditions, cell heterogeneity at the different time points may have masked modulation of type X collagen expression. The fact that Welting *et al.* (2011), instead, did find an effect of cycloxygenase-2 (a canonical IL-1β target gene) on hypertrophic differentiation of chondroprogenitors could be due to the use of cells/tissues from animal species other than human. The IL-1β mediated increase in MMP-13 expression and activity is particularly relevant for fracture callus remodelling into bone, since MMP-13 is required for proper resorption of hypertrophic cartilage (Behonick *et al.*, 2007) and subsequent endochondral bone development (Stickens *et al.*, 2004; Kosaki *et al.*, 2007).

As a result of the enhanced *in vitro* extracellular matrix (ECM) pre-processing by MMP-13, we described a faster remodelling of the hypertrophic cartilage, with less cartilaginous ECM and more abundant bone marrow engraftment at the intermediate *in vivo* time point. This last experiment was designed as a proof-of-principle of the *in vivo* effect of *in vitro* pre-treatment of hypertrophic cartilage with IL-1β. Our observation that higher amounts of TRAP-positive cells were present in the IL-1β treated samples reinforces a previous observation on the role of inflammation in chondroclastogenesis and cartilage resorption (Ota *et al.*, 2009). Limitations of this study include: (i) the use of expanded BM-MSC which may not reflect normal behaviour of progenitor cells of the bone marrow; (ii) the *in vitro* nature of the first series of experiments, which again limits its relevance to the normal behaviour of BM-MSC; (iii) the ectopic site of implantation for the *in vivo* experiments which is not the physiological site of bone repair.

The results of this study indicate the concentration-dependent role of IL-1β in regulating the chondrogenic and osteogenic differentiation of human BM-MSC and the remodelling of resulting cartilaginous templates into bone and bone marrow elements. A controlled delivery of IL-1β (e.g., by smart scaffolds) could enhance bone healing by resident MSC as well as improve the engineering of implantable tissues. Further studies are needed to extend the system with the presence of inflammatory cells and other cytokines (Liu *et al.*, 2011), in order to more comprehensively study bone regeneration in an immunocompetent animal model.

Acknowledgements

We are grateful to Dr. Stefan Schären (University-Hospital Basel, Switzerland) for the kind supply of bone marrow samples. We would like to acknowledge the European Union (OPHIS; #FP7-NMP-2009-SMALL-3-246373) and the Swiss National Foundation (NMS1725) for financial support.

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Discussion with Reviewers

Reviewer I: Given the large increase in caspase-3, it would be interesting to know whether cell death increases in pellets treated with high IL-1β. Please comment!
Authors: We did not perform specific analyses to assess IL-1β mediated cell death in BM-MSC during pellet cultures. However the reduction in size and DNA contents of pellets cultured with increasing IL-1β concentrations, the necrotic appearance of the pellets treated with the highest doses of IL-1β as well as the large increase in caspase-3 suggest that IL-1β (at high concentration) induces cell death of chondrogenically cultured BM-MSC.

Reviewer I: What can be inferred from this study on the relationship between IL-1β, FGF and ERK signalling in modulating endochondral ossification?
Authors: The current work was not aimed at studying the relationship between IL-1β, FGF and ERK signalling in modulating endochondral ossification. Our results however suggest that FGF-2 and ERK signalling might modulate some BM-MSC responses to IL-1β. We have in fact shown that: (i) FGF-2 counteracted the IL-1β-mediated proliferation of osteoprogenitor cells (ii) inhibition of ERK counteracted both IL-1β mediated mineralisation increase and BMP-2 upregulation. Since both IL-1β and FGF-2 are present at a bone fracture site, future studies will have to be undertaken to investigate the interaction between these two factors in the endochondral differentiation of BM-MSC.

Reviewer II: What is the impact of your findings for translational medicine?
Authors: Local inflammation is known to play a pivotal role in tissue regeneration, whereby absence, excess or disregulation of inflammatory processes may negatively affect bone repair. In accordance with this clinical evidence, our experimental data suggest that low levels of inflammatory cytokines may enhance the process of fracture healing by promoting chondrogenesis and the subsequent phases of callus formation/remodelling. A local control of inflammation might therefore improve the results of bone regeneration strategies.

Reviewer III: How do you think these findings relate to natural phenomena such as, for instance, fracture repair? May this constitute evidence that very low local amounts of IL-1 may have a role in priming chondrogenesis in the callus?
Authors: Local inflammation is known to play a pivotal role in tissue regeneration, whereby absence, excess or disregulation of inflammatory processes may negatively affect bone repair. In accordance with this clinical evidence, our experimental data suggest that low levels of inflammatory cytokines may enhance the process of fracture healing by promoting chondrogenesis and the subsequent phases of callus formation/remodelling. The work is in line with the increasing recognition that controlled management of inflammation is a crucial target towards enhancement of fracture healing. Our study on the one hand prompts for further investigations to better dissect the role of IL-1 and other cytokines in the different phases of bone regeneration, and on the other hand proposes the *in vitro*/ectopic replication of endochondral ossification as a model to address those critical processes.