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Proinflammatory cytokines inhibit osteogenic differentiation from stem cells: implications for bone repair during inflammation

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Summary

Objective: The effects of inflammation on bone development from mesenchymal stem cells (MSC) are unclear due to the difficulty in isolating MSC. The aim of this study was to develop a MSC isolation method and to determine the *in vitro* effects of interleukin-1β (IL-1β) and tumor necrosis factor α (TNFα) on their osteogenic differentiation.

Methods: Murine MSC were isolated from the limbs of C57/Bl6 mice through collagenase digestion of bone and enriched as the Stem cell antigen (Sca-1)$^+$ CD31$^-$ CD45$^-$ population, using lineage immunodepletion, followed by fluorescence-activated cell sorting (FACS). They were differentiated along the osteoblast linage in the presence or absence of IL-1β and TNFα. Mineralization was measured as was the expression of a number of osteogenic genes by quantitative polymerase chain reaction (PCR).

Results: We show that osteogenic differentiation from the MSC population is suppressed by IL-1β and TNFα. In addition to suppression of bone mineralization, both cytokines inhibited the differentiation-associated increases in alkaline phosphatase (ALP) activity and the gene expression for ALP, α1(I) procollagen, runt-related transcription factor 2 (Runx2) and osterix. However, only TNFα inhibited osteonectin and osteopontin mRNA expression and only IL-1β reduced cell proliferation.

Conclusions: The convenient isolation technique enables the easy generation of sufficient MSC to permit the molecular analysis of their differentiation. We were thus able to show that the proinflammatory cytokines, IL-1β and TNFα, can compromise bone development from this primary MSC population, although with some significant differences. The potential involvement of specific inflammatory mediators needs to be taken into account if optimal bone repair and presumably that of other tissues are to be achieved with MSC.

Key words: Mesenchymal stem cells, IL-1, TNF, Osteoblasts and differentiation.

Initial studies by Friedenstein *et al.*[2] identified cells colony forming unit fibroblasts (CFU-F) in bone marrow stromal populations which were capable of forming colonies of cells morphologically similar to fibroblasts. These mesenchymal stem cells (MSC) were rapidly adherent, non-phagocytic, clonogenic, capable of extended proliferation *in vitro*[2] and had tri-lineage (bone/fat/cartilage) potential[3]. MSC have generated a great deal of recent interest for tissue engineering and therapeutic applications because of their multipotentiality and relative ease of isolation from many tissues[4–7].

Our current understanding of how MSC behave in various environments is limited at best. Their behavior in stem cell niches is a rapidly expanding field of study[7] but MSC interaction within a disease microenvironment is poorly understood. Prior studies have investigated the *in vitro* effects of cytokines, including those with proinflammatory activity, on mesenchymal progenitor populations to determine what influence an inflammatory reaction might have, for example, on bone formation. This question is of potential significance since tissue regeneration is often needed in areas of the body where there is significant damage and hence an inflammatory reaction. Much of this prior *in vitro* analysis has utilized immortalized cell lines or quite heterogeneous populations of bone marrow stromal cells isolated by adherence to plastic[8–10]. As an example of a problem which can arise from the use of cell lines, a number of studies have suggested that MSC populations from normal tissue exhibit anti-proliferative, immunomodulatory and anti-inflammatory effects which have not been observed with precursor cell lines[11,12].

Stem cell antigen (Sca-1), a member of the Ly-6 multigene family, has been used, in combination with other lineage-specific antigens, for the purification of murine MSC[13–15]. Such isolated cells can subsequently be cultured and differentiated into osteoblasts, chondrocytes and adipocytes *in vitro*[16]. We have used an adaption of this approach to isolate and expand a highly enriched population of murine MSC and then compared the effects of the

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key proinflammatory cytokines, interleukin-1β (IL-1β) and tumor necrosis factor α (TNFα), on their osteogenic capacity. We report significant effects of these cytokines in suppressing osteoblast development, both at the differentiation and proliferation levels, although with some specificity in the responses to a particular cytokine.

Materials and methods

All animal experiments were preformed in accordance with Australian law and under the approval of the ethics committee of The University of Melbourne.

MSC ISOLATION AND CULTURE

The isolation protocol for murine MSC was adapted from Short *et al.* and Wong *et al.*[13,17] Briefly, using this protocol femur, tibia and iliac crest were removed from male C57Bl/6 mice aged 4–6 weeks, purchased from Monash University (Clayton, Australia). Following marrow release (mortar and pestle), the bones were washed with phosphate buffered saline (PBS), supplemented with 2% fetal bovine serum (FBS), until all the marrow was removed. Cells were released from bone following collagenase digestion from finely chopped bone and then filtration to remove fragments. The stromal precursor cells were isolated by magnetic bead depletion of lineage-specific populations using antibodies (Pharmingen) against the following surface markers: CD3, CD4, CD5, CD8, Mac-1, B220 and Gr-1; the remaining cells were sorted as Sca-1$^+$ CD31$^-$ CD45$^-$ cells using a fluorescence-activated cell sorting (FACS) Vantage SE flow cytometer (BD Bioscience). In order to increase numbers prior to osteoblast differentiation, the cells were cultured for approximately 14 days in α-MEM supplemented with 20% FBS (Hyclone) and usually passaged 2–4 times. Sca-1$^+$ CD31$^-$ CD45$^-$ cells were also isolated from bone marrow in a similar manner. Photographs of MSC were taken with an AxioCam MRc5 camera on a Axiovert 25 microscope (Zeiss) at 100× magnification.

OSTEOBLAST DIFFERENTIATION ASSAY

Expanded MSC were used between passage 2 and 4 (see above). Triplicate cultures of 1×10^4 cells per well were seeded onto a 12 well plate overnight at 37°C in 5% O_2/10% CO_2. The cells were then cultured in osteogenic medium (OM) (i.e., normal medium (see above) but supplemented with dexamethasone (10 nM), ascorbate-2-phosphate (0.1 mM) and β-glycerol-phosphate (1 M) (Sigma)) and cultured for 4 or 6 weeks at 37°C in 5% O_2/10% CO_2, with the medium being replaced every 3–4 days[18,19]. Cells were also treated with murine IL-1β (0.001–1 ng/ml) or murine TNFα (0.01–10 ng/ml) (R&D Systems) throughout the differentiation assay, and fresh cytokines added at every medium change.

Osteoblast differentiation was determined by alkaline phosphatase (ALP) activity and Von Kossa staining. ALP activity was determined as described elsewhere[20]. In brief, cultures were washed with PBS and fixed in 10% buffered formalin for 10 min, stained for 30 min with fresh, 0.22 μm-filtered, 0.1 M Tris–HCl, pH 8.3, containing 0.6 mg/ml red violet LB salt and 0.1 mg/ml naphthol ASMX-PO_4 dissolved in *N,N*-dimethylformamide (Sigma). Plates were washed and pictures taken by scanning on a GS-710 scanner (Bio-Rad). For the Von Kossa staining, cells were covered with 5% aqueous silver nitrate and placed under UV for 30 min. Cells were washed in water and covered with 5% sodium thiosulphate for 5 min, before washing with water and scanning the plate.

CHONDROCYTE DIFFERENTIATION ASSAY

Expanded MSC were used between passage 2 and 4. Cells were removed from the plate with 0.05% w/v trypsin-EDTA, washed with PBS before being resuspended in serum-deprived medium (α-MEM with 1% final w/v of bovine serum albumin (BSA), supplemented with transferring (100 μg/ml), insulin (10 μg/ml) and low-density lipoprotein (LDL) (20 μg/ml)). Micro-mass cultures were established by pipetting 5×10^5 cells in 45 μl into a 12 well plate and allowing the cells to adhere to the plate for 1 h before adding 1 mL serum-deprived medium with addition of the growth factors transforming growth factor (TGF)-β_3 (10 ng/ml), bone morphogenic protein (BMP)-6 (10 ng/ml) and platelet-derived growth factor (PDGF)-BB (50 ng/ml)[20–23]. Medium was replaced on the micro-mass every 3 days and fresh growth factors were added with

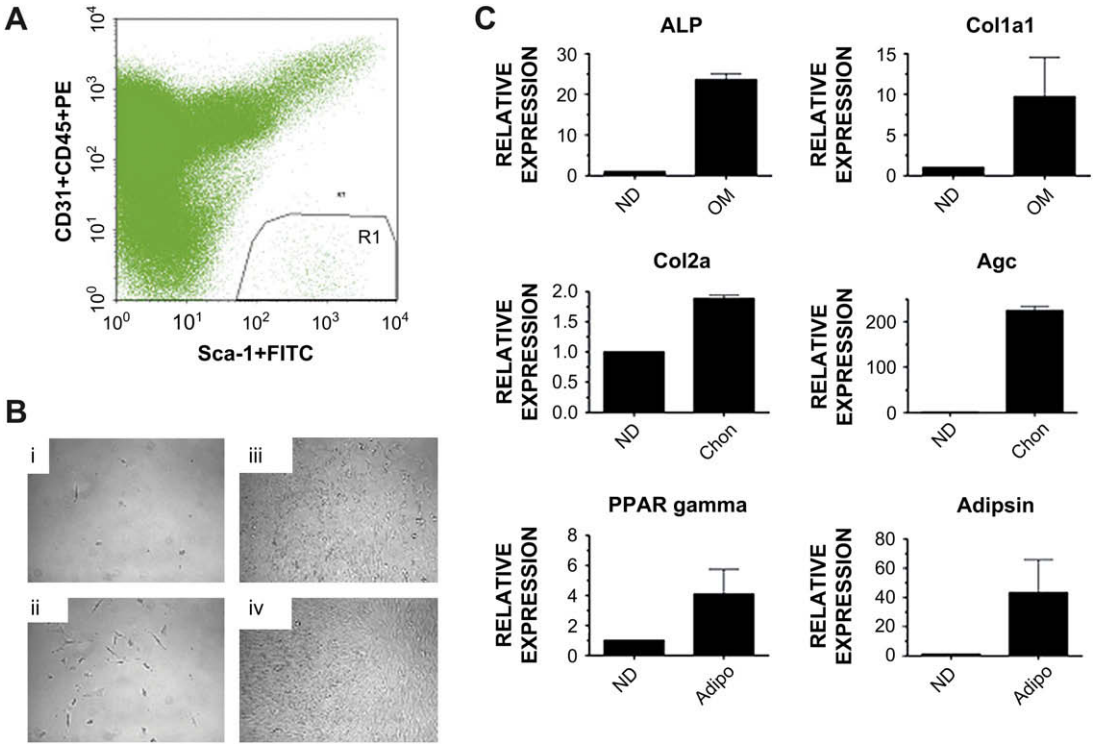

Fig. 1. MSC isolation, expansion and differentiation. (A) MSC were isolated by flow cytometry using positive selection in the final stage as Sca-1$^+$ CD31$^-$ CD45$^-$ cells from murine bone. A representative profile at this stage is provided; cells in R1 were collected. (B) MSC were expanded *in vitro* in normal medium. The cultures of the rapidly growing population can be seen at various stages: (i) initial plating as single cells; (ii) after 3 days; (iii) after 10 days; and (iv) after 12 days. Photographs were taken at 100× magnification on an Axiovert microscope. (C) MSC were differentiated for 7 days in OM or 14 days in either chondrogenic or adipogenic conditions and compared to ND. The expression of the genes associated with (i) osteogenesis, namely ALP and Col1a1, (ii) chondrogenesis, namely Col2a and aggrecan (Agc), and (iii) adipogenesis, namely PPAR gamma and adipisin, was measured by real-time PCR. The expression levels were normalized to those for 18S. Representative examples of three separate experiments; bars represent mean values ± S.E.M.

Table I
Sca-1$^+$ CD45$^-$ CD31$^-$ sorted cell counts from digested bone and bone marrow

Source of cells	Starting cell no. ($\times 10^6$)	Sca-1$^+$ CD45$^-$ CD31$^-$ cells sorted
Bone	54 ± 6	4900 ± 690
Bone marrow	140 ± 20	27 ± 6

each medium change. Assays were run for 2 weeks and then the pellets were washed twice in PBS at which point RNA was isolated.

ADIPOGENIC DIFFERENTIATION ASSAY

Expanded MSC were used between passage 2 and 4. Triplicate cultures of 1×10^4 cells per well were seeded onto a 12 well plate overnight at 37°C in 5% O_2/10% CO_2. The medium was then replaced on the cells to induce differentiation as follows. Initial inductive medium was added for the first 3–4 days of the assay, consisting of Dulbecco's modified essential medium (DMEM) supplemented with 10% v/v horse serum (Gibco) and hydrocortisone (10^{-8} M), indomethacin (60 μM) and 1-isobutyl-3-methylxanthine (IBMX) (500 μM)[24,25]. Medium was replaced after the inductive period with DMEM + 10% horse serum for the remainder of the assay, (typically 2 weeks) at which point RNA was isolated.

RNA COLLECTION AND Q-POLYMERASE CHAIN REACTION (PCR)

Total RNA was isolated from cytokine-treated osteoblast differentiation cultures (day 7), chondrocyte differentiation cultures (day 14) or adipocyte differentiation cultures (day 14) using RNeasy® according to the manufacturer's instructions (Qiagen). Approximately 2 μg total RNA was converted to cDNA using Omniscript RT kit (Qiagen) according to the manufacturer's instructions. PCR was performed on an ABI 7900HT real-time PCR machine (Applied Biosystems). PCR reactions were performed in a total volume of 10 μl in 1× Taqman master mix (Applied Biosystems) with 4 μl cDNA, 1 μl 18S primer, or 1 μl of the following primer sets from Applied Biosystems: runt-related transcription factor 2 (Runx2) (Assay ID Mm00501578_m1, reference sequence NM_009820.3), Osterix (Assay ID Mm00504574_m1, reference sequence mCT52977.2), ALP (Assay ID Mm00475831_m1, reference sequence NM_007431.2), α1(I) procollagen (Col1a1) (Assay ID Mm0801666_g1, reference sequence NM_007742.2), osteopontin (Opn) (Assay ID Mm00436767_m1, sequence reference NM_009263.1), osteonectin (SPARC) (Assay ID Mm00486332_m1, sequence reference NM_009242.1), collagen type II alpha 1 (Col2a1) (Assay ID Mm00491889_m1, sequence reference NM_031163.2) and aggrecan (Agc) (Assay ID Mm00545794_m1, sequence reference NM_007424.2).

Alternatively, PCR reactions for adipocyte genes were performed in a total volume of 10 μl in 1× SYBR green master mix (Applied Biosystems) with 4 μl cDNA and 1 μl of the following primer sets (Geneworks, SA, Australia): adipsin, forward 5′-AGACCCCTACCCTTGCAATACG-3′, reverse 5′-TGTTACCA TTTGTGATGTTTTCGATC-3′; peroxisome proliferator-activated receptor (PPAR) gamma, forward 5′-TTTTCCGAAGAACCATCCGAT-3′, reverse 5′-ACAAATGGTGATTTGTCCGTTG-3′. Gene expression was quantitated relative to *18S* using an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA). Each cDNA sample was detected in experimental triplicate. Negative control reactions were cycled alongside test samples to ensure the absence of contaminating genomic DNA. Data were analyzed using the ABI Prism software, and expression was determined relative to *18S* mRNA. Transcript abundance (*gene*/*18S*) and SD were calculated as recommended by Applied Biosystems. The values for non-differentiated cells (ND) (Fig. 1) or cells treated only with OM (Fig. 3) were set as 1.

CELL PROLIFERATION

Expanded MSC were seeded onto 12 well plates in triplicate cultures at 1×10^4 cells per well overnight at 37°C in 5% O_2/10% CO_2. They were cultured as for the osteoblast differentiation assay (see above) for 7 days. Cells were removed from the plates with trypsin and viable cell numbers counted by trypan blue exclusion.

STATISTICAL ANALYSIS

Statistical analysis was performed using Students' *t* test; $P \leq 0.05$ was considered significant.

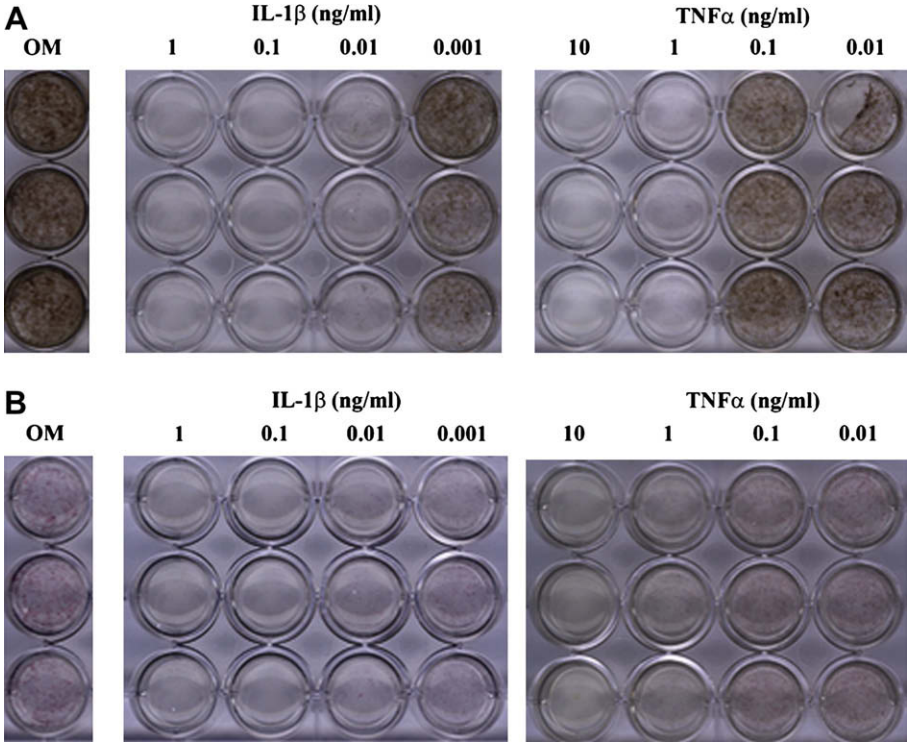

Fig. 2. Effect of IL-1β and TNFα on mineralization and ALP activity in differentiating MSC. Expanded MSC were seeded onto 12 well tissue culture plates and grown in OM and treated with IL-1β (0.001–1 ng/ml) or TNFα (0.01–10 ng/ml) for 4 weeks and stained for (A) calcium deposition (Von Kossa staining) or (B) ALP activity. Representative example of three separate experiments.

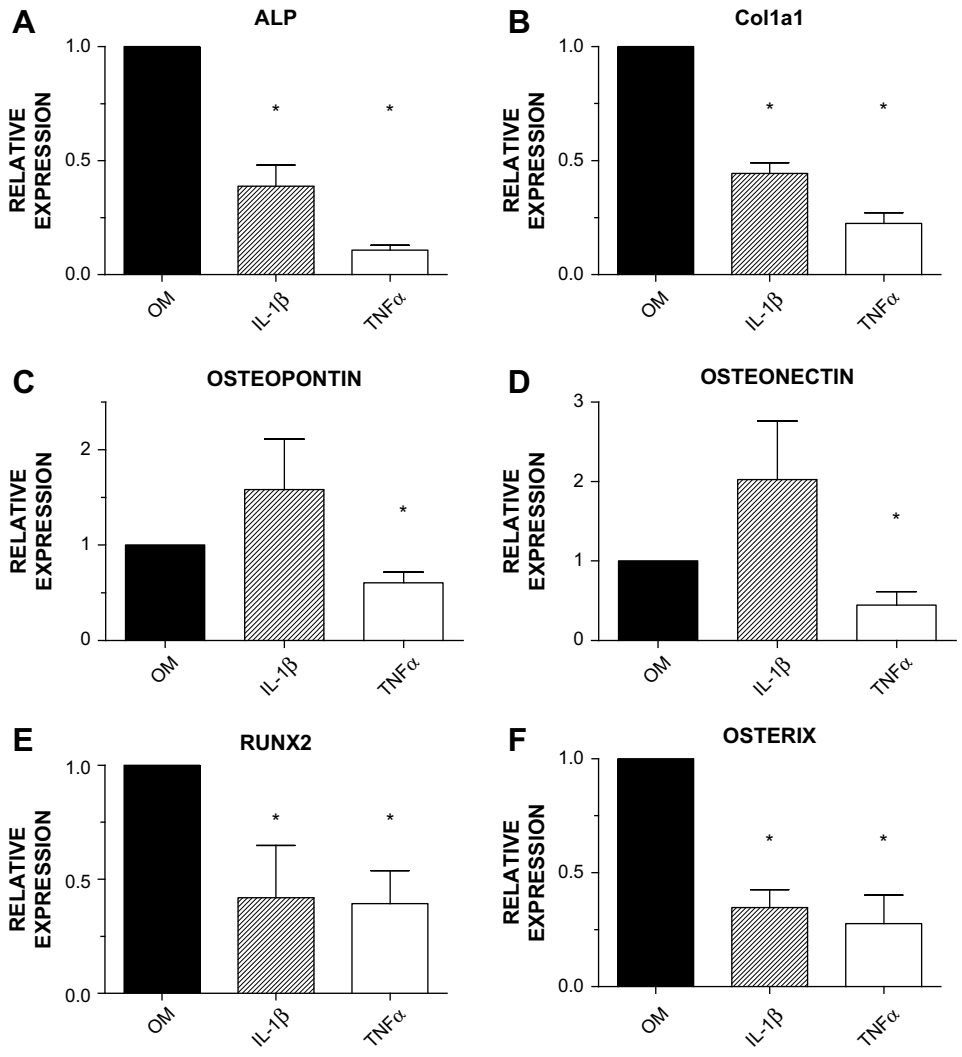

Fig. 3. Effect of IL-1β and TNFβ on osteoblast lineage gene expression in differentiating MSC. Expanded MSC were seeded onto 10 cm tissue culture plates and grown in OM and treated with IL-1β (1 ng/ml) or TNFα (10 ng/ml) for 7 days at which time RNA was isolated and the expression of the osteoblast lineage genes, (A) ALP, (B) Col1a1, (C) osteonectin, (D) osteopontin, (E) Runx2 and (F) osterix, measured by real-time PCR. The expression levels were normalized to those for 18S. Values are presented relative to the control value in OM. Bars represent mean values ± S.E.M. ($n = 5$). *$P < 0.05$.

Results

ISOLATION AND DIFFERENTIATION OF STROMAL PRECURSOR CELLS

The isolation of murine stromal precursor cells is particularly challenging because of, for example, the extremely low incidence of CFU-F (typically in the range of 0.001−0.0001% in bone marrow[3,26]), the relative lack of cell surface markers to aid in their isolation and the difficulties in propagating them in culture[17]. Studies in one of our laboratories have shown that the major reservoir of CFU-F in the adult mouse is the surrounding bone tissue and not the marrow which has allowed the isolation of a highly enriched population of stromal progenitors based on the composite phenotype Sca-1$^+$ Lin$^-$ CD31$^-$ CD45$^-$ that has a plating efficiency for CFU-F of approximately 30%[17]. As an illustration of the final stage of isolation, the FACS profile of the sorted Sca-1$^+$ CD31$^-$ CD45$^-$ population (Materials and methods) is provided in Fig. 1(A); the cells represent approximately 0.01% of the original digested bone cells with an absolute yield between 3000 and 5000 cells from six mice. As a population and at the clonal level, these cells exhibit considerable proliferative potential *in vitro* [Fig. 1(B)] (>50 population doublings) while maintaining their tri-lineage differentiation (bone/fat/cartilage) capacity (see below). We refer for convenience to this enriched population as MSC throughout the paper. That this Sca-1$^+$ CD31$^-$ CD45$^-$ population is more abundant in the bone (1 in 12,000) than in the bone marrow (1 in 6 million) can be seen in Table I. In order to confirm and illustrate that the expanded Sca-1$^+$ CD31$^-$ CD45$^-$ population used in this paper maintained tri-lineage potential, osteoblast, chondrocyte and adipocyte marker genes was measured following differentiation with appropriate stimuli [Fig. 1(C)]. When these cells were differentiated down a particular lineage there was an increase in the expression of genes associated with osteogenesis (ALP and α1(I) procollagen (Col1a1))[19],

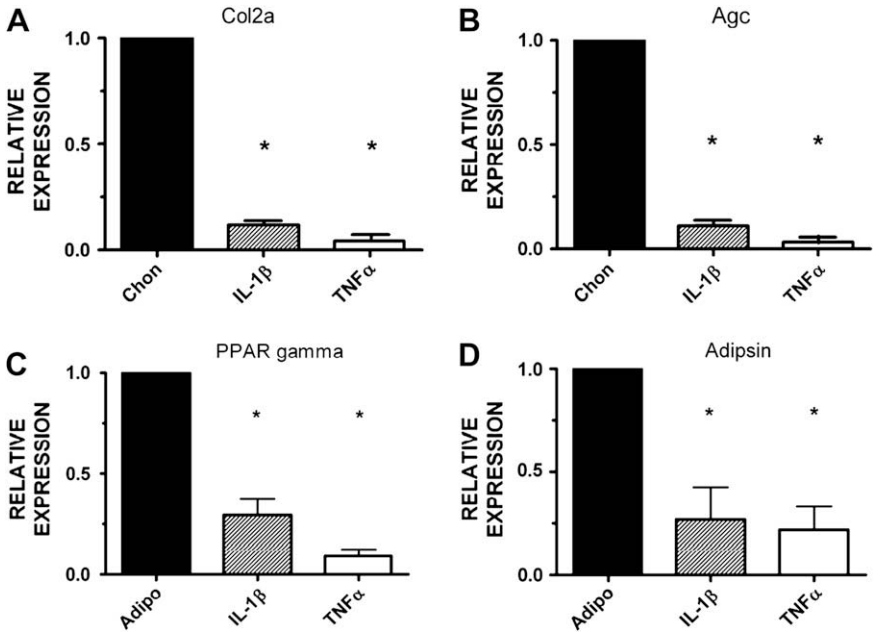

Fig. 4. Effect of IL-1β and TNFβ on chondrocyte and adipocyte lineage gene expression in differentiating MSC. Expanded MSC were differentiated in either chondrogenic (Chon) or adipogenic (Adipo) conditions and treated with IL-1β (1 ng/ml) or TNFα (10 ng/ml) for 14 days at which time RNA was isolated and the expression of lineage-specific genes, (A) Col2a, (B) aggrecan (Agc), (C) PPAR gamma, and (D) adipisin, measured by real-time PCR. The expression levels were normalized to those for 18S. Values are presented relative to the control values. Bars represent mean values ± S.E.M. ($n = 3$). *$P < 0.05$.

chondrogenesis (collagen type IIa (Col2a) and aggrecan)[19], and adipogenesis (PPAR gamma and adipisin)[25], respectively. The comparator for each treatment is undifferentiated cells.

EFFECT OF IL-1 AND TNFα ON OSTEOGENESIS

Mineralization and ALP activity

Osteogenic differentiation of the MSC was carried out, in the absence and presence of IL-1β or TNFα, for 4 weeks after which time mineralization (Von Kossa staining) and ALP activity were measured. MSC cultured in OM alone (Materials and methods) demonstrated significant mineralization [Fig. 2(A)] and had high ALP activity [Fig. 2(B)] indicating extensive differentiation along the osteoblast lineage; treatment of the MSC with either IL-1β or TNFα dose dependently inhibited this differentiation [Fig. 2(A and B)] with the former being more potent on a molar basis.

Osteoblast lineage gene expression

ALP and Col1a1 are widely used markers of osteoblasts, the latter being the most abundant protein in bone[19]. Figure 3(A and B) shows that both IL-1β and TNFα suppress ALP mRNA expression and that for Col1a1. The skeletal specific matrix protein, osteonectin, and osteopontin are also widely used markers of osteoblasts. Figure 3(C and D) shows that TNFα suppressed mRNA expression for both but, interestingly, IL-1β did not.

The expression of the transcription factors, Runx2 and osterix, has been shown to be necessary for osteoblast differentiation at a relatively early stage[17]. In order to begin to explore the mechanism for the effect of the cytokines on osteogenesis in our system, the levels of gene expression for these key transcription factors were monitored. As seen in Fig. 3(E and F), both IL-1β and TNFα reduced mRNA expression for both Runx2 and osterix; IL-1β and TNFα were active even at 0.1 ng/ml and 1 ng/ml, respectively (data not shown).

As a check on how general the suppressive effects of IL-1β and TNFα are on the differentiation of other cell types from MSC, chondrogenic and adipogenic differentiation was carried out, in the absence and presence of IL-1β or TNFα, for 2 weeks and gene expression again measured. Figure 4(A and B) shows that both IL-1β and TNFα suppress Col2a and aggrecan (Agc) mRNA expression as markers of chondrogenesis; likewise both inflammatory cytokines also suppress mRNA expression of the adipocyte markers, PPAR gamma and adipsin [Fig. 4(C and D)].

MSC proliferation

A key element in tissue development is the proliferation from mesenchymal precursors. Alterations in the differentiation along a cell lineage may or may not be accompanied by changes in the rate of cell proliferation and both IL-1β and TNFα can influence this parameter in other cell types[27,28]. We therefore monitored the effects of these cytokines on cell number during MSC osteogenesis. IL-1β treatment inhibited MSC proliferation in a dose dependent manner down to 0.01 ng/ml [Fig. 5(A)]; interestingly, in contrast, TNFα, even at a concentration of 10 ng/ml, had no effect [Fig. 5(B)].

Discussion

MSC have been isolated from many different tissues using numerous techniques; here we have isolated MSC from cortical bone which is different from many reported studies. The most resent study by Sorrentino *et al.* used human

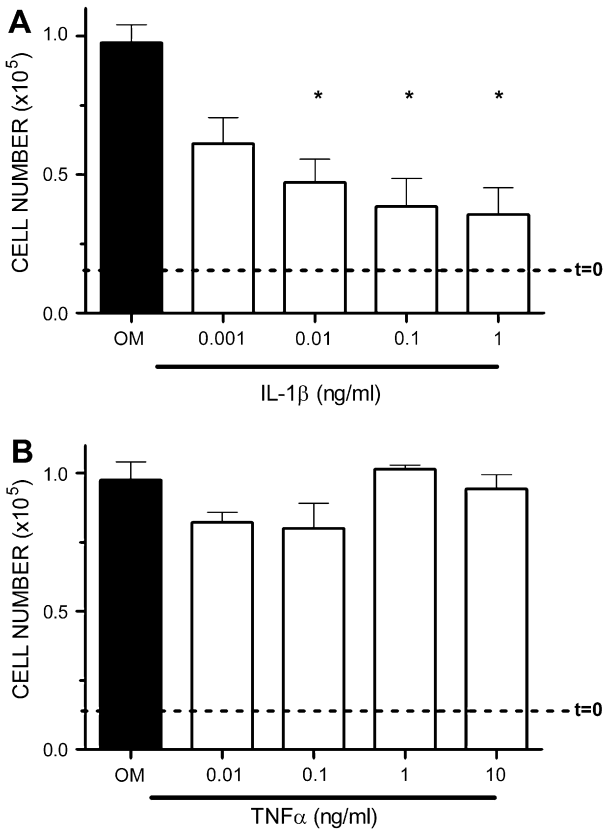

Fig. 5. Effect of IL-1β and TNFα on the proliferation of differentiating MSC. Expanded MSC were seeded onto 12 well tissue culture plates and grown in triplicate cultures in OM and treated with (A) IL-1β (0.001−1 ng/ml) or (B) TNFα (0.01−10 ng/ml) for 7 days. Cells were removed and viable cells counted by trypan blue exclusion. Data from a representative experiment are shown which was repeated three times. Bars represent mean values ± S.E.M. ($n = 3$). *$P < 0.01$.

bone marrow cells and CD146 as a marker of MSC[29]. Their method varies from ours since they first immuno-depleted human bone marrow using anti-CD3, CD14, CD19, CD38, CD66b, and CD34 antibodies before culturing the enriched cells for 2 weeks, at which point $CD146^+$ cells were used in experiments. In comparison we have used mouse cells from cortical bone as opposed to bone marrow; in addition, we have used a wider selection of immuno-depleting antibodies and positively selected $Sca\text{-}1^+$ cells before culturing our enriched population.

The aim of our study was to determine how a highly defined MSC population might behave in an inflammatory environment and therefore to determine the feasibility of using MSC to repair tissue in an inflammatory microenvironment. In general, even though the receptors for IL-1β and TNFα are quite different they can still activate some common intracellular pathways leading to similar but not identical cellular responses[30]. Prior studies examining the effects of IL-1β and TNFα on osteogenesis have usually not directly compared these proinflammatory cytokines and have usually used stem cell lines or heterogeneous populations of bone marrow stromal cells. We used primary cultures of an enriched, bone-derived population of highly proliferative $Sca\text{-}1^+$ $CD31^-$ $CD45^-$ progenitor cells and have shown for the first time with such cells that both IL-1β and TNFα can inhibit their osteogenesis, albeit with some differences. Both cytokines suppressed mineralization, ALP activity and mRNA expression for Col1a1, Runx2 and osterix, with IL-1β being generally effective at a lower concentration than TNFα. However, IL-1β, unlike TNFα, could not prevent osteonectin and osteopontin gene expression during differentiation; in contrast, IL-1β, but not TNFα, reduced the number of osteoblast-like cells able to be generated by proliferation in the OM. Thus the effects of IL-1β and TNFα on osteogenesis from the progenitor cells need to be considered individually and our data indicate that they are likely therefore to influence osteogenesis differently at sites of inflammation (see below). As the cell population used is an enriched population, the possibility that the differences seen are due to alternate effects on different cell types cannot be excluded.

There are data suggesting that Runx2 is upstream of osterix and that this pathway governs osteoblast differentiation[1]. Our data showing that the suppressive effects of IL-1β and TNFα on the gene expression of both transcription factors are consistent with a link between them and with the suppressive effects of TNFα on their gene transcription in calvaria and in cell lines[8,10,31–35]. The discordant effect of the cytokines on osteopontin gene expression is surprising given the evidence from Kim *et al.* where over-expression of osterix in NIH3T3 fibroblasts increased osteopontin expression[36] and highlights the importance of using relevant primary cell cultures where possible. Our data indicate that for some reason the suppressive effect of IL-1β on the Runx2/osterix pathway does not lead to reduced osteonectin or osteopontin gene expression. We observed similar inhibitory effects of IL-1β and TNFα on the gene expression for chondrocyte (Col2a and Agc) and adipocyte (PPAR gamma and adipisin) differentiation.

There are many situations where bone needs to be regenerated following damage, for example, during fracture, localized osteolysis around prosthetic implants, and joint disease. The regenerative capabilities of MSC make them ideal for such repair in these scenarios. However, in many such situations where new bone needs to be formed, the milieu is likely to contain many cytokines, including those with proinflammatory activity, such as IL-1β and TNFα, due to the concomitant damage and host response to such damage[37]. Therefore it is likely that the successful regeneration of bone will require a consideration and hence control of this associated inflammatory milieu. Our findings above support this contention by showing that the key proinflammatory cytokines, IL-1β and TNFα, can have profound consequences for normal bone development from progenitor cells both at the differentiation and proliferation levels. Therefore we suggest that much more understanding of the environment surrounding osteoblast progenitors, such as MSC, is required before successful bone regeneration becomes a routine reality. The development of this isolation technique accelerates the generation of reasonable numbers of MSC to aid in this goal. These MSC should be useful for understanding further the molecular mechanisms governing specific cytokine modulation of MSC differentiation *in vitro* and hopefully *in vivo*.

Patients suffering from rheumatoid arthritis, for example, can experience two forms of bone loss, namely that of a generalized nature[38], leading to osteoporosis, and periarticular osteolysis in areas in close proximity to inflamed joints[39,40]. Osteoclasts are commonly considered to be mainly responsible for such reduced bone mass; however, our work highlights the potential inhibitory effects of proinflammatory cytokines on the anabolic actions of MSC.

This inhibition of MSC differentiation to osteoblasts by IL-1β and TNFα could play a role in both types of bone loss and may have relevance for inflammatory conditions, such as rheumatoid arthritis.

Author contribution

Contributions: DCL designed the research, performed experiments, analyzed results and composed this manuscript. PJS initiated this project and supervised the project. SEG initiated this project, supervised the project and edited the manuscript. JAH initiated this project, supervised the project, designed the research and composed this manuscript.

Conflict of interest

The authors indicate no potential conflict of interest.

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