

EXTENDED REPORT

FGF2 induces RANKL gene expression as well as IL1 β regulated MHC class II in human bone marrow-derived mesenchymal progenitor stromal cells

Chiara Bocelli-Tyndall,^{1,2} Emanuele Trella,¹ Audrey Frchet,³ Paul Zajac,¹ Dennis Pfaff,³ Jeroen Geurts,⁴ Stefan Heiler,^{1,5} Andrea Barbero,¹ Marcus Mumme,¹ Therese J Resink,³ Stefan Schaeeren,¹ Giulio C Spagnoli,¹ Alan Tyndall²

Handling editor Tore K Kvien

► Additional material is published online only. To view please visit the journal online (<http://dx.doi.org/10.1136/annrheumdis-2013-204235>).

¹Departments of Surgery and of Biomedicine, University Hospital Basel, Basel, Switzerland

²Department of Rheumatology, University Hospital Basel, Basel, Switzerland

³Department of Biomedicine, Signal Transduction, University Hospital Basel, Basel, Switzerland

⁴Orthopaedic Department, Osteoarthritis Research Center, University Hospital Basel, Basel, Switzerland

⁵Department of Biomedicine, Developmental and Molecular Immunology, University of Basel, Basel, Switzerland

Correspondence to

Dr Chiara Bocelli-Tyndall, Department of Rheumatology, University Hospital Basel, Basel 4031, Switzerland; Chiara.Tyndall@usb.ch

Received 5 July 2013

Revised 18 September 2013

Accepted 26 October 2013

Published Online First

18 November 2013

ABSTRACT

Objective Human bone marrow mesenchymal stromal cells (hBM-MSC) are being applied in tissue regeneration and treatment of autoimmune diseases (AD). Their cellular and immunophenotype depend on isolation and culture conditions which may influence their therapeutic application and reflect their in vivo biological functions. We have further characterised the phenotype induced by fibroblast growth factor 2 (FGF2) on healthy donor hBM-MSC focusing on the osteoimmunological markers osteoprotegerin (OPG), receptor activator of nuclear factor κ B (RANK), RANK ligand (RANKL) and HLA-DR and their regulation of expression by the inflammatory cytokines IL1 β and IFN γ .

Methods RANK, RANKL, OPG and HLA-DR expression in hBM-MSC expanded under specific culture conditions, were measured by RT-PCR and flow cytometry. MAPKs induction by FGF2, IL1 β and IFN γ in hBM-MSC was analysed by immunoblotting and RT-PCR.

Results In hBM-MSC, OPG expression is constitutive and FGF2 independent. RANKL expression depends on FGF2 and ERK1/2 activation. IL1 β and IFN γ activate ERK1/2 but fail to induce RANKL. Only IL1 β induces p38MAPK. The previously described HLA-DR induced by FGF2 through ERK1/2 on hBM-MSC, is suppressed by IL1 β through inhibition of CIITA transcription. HLA-DR induced by IFN γ is not affected by IL1 β in hBM-MSC, but is suppressed in articular chondrocytes and lung fibroblasts.

Conclusions RANKL expression and IL1 β regulated MHC-class II, both induced via activation of the ERK1/2 signalling pathway, are specific for progenitor hBM-MSC expanded in the presence of FGF2. HLA-DR regulated by IL1 β and ERK1/2 is observed on hBM-MSC during early expansion without FGF2 suggesting previous in vivo acquisition. Stromal progenitor cells with this phenotype could have an osteoimmunological role during bone regeneration.

INTRODUCTION

Human mesenchymal progenitor stromal cells (hMSC) isolated from bone marrow (BM) or other sources, have been studied in the laboratory for their multilineage cell differentiation potential and their ability to suppress lymphocyte proliferation.^{1,2} Due to these properties, mesenchymal stromal cells (MSC) are being investigated for therapeutic and commercial applications.^{3–8}

In most cases, the hMSC are expanded in culture. Despite multiple cell divisions, they retain in vitro cell differentiation and antiproliferative properties. Fibroblast growth factor 2 (FGF2) is a pleiotropic growth factor involved in several cellular pathways during embryogenesis and in adult life.^{9–10} FGF2, although described as an inhibitor of cellular senescence,¹¹ is also a potent cell mitogen. The addition of FGF2 to hBM-MSC cultures achieves high cell yields and favours enrichment of more progenitor cells.¹²

FGF2 participates in several bone metabolic processes, including skeletogenesis,¹³ osteocyte differentiation^{14–15} and osteogenic differentiation of MSC.¹⁶ FGF2 also selects MSCs best suited for ectopic bone formation.¹⁷

Healthy bone is a balance between bone formation by osteoblasts and bone resorption by osteoclasts. The alteration of this balance is a feature of some autoimmune diseases (AD) such as rheumatoid arthritis (RA) and of degenerative bone conditions. In the cells involved in bone metabolism, the expression of the receptor activator of nuclear factor κ B (RANK), its ligand (RANKL) and osteoprotegerin (OPG), the RANKL decoy receptor, is central in bone remodelling by the immune system in health and disease.^{18–19} In the BM niche, stromal cells expressing OPG and RANKL participate in bone homeostasis as precursors of osteoblasts while supporting haematopoietic cell differentiation and osteoclast precursors.²⁰ In the BM, activated T cells, whether cytokine or antigen driven, express RANKL, whereas BM macrophages and osteoclasts express RANK.^{20–22} RANKL binding to RANK on preosteoclasts activates osteoclast maturation and osteoclastogenesis. Progenitor hBM-MSC constitutively express OPG.^{21–23}

Inflammatory cytokines such as IL1 β and interferon gamma (IFN γ) regulate bone homeostasis as mediators of cross talk between the bone and the immune system.¹⁸ In differentiated cells, IL1 β can suppress IFN γ induced HLA-DR,²⁴ and IFN γ can inhibit IL1 β binding and signalling with implications for immunological responses and cytokine-induced tissue destruction.²⁵

We^{26–27} and others^{28–29} have reported that in cultured hBM-MSC FGF2 induces expression of major histocompatibility complex (MHC)-classII (HLA-DR), a phenotypic marker of antigen presentation in haematopoietic and non-haematopoietic



CrossMark

To cite: Bocelli-Tyndall C, Trella E, Frchet A, et al. *Ann Rheum Dis* 2015;**74**:260–266.

cells, constitutive in professional antigen-presenting cells and inducible by IFN γ in most cells. The induction of HLA-DR by FGF2 in vitro occurs only in hMSC (but not in mouse MSC) and requires the activation of ERK1/2 and cell proliferation. Neither of these conditions are required for the induction of HLA-DR by IFN γ .²⁷ Expression of HLA-DR is observed also in some hBM-MS-C during early in vitro expansion without FGF2²⁷ and could represent a residual in vivo FGF2 or IFN γ induced phenotype.

Although the HLA-DR on the hBM-MS-C is functional, its significance when induced by FGF2 is not known. Adipose derived hMSC also respond to FGF2 with HLA-DR expression,²⁷ but stromal cells from the Wharton's jelly of the umbilical cord do not express HLA-DR under induction of either FGF2 or IFN γ .^{30 31}

In this study, we have investigated RANK, RANKL and OPG gene expression in hBM-MS-C expanded in vitro with or without FGF2 and in the presence of IL1 β and IFN γ . We also investigated whether BM-MS-C HLA-DR expression induced by FGF2 and by IFN γ could be affected by IL1 β .

MATERIALS AND METHODS

Cells, growth factors and cytokines

Human BM-MS-C were obtained from healthy donors, and in vitro expanded as previously described in complete 10% fetal bovine serum (FBS) α MEM, defined as control (CTR) medium.²⁷ To prevent senescence-related effects,³² the cells were not expanded for more than three passages. FGF2 (5 ng/mL, R&D) was added when stated.

IL1 β (Sigma-Aldrich), IFN γ (R&D), IL1Ra (R&D) and the inhibitors UO126 (Sigma-Aldrich), PTDC (Sigma-Aldrich) and SB203580 (Cell Signaling Technology) were used as described in the figure legends.

RT-PCR

RT-PCR was performed as previously reported²⁷ from cDNA isolated from hBM-MS-C expanded under the conditions

described in the figure legends. RT-PCR primers and probes for the human genes: β -actin, indoleamine 2,3-dioxygenase (IDO), CIITA, RANK, RANKL and OPG were purchased from Assays on Demand, Gene Expression products (Applied Biosystems).

Western blotting (WB)

BM-MS-C were grown to semiconfluence in complete (10% FBS) α MEM, then maintained for 24 h in α MEM without FBS, before pulsing with FGF2, IL1 β and IFN γ . Immunoblotting of whole cell lysates was performed as described previously.³³ The following primary antibodies were used: rabbit antiphospho^{thr202/tyr204}-ERK1/2, rabbit antiphospho^{ser473}-Akt, rabbit antiphospho^{thr180/tyr182}-p38MAPK (Cell Signaling Technology) and goat anti-GAPDH (Abcam, UK) as the internal protein loading control. Secondary HRP-conjugated goat antirabbit IgG (Southern Biotechnology) or donkey antigoat IgG (Santa Cruz Biotechnology) together with Amersham ECL (Amersham Biosciences, UK) were used to detect immunoreactive proteins. Scanned images of autoradiograms were analysed using AIDA Image or Scion (NIH) Image software.

Fluorescence-activated cell sorting (FACS)

This was performed as previously described.³⁴ All antibodies were from BD (Switzerland).

Statistical analysis

Data are reported as the mean \pm SD. Comparisons between two groups were performed by Student 2-tailed t test.

RESULTS

FGF2 induces RANKL expression via the ERK1/2 signalling pathway in hBM-MS-C

The expression of OPG, RANKL and RANK was tested by RT-PCR in hBM-MS-C (from five healthy donors) which had been expanded as follows: without FGF2 (CTR, figure 1A), in the continuous presence of FGF2 (FGF, figure 1B), or with addition of FGF2 after initial expansion under CTR conditions²⁷

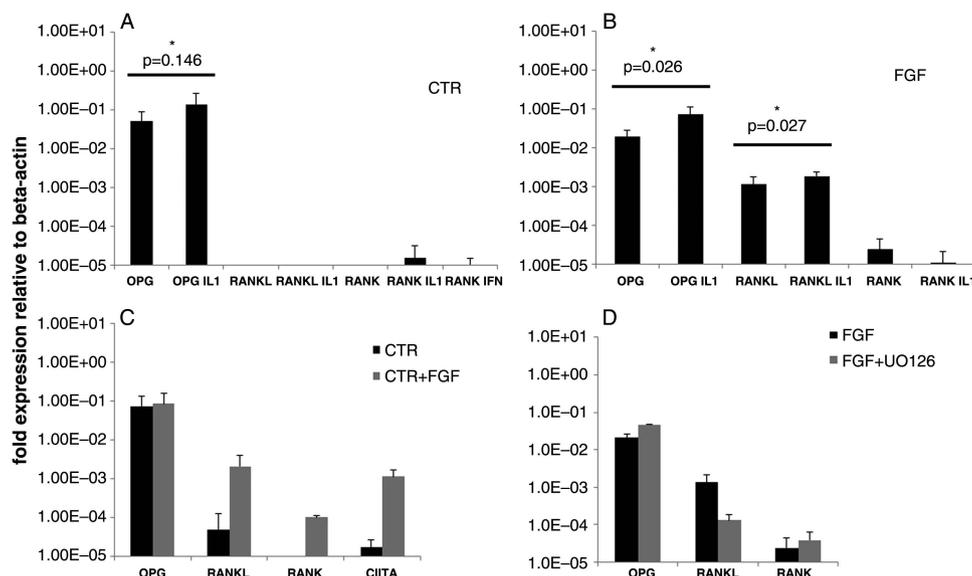


Figure 1 Modulation of receptor activator of nuclear factor κ B (RANK), RANK ligand (RANKL) and osteoprotegerin (OPG) mRNA expression (measured by RT-PCR) in bone marrow mesenchymal stromal cells (BM-MS-C) expanded: (A) without fibroblast growth factor 2 (FGF2) (CTR), (B) with FGF2 (FGF) (5 ng/mL) or (C) without FGF2 as in (A) with successive addition of FGF2 (CTR+FGF). Incubation with FGF2 (in CTR+F, (C)) and IL1 β (IL1) (1 ng/mL) or IFN γ (IFN) (10 ng/mL) was for 96 h. (D) Effect of the MEK1-2 inhibitor UO126 (10 μ M) on RANK, RANKL and OPG mRNA expression in FGF2-expanded BM-MS-C as in (B). Cells were incubated with UO126 for 96 h. Each histogram represents the relative expression of each gene under every cell culture condition. * p <0.05=significant.

(CTR+F, figure 1C). OPG was constitutively expressed at similar levels under all three culture conditions. RANKL and RANK were expressed only in the presence of FGF2 although RANK was induced at much lower levels ($\leq 10\times$) than RANKL (figure 1B,C). IL1 β moderately increased OPG (figure 1A,B) and RANKL expression (figure 1B). In the absence of FGF2, IL1 β was unable to induce either RANKL or RANK expression (figure 1A). FGF2 also induced CIITA in the CTR+FGF2 cells (figure 1C) as expected.²⁷ In hBM-MSC expanded without FGF2, IFN γ did not induce RANK (figure 1A) nor significantly modified OPG and RANKL expression (not shown). UO126, an inhibitor of MEK1-2³⁵ (figure 2E), reduced significantly the expression of RANKL but not of RANK in FGF2 expanded hBM-MSC (figure 1D).

IL1 β lowers CIITA transcription and HLA-DR expression induced by FGF2 in hBM-MSC

Addition of IL1 β during hBM-MSC expansion (from five donors) with FGF2, reduces the expression of HLA-DR (figure 2A). The IL1 β doses chosen, (50–1000 pg/mL), which are physiologically relevant, have previously been shown to induce *in vivo* and *in vitro* mineralisation enhancement during osteogenic differentiation.³⁶ Competition for IL1R1 binding with IL1Ra, the IL1R1 antagonist, shows that IL1 β reduces HLA-DR by binding to its own receptor (figure 2B). Intercellular adhesion molecule (ICAM) (CD54), important in immunological intercellular interactions, is expressed in hBM-MSC independently of FGF2 and

is upregulated by IL1 β (figures 2C and 3C). IL1Ra inhibits the upregulation of CD54 by IL1 β , confirming the specific binding of IL1 β to IL1R1. Expression of FGFR1, the main FGF2 receptor on these cells under these conditions,²⁷ is not downregulated by IL1 β (figure 2F). Cell proliferation, a necessary condition for the maintenance of FGF2-induced HLA-DR,²⁷ is not reduced.

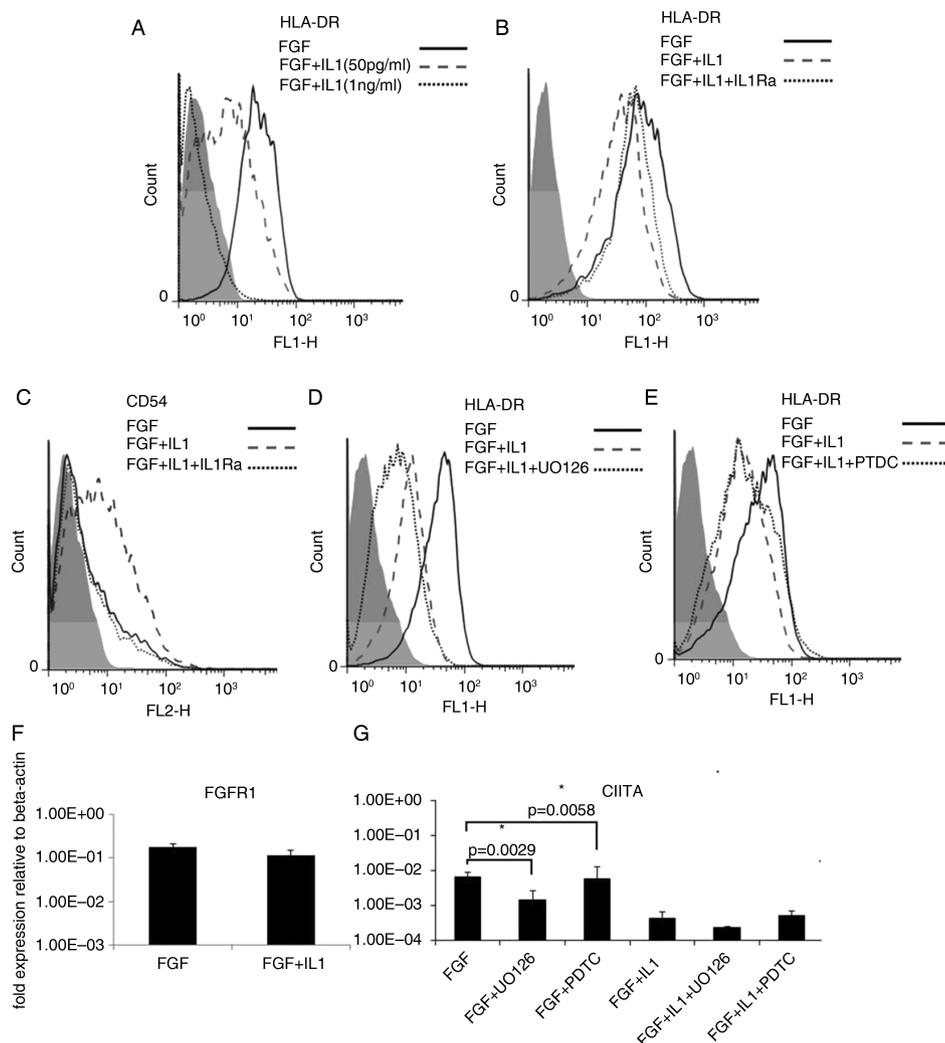
CIITA expression is necessary for the induction of HLA-DR in all cells. We have previously shown that this is the case also when HLA-DR is induced by FGF2 in hBM-MSC.²⁷ IL1 β significantly inhibits CIITA expression in these cells (figure 2G).

The expression of CIITA by FGF2 in hBM-MSC occurs through the activation of the ERK1/2 signalling pathway.²⁷ Inhibition of MEK1-2/ERK1/2 with UO126 significantly reduces the expression of CIITA (figure 2G) and HLA-DR (figure 2D). Simultaneous addition of IL1 β and UO126 further reduced CIITA transcription and HLA-DR expression (figure 2G,D), while simultaneous addition of PTDC, an inhibitor of NF- κ B, induced by IL1 β in most cells, did not further reduce either CIITA (figure 2G) or HLA-DR (figure 2E) expression.

IL1 β lowers neither HLA-DR nor indoleamine 2,3-dioxygenase (IDO) expression induced by IFN γ in hBM-MSC

Induction of HLA-DR by IFN γ in hBM-MSC occurs via activation of the Jak/Stat signalling pathway,³⁷ does not require cell proliferation²⁷ and is independent of MEK1-2/ERK1/2.²⁷ IFN γ

Figure 2 HLA-DR expression, measured by FACS, in fibroblast growth factor 2 (FGF2) expanded bone marrow mesenchymal stromal cells (BM-MSC) (FGF) in presence of (A) IL1 β (IL1), (B) IL \pm IL1Ra, (D) IL1 \pm MEK1-2 inhibitor UO126 or (E) IL1 \pm NF κ B inhibitor PTDC. (C) CD54 (ICAM) expression, measured by FACS, in FGF BM-MSC in presence of IL1 \pm IL1+Ra. Filled grey histograms are control IgG. RT-PCR analysis of relative expression of (F) FGF2 receptor 1 (FGFR1) and (G) CIITA in FGF BM-MSC \pm IL1 \pm UO126 or PTDC. Each histogram represents the relative expression of each gene under every cell culture condition. FGF2: 5 ng/mL; IL1: 1 ng/mL; IL1Ra: 1–10 ng/mL; UO126, PTDC: 10 μ M. Incubation times in all cases was for 96 h. * $p < 0.05$ =significant.



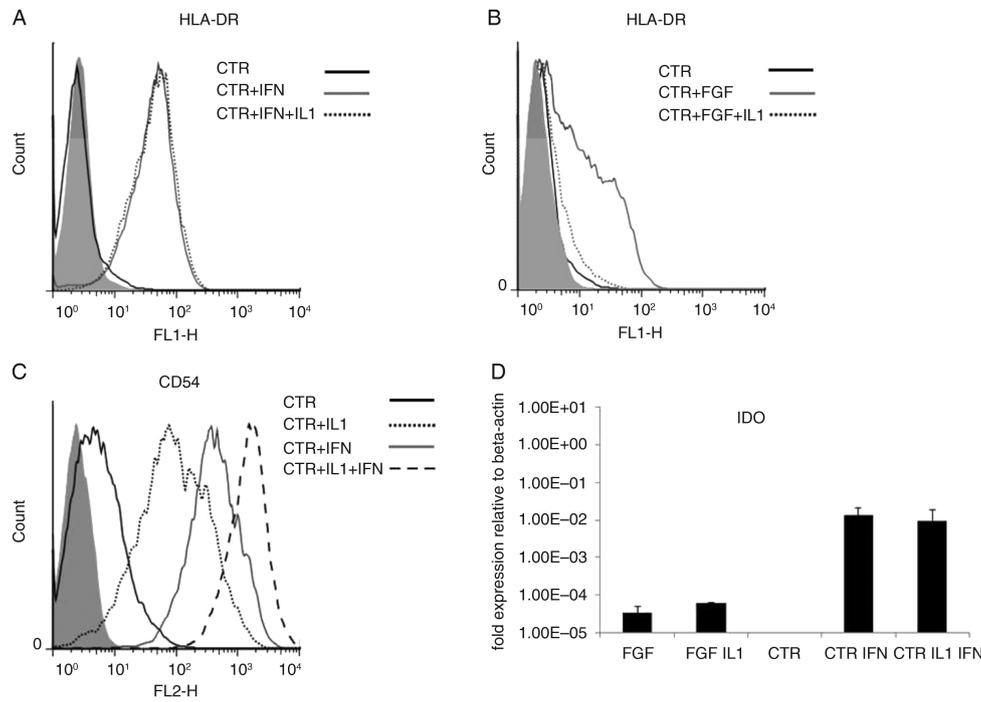


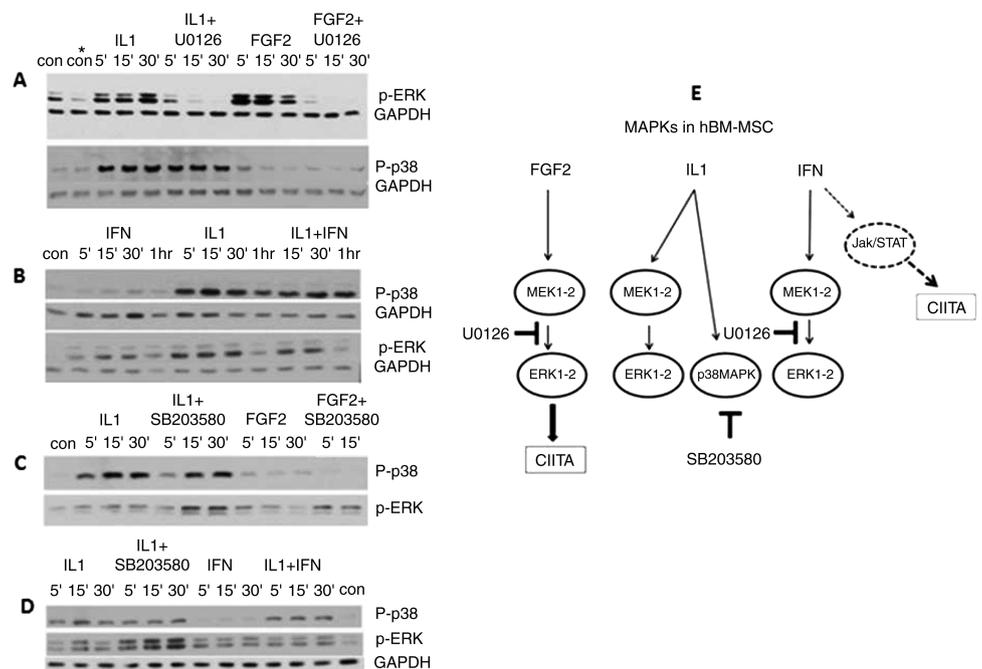
Figure 3 Effect of IL1 β (IL1) on HLA-DR expression, measured by FACS, in human bone marrow mesenchymal stromal cells (hBM-MSC) expanded without fibroblast growth factor 2 (FGF2) (CTR condition), to which (A) IFN γ (IFN)(5–10 ng/mL) or (B) FGF2(CTR+FGF)(5 ng/mL) were subsequently added with or without IL (1–5 ng/mL). Simultaneous incubation with each cytokine and FGF2 (in CTR+F) was for 96 h. Alternatively, the cells were preincubated with IL1 for 24 h before addition of IFN. (C) CD54(ICAM) expression in BM-MSC with or without IL1 \pm IFN. Filled grey histograms are control IgG. (D) Relative expression, measured by RT-PCR, of indoleamine 2,3-dioxygenase (IDO) enzyme in: FGF2 expanded BM-MSC alone(FGF) or with IL1 addition (FGF IL), and BM-MSC expanded without FGF2 (CTR), to which IFN (CTR IFN) or IFN+IL1 (CTR IL1 IFN) were added for 96 h at concentrations as above.

induces in hBM-MSC also IDO, an inhibitor of lymphocyte proliferation and, possibly, a mediator of the immunosuppressive effect of the hBM-MSC.³⁸

We queried whether IL1 β would suppress the induction of HLA-DR and of IDO by IFN γ in hBM-MSC. The IFN γ concentrations used (5–10 ng/mL) were chosen for their physiological

significance, consistent with already published literature.^{24–27} The addition of IL1 β and IFN γ , either simultaneous or with addition of IFN γ after preincubation with IL1 β , to hBM-MSC (from five different donors) expanded without FGF2 in CTR conditions, did not lower the HLA-DR induced by IFN γ , as exemplified in figure 3A. IL1 β alone did not induce any

Figure 4 (A–D) WB analysis of the MAPK proteins (ERK1/2 and p38) induced in bone marrow mesenchymal stromal cells (BM-MSC) by fibroblast growth factor 2 (FGF2) (5 ng/mL), IL1 β (IL1)(1 ng/mL) and IFN γ (IFN)(10 ng/mL). UO126: MEK1-2 inhibitor (10 μ M), SB203580: p38 inhibitor (10 μ M); P-p38: phosphorylated p38 (MW: 40 kDa), P-ERK1/2: phosphorylated ERK1-2 (MW: 42–44 kDa). GAPDH (MW: 35 kDa): internal protein control. Time intervals of exposure to each factor are indicated above each panel. In experiments with kinase inhibitors, the cells were preincubated with these (4–18 h) before being pulsed with either FGF2 or the cytokine. (E) Schematic diagram of the MAPKs activated by FGF2, IL1 and IFN in BM-MSC and of the inhibitory activity of UO126 or SB203580 in the ERK1/2 signalling pathway. Elements drawn in broken line represent published data not included in this study.



HLA-DR (not shown). When FGF2 rather than IFN γ was added to the same cells, HLA-DR was expressed and suppressed by IL1 β as expected (figure 3B). RT-PCR experiments showed also no reduction of CIITA expression due to IFN γ in the presence of IL1 β (data not shown). The failed suppression by IL1 β of IFN γ -induced HLA-DR was not due to the lowering by IFN γ of the expression of the IL1 β receptor IL1R1, since CD54, upregulated by either IL1 or IFN γ , was synergistically increased when both cytokines were present (figure 3C).

Additionally, IL1 β does not suppress IFN γ -induced expression of the IDO enzyme (figure 3D) and, therefore, the subsequent BM-MSc associated lymphocyte antiproliferative effect.

MAPKs activated in BM-MSc by IL1 β , FGF2 and IFN γ

IL1 β is recognised to activate members of the MAPK family of serine/threonine kinases, and in a cell context manner.³⁹ Activation of the ERK1/2 signalling pathway is required by IL1 β for in vitro osteogenic differentiation of hBM-MSc and to upregulate mineralisation genes,³⁶ and by FGF2 to induce CIITA/HLA-DR and RANKL. At the same time, IL1 β suppresses CIITA expression induced by FGF2 as shown above. One publication using WB analysis, shows that IL1 β does not activate ERK1/2 in hBM-MSc.⁴⁰ To our knowledge, no data exist with respect to the MAPKs induced by IFN γ in hBM-MSc. Therefore, we investigated by WB analysis whether the ERK1/2, p38 and JNK MAPKs were activated by FGF2, IL1 β and IFN γ in hBM-MSc. Figure 4 shows representative results from six experiments, which were consistent, although quantitative variations occurred in the amount of phosphorylated kinase induced each time due to donor and cell culture variations.

FGF2, IL1 β and IFN γ activate ERK1/2

JNK activation was not induced by either FGF2 or IL1 β (data not shown). Its induction by IFN γ was not tested. Figure 4 shows that FGF2, IL1 β (4A) and IFN γ (4B,D) all activated ERK1/2 within 5 min of addition to the cells. The presence of the MEK1-2 inhibitor UO126 severely impaired ERK1/2 phosphorylation induced by both FGF2 and IL1 β , as expected (figure 4A).

Only IL1 β activates p38MAPK

Phosphorylation of the p38MAPK was induced by IL1 β (figure 4A–D) but not FGF2 (figure 4A,C) and IFN γ (figure 4B,D). IFN γ does not affect p38-induced phosphorylation induced by IL1 β . SB203580 inhibits only the catalytic activity of

phosphorylated p38, but not its phosphorylation by upstream kinases.⁴¹ However, SB203580 increases the amount of phosphorylated pERK1/2 induced by IL1 β or FGF2 (figure 4C,D).

The HLA-DR expressed on BM-MSc expanded without FGF2 is suppressed by IL1 β and the MEK1-2 inhibitor UO126

HLA-DR positive (≤ 5 –10%) hBM-MSc have been observed in about 10% of the donors, also at the earliest expansion passage without FGF2. Such positivity disappears with further passaging or increases if FGF2 is added.²⁷ Whether such positivity derives from previous in vivo exposure to IFN γ or FGF2 is not known. However, the discriminatory effect of MEK1-2 inhibition and IL1 β on the HLA-DR induced by either factor should provide now some clarity.

BM-MSc from three healthy donors (32–50 years old) showed at the first expansion passage in the absence of FGF2 (or IFN γ) a significant HLA-DR positivity (20–30% positive cells), which diminished over successive expansion passages.

Addition of FGF2 amplified the original HLA-DR positive population (figure 5A) which was suppressed by both UO126 and IL1 β as exemplified in figure 5B,C respectively. These results suggest that the original HLA-DR was induced via the ERK1/2 signalling pathway and was not due to IFN γ .

DISCUSSION

Although hBM-MSc have rapidly gone from the bench to the patient bedside for the treatment of a variety of AD and tissue degenerative conditions, knowledge as to their biology and the mechanisms underlying their functions is incomplete. The definition of the progenitor phenotype of the cells which are infused in vivo is arbitrary and dependent on the isolation method, culture conditions and cell source.

In view of the above translational applications, the osteoimmunological profile of the cells will be critical. This will depend on the combination of cytokines and growth factors encountered in the eventual inflammatory niche.

We^{26, 27} and others³⁷ have reported that hBM-MSc expanded with FGF2 express HLA-DR. This HLA-DR is functional.²⁷

The OPG/RANKL/RANK axis and its modulation is pivotal in bone formation in health and disease and is crucial for the development of new drugs against bone-related disorders.⁴²

Human osteoblasts, but not BM-MSc, express RANKL.^{21, 23} However, BM-MSc produce constitutively the decoy receptor of RANKL, OPG, which competes with RANK for RANKL

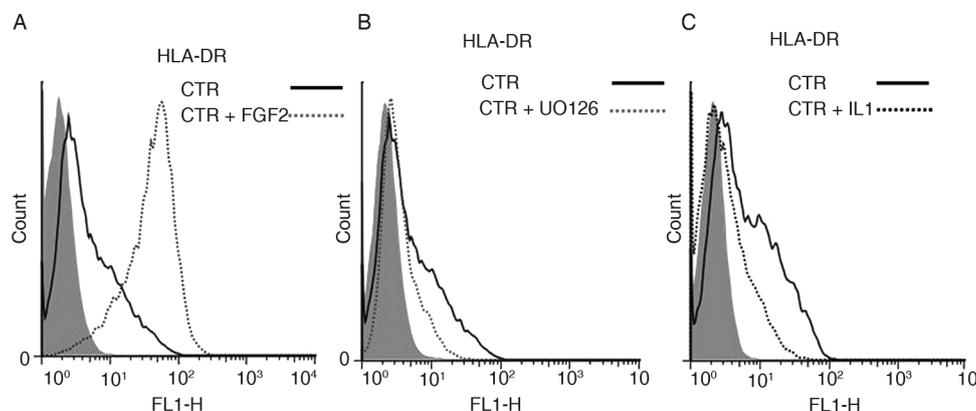


Figure 5 HLA-DR expression, measured by FACS, in human bone marrow mesenchymal stromal cells (hBM-MSc) expanded without fibroblast growth factor 2 (FGF2) (CTR condition), to which (A) FGF2, (B) the MEK1-2 inhibitor UO126 and (C) IL1 β (IL1) were subsequently added. In (C) and (A): same donor CTR BM-MSc but at different expansion passage and different baseline HLA-DR expression. Filled grey histograms are control IgG.

binding thereby controlling RANKL-induced osteoclastogenesis.²³ We show for the first time that hBM-MSC express RANKL and, to a much lower level, RANK, when FGF2 is added to the cells (figure 1B,C). RANKL, but not RANK, expression depends on ERK1/2 activation by FGF2 (figure 1D). Induction of RANKL/RANK expression was observed by others in hBM-MSC exposed to conditioned medium from activated T cells²¹ suggesting a role of inflammatory T cells in osteoblastic differentiation. Annexin II, a factor promoting osteoclast formation, has been reported also to stimulate in hBM-MSC RANKL mRNA and protein via ERK1/2.⁴³ The significance, if any, of low RANK mRNA expression in hBM-MSC is unknown. We confirm that OPG expression is constitutive and unaffected by FGF2. FGF2-induced RANKL mRNA is moderately increased by IL1 β (figure 1B), a cytokine often associated with osteoclastogenesis and bone erosion. IFN γ , which exerts an antiosteoclastogenic activity,⁴⁴ does not significantly affect expression of either OPG or RANKL (data not shown).

In hBM-MSC, in parallel with the induction of RANKL, FGF2 induces via ERK1/2 also the expression of CIITA and HLA-DR.²⁷ We then asked whether IL1 β , secreted by monocytes, osteoclast precursors and in inflammation, would affect the expression of HLA-DR induced by either FGF2 or IFN γ .

We report for the first time that, in hBM-MSC, IL1 β specifically inhibits CIITA transcription and HLA-DR expression only when FGF2-induced. IL1 β suppresses neither HLA-DR (figure 3A) nor IDO enzyme expression induced by IFN γ (FGF2 induces no IDO expression in BM-MSC) (figure 3D), but upregulates CD54 (ICAM) in both FGF2 and IFN γ treated cells (figure 3C).

The ERK1/2 signalling pathway appears central for the induction of RANKL and CIITA by FGF2 in hBM-MSC. It mediates also the upregulation by IL1 β of mineralisation genes in osteogenic differentiations.³⁶ We therefore investigated by immunoblotting the MAPKs activated by FGF2, IL1 β and IFN γ in hBM-MSC. We show for the first time that FGF2, IL1 β and IFN γ all activate ERK1/2 (figure 4A–D). Yet, only FGF2 induces RANKL and CIITA expression via ERK1/2; IFN γ does not require ERK1/2 for CIITA/HLA-DR expression.²⁷

Among FGF2, IL1 β and IFN γ , only IL1 β activates p38MAPK (figure 4C). In hBM-MSC, inhibition of cellular p38MAPK results in increased levels of pERK1/2 activated by FGF2 (figure 4C,D) or endogenous (not shown). Yet, in FGF2 expanded hBM-MSC, inhibition of cellular p38MAPK does not affect CIITA mRNA, but lowers HLA-DR expression. This occurs also when HLA-DR is induced by IFN γ in cells expanded without FGF2 (see online supplementary figure S2, supplementary data). These data suggest that p38 controls HLA-DR expression in hBM-MSC at an unidentified post-CIITA transcriptional level. P38 alone, when activated by IL1 β , cannot therefore be responsible for the lowering of ERK1/2 activation dependent CIITA transcription. On the other hand, the latter is suppressed by IL1 β in synergy with the inhibition of MEK1-2 (by UO126, figure 2G) and of p38 (by SB203580, online supplementary figure S2B, supplementary data), suggesting an additional control by IL1 β on the CIITA transcription induced by FGF2 in hBM-MSC.

By inducing RANKL mRNA, FGF2 seems to skew the hBM-MSC toward an osteoblastic phenotype. The characterisation of the correspondent RANKL protein with respect to conditions of expression, cellular location and ratio to OPG, is necessary for better evaluating its biological and translational significance. These results, however, suggest caution in the use of hBM-MSC as therapeutic tools in the treatment of

inflammatory disorders involving bone loss, such as RA.^{23 45} They could express RANKL in vivo in the presence of either FGF2, which is ubiquitously present especially during new bone and vascular formation, or of inflammatory lymphocytes.²¹ This effect of FGF2 should be investigated also on adipose tissue-derived MSC, which do require FGF2 for their expansion in vitro⁴⁶ and are often used in translational applications.

In hBM-MSC, the expression via ERK1/2 of RANKL and CIITA/HLA-DR are specific to FGF2. Platelet derived growth factor (PDGF) type BB, often simultaneously present in vivo with FGF2, also induces HLA-DR in hBM-MSC,²⁷ but has not been tested either for RANKL induction or HLA-DR sensitivity to IL1 β .

Mitogenic proliferation via ERK1/2 activation, is necessary for HLA-DR expression induced by FGF2; whether it is necessary also for the expression of RANKL is not known. Although in the presence of FGF2, CIITA/HLA-DR and RANKL are simultaneously expressed and decline in parallel with cell passing, there is no causal relationship between them because CIITA gene silencing with shRNA does not inhibit RANKL induction by FGF2 (unpublished results).

The expression of RANKL and IL1 β -regulated CIITA/HLA-DR on progenitor hBM-MSC proliferating under the effect of FGF2, could represent the osteoimmunological phenotype of BM-MSC populations building cell mass at a specific phase of bone regeneration. T cells activated by antigen receptor engagement express RANKL and influence bone metabolism.⁴⁷ The HLA-DR/RANKL/OPG expressing hBM-MSC could interact with the CD4 T-cells, and influence the osteoblasts/osteoclasts balance. HLA-DR on hBM-MSC due to either FGF2 or IFN γ is equally functional,²⁷ and its discriminate suppression by IL1 β may reflect different temporal and/or cell differentiation stages. In differentiated cells of mesodermal origin, chondrocytes and fibroblasts, FGF2 cannot induce CIITA/HLA-DR²⁷ and IL1 β suppresses HLA-DR due to IFN γ (see online supplementary figure S1A and B, supplementary data).

Finally, the hypothesis that HLA-DR positive hBM-MSC may occur in vivo, has been suggested by reports that non-expanded (though selected for a stem cell marker expression) hBM-MSC are HLA-DR positive but lose their positivity on passaging in culture.^{27 28} Our results showing that the HLA-DR positivity found on some hBM-MSC expanded without FGF2 requires the activation of the ERK1/2 (figure 5B) signalling pathway and is sensitive to IL1 β (figure 5C), further support this hypothesis.

Acknowledgements We thank Professor Ivan Martin, Department of Surgery and Biomedicine, University Hospital Basel, for his constructive discussions and logistics support.

Competing interests None.

Provenance and peer review Not commissioned; externally peer reviewed.

REFERENCES

- 1 Le Blanc K. Mesenchymal stromal cells: tissue repair and immune modulation. *Cytotherapy* 2006;8:559–61.
- 2 Uccelli A, Moretta L, Pistoia V. Mesenchymal stem cells in health and disease. *Nat Rev Immunol* 2008;8:726–36.
- 3 Liang J, Gu F, Wang H, et al. Mesenchymal stem cell transplantation for diffuse alveolar hemorrhage in SLE. *Nat Rev Rheumatol* 2010;6:486–9.
- 4 Tolar J, Le Blanc K, Keating A, et al. Concise review: hitting the right spot with mesenchymal stromal cells. *Stem Cells* 2010;28:1446–55.
- 5 Keyszer G, Christopheit M, Fick S, et al. Treatment of severe progressive systemic sclerosis with transplantation of mesenchymal stromal cells from allogeneic related donors: report of five cases. *Arthritis Rheum* 2011;63:2540–2.
- 6 Connick P, Kolappan M, Crawley C, et al. Autologous mesenchymal stem cells for the treatment of secondary progressive multiple sclerosis: an open-label phase 2a proof-of-concept study. *Lancet Neurol* 2012;11:150–6.

- 7 Liang J, Zhang H, Wang D, *et al.* Allogeneic mesenchymal stem cell transplantation in seven patients with refractory inflammatory bowel disease. *Gut* 2012;61:468–9.
- 8 Martin I, Baldomero H, Bocelli-Tyndall C, *et al.* The survey on cellular and engineered tissue therapies in Europe in 2010. *Tissue Eng Part A* 2012;18:2268–79.
- 9 Eswarakumar VP, Lax I, Schlessinger J. Cellular signaling by fibroblast growth factor receptors. *Cytokine Growth Factor Rev* 2005;16:139–49.
- 10 Thisse B, Thisse C. Functions and regulations of fibroblast growth factor signaling during embryonic development. *Dev Biol* 2005;287:390–402.
- 11 Couto DL, Francois M, Galipeau J. Inhibition of cellular senescence by developmentally regulated FGF receptors in mesenchymal stem cells. *Blood* 2011;117:6801–12.
- 12 Bianchi G, Banfi A, Mastrogiacomo M, *et al.* Ex vivo enrichment of mesenchymal cell progenitors by fibroblast growth factor 2. *Exp Cell Res* 2003;287:98–105.
- 13 Marie PJ, Miraoui H, Severe N. FGF/FGFR signaling in bone formation: progress and perspectives. *Growth Factors* 2012;30:117–23.
- 14 Kyono A, Avishai N, Ouyang Z, *et al.* FGF and ERK signaling coordinately regulate mineralization-related genes and play essential roles in osteocyte differentiation. *J Bone Miner Metab* 2012;30:19–30.
- 15 Ling L, Murali S, Dombrowski C, *et al.* Sulfated glycosaminoglycans mediate the effects of FGF2 on the osteogenic potential of rat calvarial osteoprogenitor cells. *J Cell Physiol* 2006;209:811–25.
- 16 Miraoui H, Oudina K, Petite H, *et al.* Fibroblast growth factor receptor 2 promotes osteogenic differentiation in mesenchymal cells via ERK1/2 and protein kinase C signaling. *The J Biol Chem* 2009;284:4897–904.
- 17 Tasso R, Gaetani M, Molino E, *et al.* The role of bFGF on the ability of MSC to activate endogenous regenerative mechanisms in an ectopic bone formation model. *Biomaterials* 2012;33:2086–96.
- 18 Takayanagi H. Osteoimmunology: shared mechanisms and crosstalk between the immune and bone systems. *Nat Rev Immunol* 2007;7:292–304.
- 19 Lorenzo J, Horowitz M, Choi Y. Osteoimmunology: interactions of the bone and immune system. *Endocr Rev* 2008;29:403–40.
- 20 Pacifici R. T cells: critical bone regulators in health and disease. *Bone* 2010;47:461–71.
- 21 Rifas L, Arackal S, Weitzmann MN. Inflammatory T cells rapidly induce differentiation of human bone marrow stromal cells into mature osteoblasts. *J Cell Biochem* 2003;88:650–9.
- 22 Di Rosa F, Pabst R. The bone marrow: a nest for migratory memory T cells. *Trends Immunol* 2005;26:360–6.
- 23 Oshita K, Yamaoka K, Udagawa N, *et al.* Human mesenchymal stem cells inhibit osteoclastogenesis through osteoprotegerin production. *Arthritis Rheum* 2011;63:1658–67.
- 24 Rohn W, Tang LP, Dong Y, *et al.* IL-1 beta inhibits IFN-gamma-induced class II MHC expression by suppressing transcription of the class II transactivator gene. *J Immunol* 1999;162:886–96.
- 25 Hu X, Ivashkiv LB. Cross-regulation of signaling pathways by interferon-gamma: implications for immune responses and autoimmune diseases. *Immunity* 2009;31:539–50.
- 26 Larghero J, Farge D, Braccini A, *et al.* Phenotypical and functional characteristics of in vitro expanded bone marrow mesenchymal stem cells from patients with systemic sclerosis. *Ann Rheum Dis* 2008;67:443–9.
- 27 Bocelli-Tyndall C, Zajac P, Di Maggio N, *et al.* Fibroblast growth factor 2 and platelet-derived growth factor, but not platelet lysate, induce proliferation-dependent, functional class II major histocompatibility complex antigen in human mesenchymal stem cells. *Arthritis Rheum* 2010;62:3815–25.
- 28 Tarte K, Gaillard J, Lataillade JJ, *et al.* Clinical-grade production of human mesenchymal stromal cells: occurrence of aneuploidy without transformation. *Blood* 2010;115:1549–53.
- 29 Sotiropoulou PA, Perez SA, Gritzapis AD, *et al.* Interactions between human mesenchymal stem cells and natural killer cells. *Stem Cells* 2006;24:74–85.
- 30 Nekanti U, Mohanty L, Venugopal P, *et al.* Optimization and scale-up of Wharton's jelly-derived mesenchymal stem cells for clinical applications. *Stem Cell Res* 2010;5:244–54.
- 31 Prasanna SJ, Gopalakrishnan D, Shankar SR, *et al.* Pro-inflammatory cytokines, IFN-gamma and TNF-alpha, influence immune properties of human bone marrow and Wharton jelly mesenchymal stem cells differentially. *PLoS ONE* 2010;5:e9016.
- 32 Cipriani P, Di Benedetto P, Liakouli V, *et al.* Mesenchymal stem cells (MSCs) from scleroderma patients (SSc) preserve their immunomodulatory properties although senescent and normally induce T regulatory cells (Tregs) with a functional phenotype: implications for cellular-based therapy. *Clin Exp Immunol* 2013;173:195–206.
- 33 Kuzmenko YS, Kern F, Bochkov VN, *et al.* Density- and proliferation status-dependent expression of T-cadherin, a novel lipoprotein-binding glycoprotein: a function in negative regulation of smooth muscle cell growth? *FEBS Lett* 1998;434:183–7.
- 34 Bocelli-Tyndall C, Barbero A, Candrian C, *et al.* Human articular chondrocytes suppress in vitro proliferation of anti-CD3 activated peripheral blood mononuclear cells. *J Cell Physiol* 2006;209:732–4.
- 35 Favata MF, Horiuchi KY, Manos EJ, *et al.* Identification of a novel inhibitor of mitogen-activated protein kinase kinase. *J Biol Chem* 1998;273:18623–32.
- 36 Mumme M, Scotti C, Papadimitropoulos A, *et al.* Interleukin-1beta modulates endochondral ossification by human adult bone marrow stromal cells. *Eur Cell Mater* 2012;24:224–36.
- 37 Stagg J, Pomme S, Eliopoulos N, *et al.* Interferon-gamma-stimulated marrow stromal cells: a new type of nonhematopoietic antigen-presenting cell. *Blood* 2006;107:2570–7.
- 38 Meisel R, Zibert A, Laryea M, *et al.* Human bone marrow stromal cells inhibit allogeneic T-cell responses by indoleamine 2,3-dioxygenase-mediated tryptophan degradation. *Blood* 2004;103:4619–21.
- 39 Dinarello CA. Biologic basis for interleukin-1 in disease. *Blood* 1996;87:2095–147.
- 40 Carrero R, Cerrada I, Lledo E, *et al.* IL1beta induces mesenchymal stem cells migration and leucocyte chemotaxis through NF-kappaB. *Stem Cell Rev* 2012;8:905–16.
- 41 Cuenda A, Rouse J, Doza YN, *et al.* SB 203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1. *FEBS Lett* 1995;364:229–33.
- 42 Wada T, Nakashima T, Hiroshi N, *et al.* RANKL-RANK signaling in osteoclastogenesis and bone disease. *Trends Mol Med* 2006;12:17–25.
- 43 Li F, Chung H, Reddy SV, *et al.* Annexin II stimulates RANKL expression through MAPK. *J Bone Miner Res* 2005;20:1161–7.
- 44 Feng X. RANKing intracellular signaling in osteoclasts. *IUBMB Life* 2005;57:389–95.
- 45 Liang J, Li X, Zhang H, *et al.* Allogeneic mesenchymal stem cells transplantation in patients with refractory RA. *Clin Rheumatol* 2012;31:157–61.
- 46 Muller AM, Davenport M, Verrier S, *et al.* Platelet lysate as a serum substitute for 2D static and 3D perfusion culture of stromal vascular fraction cells from human adipose tissue. *Tissue Eng Part A* 2009;15:869–75.
- 47 Kong YY, Feige U, Sarosi I, *et al.* Activated T cells regulate bone loss and joint destruction in adjuvant arthritis through osteoprotegerin ligand. *Nature* 1999;402:304–9.