ABSTRACT

Objectives. The Spondyloarthropathies (SpAs) are genetically and therapeutically linked to IL-23, which in turn regulates IL-22, a cytokine that has been implicated in the regulation of new bone formation in experimental models. We hypothesised that IL-22, a master regulator of stem cells in other niches, might also regulate human mesenchymal stem cell (MSC) osteogenesis.

Methods. The effects of IL-22 on in vitro MSC proliferation, migration and osteogenic differentiation were evaluated in the presence or absence of IFN-γ and TNF (to ascertain IL-22 activity in pro-inflammatory environments). Colorimetric XTT assay, trans-well migration assays, quantitative real time-polymerase chain reaction (qRT-PCR) for MSC lineage markers and osteogenesis assays were used.

Results. Combined treatment of MSC with IL-22, IFN-γ and TNF resulted in increased MSC proliferation ($p=0.008$) and migration ($p=0.04$); an effect which was not seen in cells treated with IL-22 alone and untreated cells. Osteogenic, adipogenic but not chondrogenic transcription factors were up-regulated by IL-22 alone ($p<0.05$). MSC osteogenesis was enhanced following IL-22 exposure ($p=0.03$, measured by calcium production). The combination of IFN-γ and TNF with or without IL-22, suppressed MSC osteogenesis ($p=0.03$).

Conclusion. This work shows that IL-22 is involved in human MSC proliferation/migration in inflammatory environments with MSC osteogenesis occurring only in IFN-γ/TNF absence. These effects of IL-22 on MSC function is a novel pathway for exploring pathological, post-inflammation osteogenesis in human SpA.

INTRODUCTION

The spondyloarthropathies (SpAs) including ankylosing spondylitis (AS) show a propensity for florid new bone formation following bouts of inflammation [1-3]. Bone formation at insertions occurs at sites of maximal entheseal tension and histologically may exhibit endochondral, intramembranous or chondroidal metaplasia [4]. The basis for the post inflammatory new bone formation at entheses in SpA remains poorly understood. However, in other skeletal diseases, such as rheumatoid arthritis, inflammation predictably leads to diffuse bone loss and periarticular erosion. A biological explanation for these observations is lacking [5] but a clue may lie in the observation that SpAs are genetically associated with SNPs in the common p40 subunit of interleukin (IL)-12/23 and also IL-23 receptor SNPs [6]. Monoclonal therapies that target this pathway are associated with significant suppression of joint inflammation [7]. Two of the major effector cytokines downstream of IL-23R engagement are IL-17 and IL-22. Interleukin-22 is a pleiotropic cytokine produced solely by immune cells, functioning exclusively on non-immune cells [8]. The mechanism by which IL-22 influence bone formation is still not well defined [2].

Of note, IL-22 regulates stem cell function in the intestine, liver and skin, and has been dubbed a master regulator of stem cell function [9], so we hypothesised that IL-22 might likewise affect MSC function. Human MSC may play a major role in both bone repair and aberrant new bone formation at the entheses [10]. Based on these observations, we hypothesised that IL-22 may also regulate human MSC function; particularly, MSC osteogenesis in an inflammation-dependent context. In this work, we provide proof of concept that IL-22 is an important regulator of MSC function, which has implications for studying pathological bone formation in SpA.

MATERIAL AND METHODS

Isolation and expansion of human MSC

Samples were obtained following patients written consent. Sample collection was approved by the Yorkshire and Humberside ethics committee. Culture expanded MSC were isolated from bone marrow aspirates (n=10). After Lymphoprep™ preparation

(Axis-Shield), bone marrow mononuclear cells were seeded at a density of 10^4 cells/cm^2 and MSC cultures expanded by 2-4 passages.

Flow cytometry evaluation of MSCs IL-22 receptor expression.

To optimise IL-22 receptor (IL-22R) expression on MSCs, flow cytometry of culture expanded MSC (10^5, passage 3, n=5) was evaluated following stimulation in the presence or absence of the following cytokines: IFN-γ (10 ng/mL) and TNF (15 ng/mL, Miltenyi Biotec) or both combined. For IL-22Rα1 detection, cells were fixed and permeabilized using Fix/Perm Buffer (eBioscience), washed once with permeabilization buffer (eBioscience) and stained with anti-IL-22Rα1 (clone 305405; R&D systems) for 30 minutes on ice. Following staining, cells were washed and then resuspended in FACS buffer (Phosphate buffer saline (PBS) supplemented with 0.5% Bovine serum albumin (BSA), 0.5mM ethylenediaminetetraacetic acid (EDTA) and 0.05% NaN3 (all Sigma). Analysis performed on BD™ LSRII flow cytometer using BD FACSDiva software version 6.0 against the corresponding isotype controls.

To support the idea that pro-inflammatory cytokines activated MSCs, their HLA class I and II expression levels were determined using anti-HLA class I antigen (clone W6/32; Sigma-Aldrich) and anti-HLA-DR (clone G46-6; BD Biosciences) according to manufacturer's instructions.

Cell proliferation assay:

MSC proliferation was determined using the cell proliferation kit II (XTT; Roche Diagnostics). Briefly, MSC were seeded at a density of 10^3 cells/well in 96-well flat bottom cell culture plates, and grown in Dulbecco's modified eagle medium (DMEM; Thermo Fisher) supplemented with 5% heat-inactivated foetal calf serum (FCS; Sigma-Aldrich). Cultured MSC were treated with different combinations of recombinant human cytokines with optimal concentration being used: IL-22 (10 ng/mL, PeproTech), IFN-γ (10 ng/mL) and TNF (15 ng/mL) or concomitant IL-22, IFN-γ and TNF. DMEM with either 5 or 10% FCS was used as negative and positive controls respectively. After six days, the cells were incubated with XTT labelling mixture and absorbance at 450nm recorded.

Migration assay:

MSC were serum deprived in DMEM with 0.4% FCS as a basal medium overnight. After trypsinization, 10^4 cells were seeded into the upper chamber of Falcon® cell culture inserts with an 8.0µm pore polyethylene terephthalate membrane (BD Biosciences) in triplicate. In the lower chamber, media containing either: 1) IFN-γ (10 ng/mL), TNF (15 ng/mL) and IL-22 (10 ng/mL); 2) IFN-γ and TNF or 3) IL-22 alone was added. DMEM with 10% FCS and basal medium with no additional cytokines were used as positive and negative control media respectively. The plate was incubated for 4 hours at 37ºC, 5.0% CO^2.

Following removal of non-migrated cells using a cotton bud, migrated cells were fixed in 3.7% paraformaldehyde for 24 hours and stained with Mayer's haematoxylin and eosin Y, membranes were cut out and mounted on slides using DPX mountant, DBP Free (Solmedia). To quantify migrated cells, Nikon E-1000 Eclipse light microscope (Nikon, Japan) was used to capture six fields of view and the average number of migrated MSC per field was calculated.

Quantitative real-time (qRT) PCR

Standard TaqMan® assays were used to quantify gene expression of MSC tri-lineage markers in response to cytokine stimulation. MSCs were plated at a density of 10^5 cells/25 cm^2 flask for four days, then starved in serum free medium for 24 hours, prior incubation for 72 hours in DMEM with 5% FCS containing either: 1) IFN-γ (10 ng/mL), TNF (15 ng/mL) and IL-22 (10 ng/mL); 2) IFN-γ and TNF; 3) IL-22 alone or 4) No cytokines. MSCs were harvested and total RNA was extracted using Animal t-RNA Tissue kit (Norgen Biotek). Single-stranded cDNA was synthesized using High Capacity Reverse Transcription kit (ThermoFisher). TaqMan® assays for: ACAN, ALPL, BMP2, COL10A1, COL1A1, COL2A1, FABP4, HPRT, PPARG, RUNX2, SOX9, TNFRSF11B were used with 2x Gene expression mix (ThermoFisher). Gene expression was normalized to HPRT and calibrated to un-stimulated control ($2^{-\Delta\Delta Ct}$). Only mean fold changes greater than two were considered.

Osteogenic differentiation assay.

MSC were seeded at a density of 10^4 cells per well in 12 well tissue culture plates and expanded in osteogenic differentiation medium (ODM); DMEM with 5% FCS supplemented with 100 μM ascorbic acid, 10 mM β-glycerophosphate and 100 nM dexamethasone; (all from Sigma-Aldrich). MSCs were cultured for 14 days in ODM containing either 1) IFN-γ (10 ng/mL), TNF (15 ng/mL) and IL-22 (10 ng/mL); 2) IFN-γ and TNF; 3) IL-22 alone or 4) No cytokines as a control.

Mineralization of the extracellular matrix was quantified by measuring the acid soluble calcium using the cresolphthalein complexone method (Sentinel Diagnostics). Briefly, cells were washed twice with calcium free PBS and calcium was solubilised with 0.5N HCl at 4℃ for four hours. Calcium was measured colourimetrically following the manufacturer's instructions.

Statistical analysis.

Friedman's test was used to compare between different matched group data following cytokine treatment of MSC in proliferation and migration experiments. Wilcoxon matched-pairs signed rank test was used to compare each MSC cytokine treatment to the untreated MSCs in osteogenesis and gene expression experiments. GraphPad Prism 6 (GraphPad Software) was used to generate all graphs. All bar charts show mean (bar height) and standard error of mean.

RESULTS

MSC identity was verified in representative samples using flow cytometry according to ISCT phenotypic criteria [11] (data not shown). MSC functionality was confirmed for cells used in subsequent experiments using in vitro tri-lineage MSC differentiation assays in representative samples (n=6) as previously described [12] (data not shown).

IL-22 drives pro-inflammatory stimulated MSCs proliferation and migration:

Culture expanded MSC expressed the IL-22 receptor (IL-22Rα1) indicating that MSC were permissive to IL-22 signalling. IL-22R expression was detected intracellularly and increased approximately 1.5 fold following optimised concentrations of combined IFN-γ and TNF stimulation for 72 hours. The pro-inflammatory effect of this panel was confirmed by the by up-regulation of HLA class II and I) (**Fig. 1A and 1B)**.

MSC proliferation was significantly increased by combined stimulation of IL-22, IFN-γ and TNF (p=0.008), compared to unstimulated MSCs (**Fig. 1C-E)**, while IL-22 alone or the IFN-γ and TNF combination showed a non-significant increase in proliferation compared to un-stimulated MSCs.

As shown in (**Fig. 2A-C)**, MSC migration was also significantly increased by combined IL-22, IFN-γ and TNF stimulation (p=0.037), compared to unstimulated MSC (negative control), while IL-22 alone or (IFN-γ and TNF) combination showed non-significant increases in migration compared to unstimulated MSCs.

Gene expression of MSC lineage markers in response to cytokine stimulation

Using qRT-PCR to test whether IL-22 has any influence on MSC' adipo-, osteo- or chondro-genic potentials and whether inflammation altered this, it was found that the transcription factors; PPARG, RUNX2 and SOX9 were up-regulated by IL-22 alone (p=0.0313, p=0.0313 & p=0.0625 respectively) (**Fig. 2D**). Osteogenic markers ALPL, BGLAP and COL1A1 all showed upregulation following IL-22 stimulation, while ACAN also showed slight upregulation.

Inflammatory stimuli (IFN-γ +TNF±IL-22) did not affect chondrogenic or adipogenic transcription factor expression in DMEM media but did hinder the increase of pro-osteogenic RUNX2. Mature bone markers, particularly BGLAP/osteocalcin were rarely detectable in the presence of inflammation. The pro-inflammatory milieu also downregulated all tested mature chondrogenic markers ACAN, COL2A1 and COL10A1 compared to unstimulated and IL-22 stimulated MSC. Adipocyte marker; FABP4, did appear downregulated (but not statistically significant) by inflammation though remained stable in the presence of IL-22 (**Fig. 2D**).

IL-22 enhances, while combined IFN-γ **and TNF inhibit MSC osteogenesis**

In osteogenic conditions, IL-22 alone significantly increased the calcium production of MSC compared to untreated MSC (p=0.0313). To mimic the effect that a severe inflammatory environment may have on IL-22 mediated osteogenesis, we treated MSC with IFN-γ and TNF supplemented osteogenic media in the presence or absence of IL-22. We found profound suppression of osteogenesis in both conditions compared to untreated MSCs (both p=0.0313) **(Fig. 2E)**.

DISCUSSION

IL-22 is a master regulator of stem cell niches in the intestine, liver, endometrium and skin but to the best of our knowledge, no data on its influence upon human osteoprogenitors or MSC has been described [13-16]. This is especially relevant since the human SpAs are linked to IL-22 via the IL-23 pathway [6]. The pathogenesis of aberrant new bone formation in SpA is poorly understood including why anti-TNF therapy does not completely block this process [5]. Given that IL-22 is downstream of the IL-23 pathway and its role in stem cell function elsewhere [9], we explored the effects of IL-22 on normal MSC function. We found consistent effects on MSC proliferation, migration and osteogenic differentiation, with the latter being blocked in a pro-inflammatory milieu.

In this work, IL-22 enhanced the osteogenic capacity of MSC in vitro. To recreate a pro-inflammatory environment, the combined use of IFN-γ and TNF effectively prevented aforementioned IL-22 enhanced osteogenesis. This is in line with previous work showing that IFN-γ blocked osteogenesis [17]. Additionally, the effect of IL-22 on MSC proliferation and migration was tested in the presence and absence of pro-inflammatory cytokines (IFN-γ and TNF). Notably, IL-22 acting in conjunction with IFN-γ and TNF, increased MSC proliferation and migration more than IL-22 alone or IFN-γ and TNF without IL-22. This indicates a potentially important role for IL-22 in the maintenance, proliferation and migration of MSC and MSC topography in an inflammatory environment. Collectively, these findings suggest that IL-22 is a hitherto unappreciated regulator of the MSC niche in bone.

Several human diseases have been genetically linked to the IL-23 signalling pathway, which, in turn, has been linked to stem cell function. For example, intestinal damage leads to IL-23 dependent production of IL-22 by group 3 innate lymphoid cell (ILC3), with subsequent stem cell induction and mucosal protection [14]. In an IL-23 dependent murine SpA model, increased IL-22 production may contribute to bone repair via a murine ILC3 like population, but the basis for this is still unclear [2]. IL-22 is one of the members of IL-10 cytokine superfamily [18]. It has the unique feature of being produced by various immunological cells but exerts its effects on non-immune cells resulting in either pro-inflammatory, anti-inflammatory, or both effects, depending

on the microenviornment [19, 20]. Our finding that IL-22 had no effect on MSC osteogenesis in an inflammatory environment is consistent with its initial pro-inflammatory role, but a bone-forming role in the post inflammatory phase of the disease. Indeed, this is exactly what happens in vivo, in SpAs including AS, where new bone occurs in the post-inflammatory environment. However, it remains to be seen what the role of this pathway is on MSC from diseased tissue. Differentiation of MSC under the effect of cytokines was not performed for all lineages (fat and cartilage) here because IL-22 did not promote transcripts indicative of fat and cartilage maturity. However, following IL-22 stimulation, mature bone transcripts, including ALPL and COL1A1 were enhanced.

In conclusion, this work shows that IL-22 regulates MSC function, including proliferation, migration and osteogenesis in an inflammation-dependent context. These findings on the physiological effects of IL-22 on MSC open up new avenues for investigating inflammation and new bone formation in AS and SpA. Although anti-TNF therapies have failed to arrest new bone formation in axial SpA, it will be interesting to note what effects blockade of the IL-23 pathway, or its downstream partner, IL-17, has on these diseases.

Key messages:

- IL-23 is genetically and therapeutically linked to AS and SpA.
- IL-23 regulates IL-22 expression and, IL-22 is a master regulator of skin and gut stem cell niches.
- This study showed that IL-22 regulates human mesenchymal stem cell migration, proliferation and osteogenesis.

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Conflicts of interest: the authors declare no conflicts of interest.

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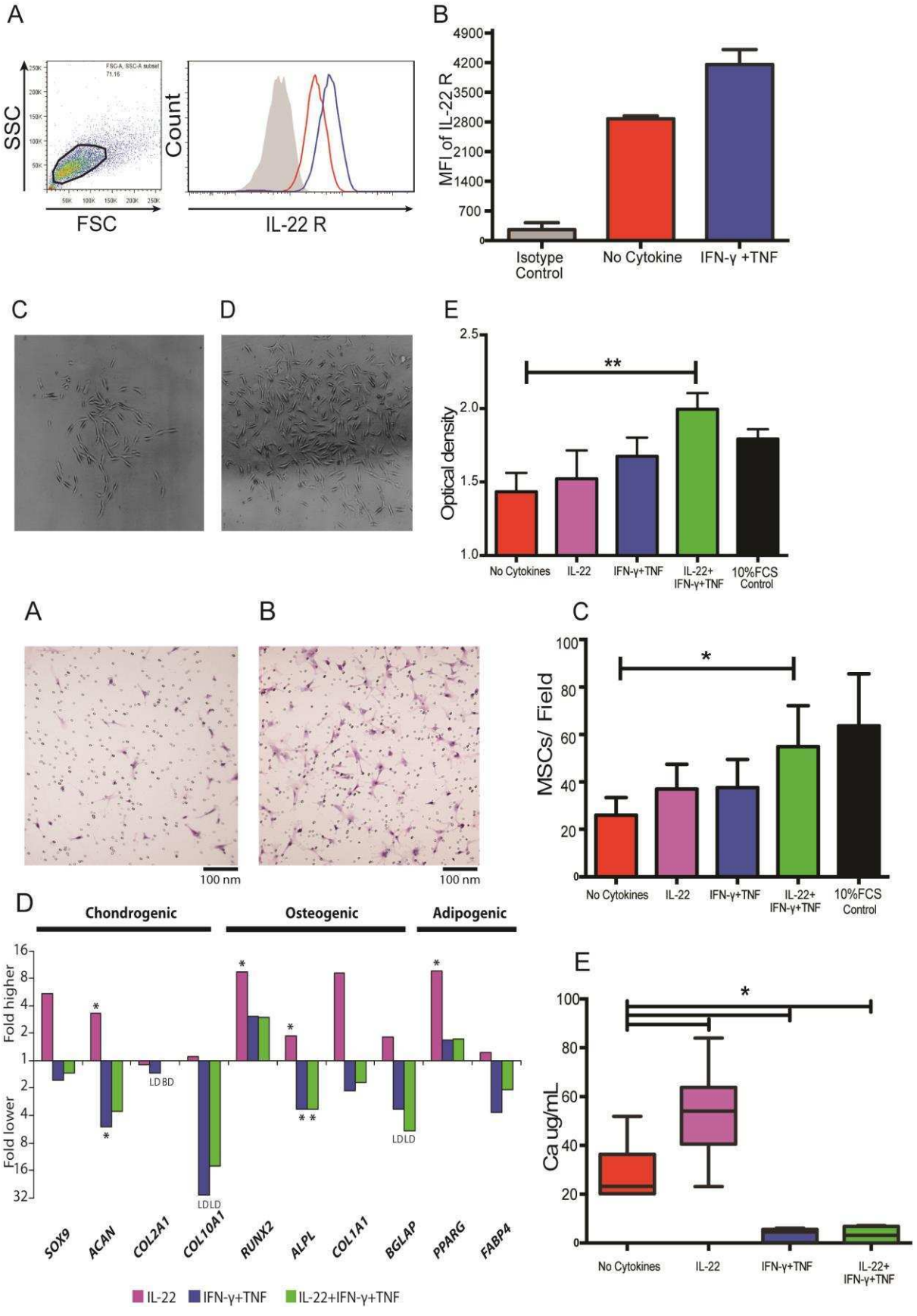

FIGURE LEGENDS:

FIG. 1: IL-22R expression and impact of pro-inflammatory cytokine combinations on MSCs proliferation. (A) Flow cytometry showing intracellular IL-22 receptor expression with IFN-γ and TNF stimulation (Blue histogram) and without stimulation (Red histogram), grey histogram shows isotype control. (B) Graph showing the geometric mean fluorescence intensity (MFI) of IL-22 receptor expression in none treated and IFN-γ/TNF cytokine treated MSCs. Error bars represent biological replicates (n=3). (C) Unstimulated cultured MSCs in control DMEM and 5% FCS media. D. Cultured MSCs after 6 days stimulation with combined IL-22, IFN-γ and TNF. (E) Graph showing the impact of IL-22, IFN-γ and TNF on MSCs proliferation. **p= 0.008. Error bars represent biological replicates (n=4).

FIG. 2: Impact of IL-22 and pro-inflammatory cytokines on MSCs migration, transcription and osteogenesis. (A and B) Transwell membranes showing that MSCs migrate towards DMEM containing 0.4% FCS and DMEM containing 0.4% FCS supplemented with IL-22, IFN-γ and TNF respectively. (C) Graph shows the effect of IL-22, IFN-γ and TNF on MSCs migration with the triple cocktail increasing MSC migration *p= 0.037. Error bars represent biological replicates (n=4). (D) Changes in relative gene expression under the influence of cytokines, data normalised to unstimulated MSCs (n=5). TF= transcription factor, BD = below detection, LD = low detection rate (1 or 2 samples). (*= significant difference, p=0.313) (E) Osteogenic differentiation of MSCs after treatment with different cytokines. MSCs were cultured for 2 weeks in osteogenic medium with under differing cytokine combinations (no cytokines, IL-22 alone, IFN-γ and TNF, and combination of IL-22, IFN-γ and TNF). IL-22 alone increased calcium production compared to untreated MSCs, while IFN-γ and TNF with or without IL-22 suppressed osteogenesis in all MSCs donors compared to untreated MSCs. *p=0.03 for all (Wilcoxon test). Errors bars represent biological replicates (n=6).