RHEUMATOLOGY

Rheumatology 2017;56:488–493
doi:10.1093/rheumatology/kew384
Advance Access publication 3 December 2016

Concise report

Interleukin-22 drives the proliferation, migration and osteogenic differentiation of mesenchymal stem cells: a novel cytokine that could contribute to new bone formation in spondyloarthropathies

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BASIC SCIENCE

Abstract

Objectives. The 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 hypothesize 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 PCR (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 that was not seen in cells treated with IL-22 alone and untreated cells. Osteogenic and adipogenic, but not chondrogenic, transcription factors were upregulated 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 the absence of IFN-γ/TNF. These effects of IL-22 on MSC function is a novel pathway for exploring pathological, post-inflammation osteogenesis in human SpA.

Key words: spondyloarthropathy, mesenchymal stem cells, IL-22, IL-23 axis, osteogenesis

Rheumatology 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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Submitted 10 March 2016; revised version accepted 19 September 2016

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Introduction

The SpAs, including 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 RA, 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 single-nucleotide polymorphisms (SNPs) in the common p40 subunit of IL-12/23 and also IL-23 receptor (IL-23R) 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. IL-22 is a pleiotropic cytokine produced solely by immune cells, functioning exclusively on non-immune cells [8]. The mechanism by which IL-22 influences 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 hypothesize that IL-22 might likewise affect mesenchymal stem cell (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 hypothesize 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.

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 MSCs were isolated from bone marrow aspirates ($n = 10$). After Lymphoprep preparation (Axis-Shield, Dundee, UK), bone marrow mononuclear cells were seeded at a density of 10^4 cells/cm^2 and MSC cultures expanded by two to four passages.

Flow cytometry evaluation of MSC IL-22R expression

To optimize IL-22R expression on MSCs, flow cytometry of culture expanded MSCs (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, Bergisch Gladbach, Germany) or both in combination. For IL-22Rα1 detection, cells were fixed and permeabilized using Fix/Perm Buffer (eBioscience, San Diego, CA, USA), washed once with permeabilization buffer (eBioscience) and stained with anti-IL-22Rα1 (clone 305405; R&D Systems, Minneapolis, MN, USA) for 30 min on ice. Following staining, cells were washed and then resuspended in FACS buffer [PBS supplemented with 0.5% BSA, 0.5 mM EDTA and 0.05% NaN_3 (all Sigma-Aldrich, St Louis, MO, USA)]. Analysis performed on an LSR II flow cytometer using FACSDiva software version 6.0 (both BD Biosciences, San Jose, CA, USA) 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 the manufacturer's instructions.

Cell proliferation assay

MSC proliferation was determined using a cell proliferation kit II (XTT; Roche Diagnostics, Basel, Switzerland). Briefly, MSCs were seeded at a density of 10^3 cells/well in 96-well flat bottom cell culture plates and grown in DMEM (Thermo Fisher, Waltham, MA, USA) supplemented with 5% heat-inactivated foetal calf serum (FCS; Sigma-Aldrich). Cultured MSCs were treated with different combinations of recombinant human cytokines with optimal concentrations being used: IL-22 (10 ng/ml; PeproTech, Rocky Hill, NJ, USA), 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 6 days, the cells were incubated with XTT labelling mixture and absorbance at 450 nm recorded.

Migration assay

MSCs were serum deprived overnight in DMEM with 0.4% FCS as a basal medium. After trypsinization, 10^4 cells were seeded into the upper chamber of Falcon cell culture inserts with an 8.0 μm pore polyethylene terephthalate membrane (BD Biosciences) in triplicate. In the lower chamber, media containing either (i) IFN-γ (10 ng/ml), TNF (15 ng/ml) and IL-22 (10 ng/ml); (ii) IFN-γ and TNF or (iii) 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 h at 37ºC in 5.0% CO_2.

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

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

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cells/25 cm^2 flask for 4 days, then starved in serum-free medium for 24 h prior incubation for 72 h in DMEM with 5% FCS containing either (i) IFN-γ (10 ng/ml), TNF (15 ng/ml) and IL-22 (10 ng/ml); (ii) IFN-γ and TNF; (iii) IL-22 alone or (iv) no cytokines. MSCs were harvested and total RNA was extracted using an Animal Tissue RNA Purification Kit (Norgen Biotek, Thoroid, ON, Canada). Single-stranded cDNA was synthesized using a High Capacity Reverse Transcription Kit (Thermo Fisher). TaqMan assays for *ACAN, ALPL, BMP2, COL10A1, COL1A1, COL2A1, FABP4, HPRT, PPARG, RUNX2, SOX9, TNFRSF11B* were used with 2× Gene Expression Mix (Thermo Fisher). Gene expression was normalized to *HPRT* and calibrated to unstimulated control ($2^{-\Delta\Delta Ct}$). Only mean fold changes >2 were considered.

Osteogenic differentiation assay

MSCs were seeded at a density of 10^4 cells/well in 12-well tissue culture plates and expanded in osteogenic differentiation medium; 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 osteogenic differentiation medium containing either (i) IFN-γ (10 ng/ml), TNF (15 ng/ml) and IL-22 (10 ng/ml); (ii) IFN-γ and TNF; (iii) IL-22 alone or (iv) 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, Milan, Italy). Briefly, cells were washed twice with calcium-free PBS and calcium was solubilised with 0.5N HCl at 4 °C for 4 h. 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 with the untreated MSCs in osteogenesis and gene expression experiments. GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA) was used to generate all graphs. All bar charts show means (bar height) and standard errors of the mean.

Results

MSC identity was verified in representative samples using flow cytometry according to International Society for Cellular Therapy 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 MSC proliferation and migration

Culture expanded MSCs expressed IL-22R, indicating that MSCs were permissive to IL-22 signalling. IL-22R expression was detected intracellularly and increased ∼1.5-fold following optimized concentrations of combined IFN-γ and TNF stimulation for 72 h. The pro-inflammatory effect of this panel was confirmed by the upregulation of HLA classes I and II) (Fig. 1A and B).

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

As shown in Fig. 2, MSC migration was also significantly increased by combined IL-22, IFN-γ and TNF stimulation ($P = 0.037$) compared with unstimulated MSCs (negative control), while IL-22 alone or the IFN-γ–TNF combination showed non-significant increases in migration compared with 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 chondrogenic potentials and whether inflammation altered this, it was found that the transcription factors *PPARG*, *RUNX2* and *SOX9* were upregulated by IL-22 alone ($P = 0.0313$, 0.0313 and 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 with unstimulated and IL-22-stimulated MSCs. Adipocyte marker *FABP4* did appear to be downregulated (but not statistically significant) by inflammation, although it 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 with 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 with 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 on human

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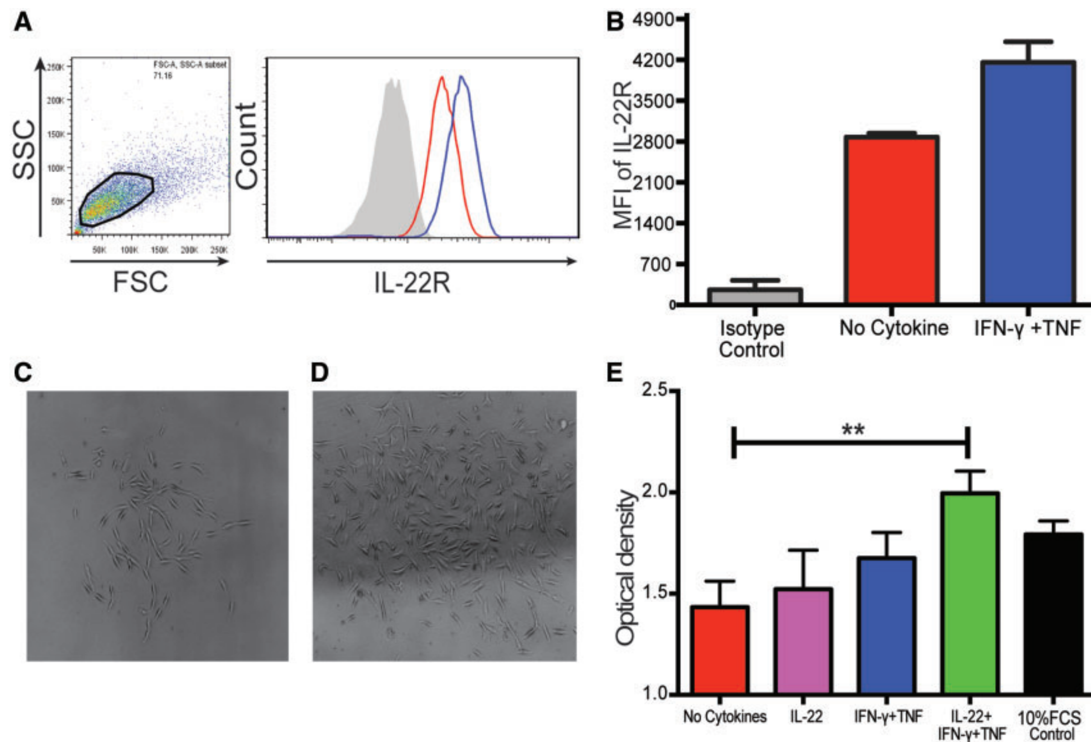
FIG. 1 IL-22R expression and impact of pro-inflammatory cytokine combinations on MSC proliferation



(**A**) Flow cytometry showing intracellular IL-22R 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-22R expression in untreated 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 of stimulation with combined IL-22, IFN-γ and TNF. (**E**) Graph showing the impact of IL-22, IFN-γ and TNF on MSC proliferation. $^{**}P = 0.008$. Error bars represent biological replicates ($n = 4$).

osteoprogenitors or MSCs 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, as well as 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 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 MSCs 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 cells, 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 innate lymphoid cell 3-like population, but the basis for this is still unclear [2]. IL-22 is one of the members of the 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 microenvironment [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 also a bone-forming role in the post-inflammatory phase

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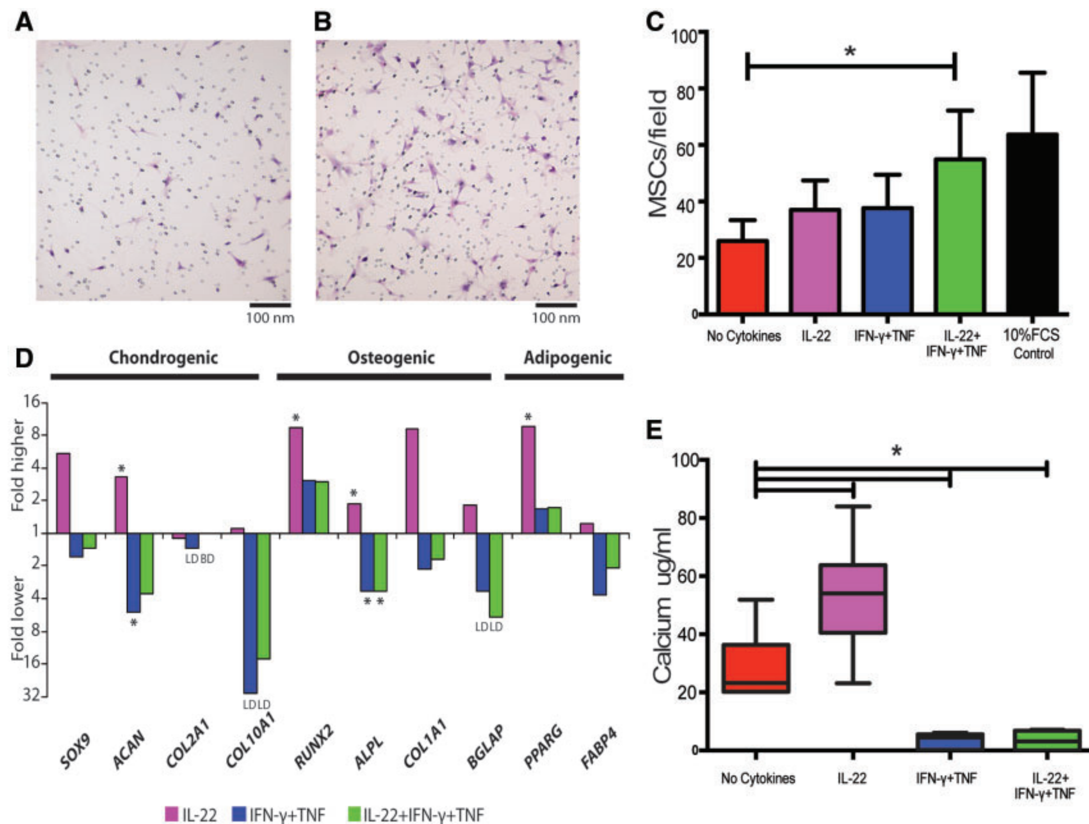
Fig. 2 Impact of IL-22 and pro-inflammatory cytokines on MSC migration, transcription and osteogenesis



Trans-well membranes showing that MSCs migrate towards (**A**) DMEM containing 0.4% FCS and (**B**) 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 MSC 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 with data normalised to unstimulated MSCs ($n = 5$). BD, below detection; LD, low detection rate (one or two samples). Significant difference, $^*P = 0.031$. (**E**) Osteogenic differentiation of MSCs after treatment with different cytokines. MSCs were cultured for 2 weeks in osteogenic medium under differing cytokine combinations (no cytokines, IL-22 alone, IFN-γ and TNF and a combination of IL-22, IFN-γ and TNF). IL-22 alone increased calcium production compared with untreated MSCs, while IFN-γ and TNF with or without IL-22 suppressed osteogenesis in all MSC donors compared with untreated MSCs. $^*P = 0.03$ for all (Wilcoxon test), (n = 6).

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 MSCs from diseased tissue. Differentiation of MSCs 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 MSCs opens 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.

Acknowledgement

We thank Dr Angelo M. Fragakis for his excellent technical assistance.

Funding: This work was supported by the National Institute of Health Research via the National Institute of Health Research, Leeds Musculoskeletal and Biomedical Research Unit and the Egyptian government scholarship scheme.

Disclosure statement: The authors have declared no conflicts of interest.

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