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# Melanoma inhibitory activity, a biomarker related to chondrocyte anabolism, is reversibly suppressed by proinflammatory cytokines in rheumatoid arthritis

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## ABSTRACT

**Objective:** In mice, melanoma inhibitory activity (MIA) is a chondrocyte-specific molecule with similar regulation to collagen type II. As MIA is a small secreted protein, its value as cartilage biomarker in human inflammatory arthritis was assessed.

**Methods:** MIA tissue distribution was studied by quantitative PCR and immunohistochemistry. The regulation of MIA production was studied in vivo in rheumatoid arthritis (RA) (n = 37) and spondyloarthritis (SpA) (n = 30) synovial fluid (SF), and in vitro in alginate embedded human chondrocytes. Therapeutic modulation of serum MIA was evaluated during tumour necrosis factor (TNF) $\alpha$  and interleukin (IL)1 blockade in RA.

**Results:** MIA was primarily expressed by chondrocytes in the human joint. SF MIA levels were lower in RA than in SpA despite similar levels of overall synovial inflammation. Further analysis indicated that these levels were inversely correlated with the degree of joint inflammation in RA, but not in SpA, and that the levels of TNF $\alpha$  and IL1 $\beta$  were significantly increased in RA versus SpA. Accordingly, these proinflammatory cytokines suppressed MIA mRNA and protein in cultured chondrocytes. This suppression was paralleled by suppression of cartilage anabolism as assessed by collagen type 2 and aggrecan mRNA. Treatment of patients with RA with TNF blockade or IL1 blockade induced an increase of serum MIA levels.

**Conclusion:** The decreased levels of MIA in the inflamed RA joint and the coregulation of MIA and cartilage matrix molecules by proinflammatory cytokines indicate that joint inflammation in RA not only drives accelerated cartilage degradation but also suppresses cartilage anabolism. This inflammation-driven suppression is reversible in vivo.

Loss of cartilage is a hallmark of chronic immune-mediated arthritides such as rheumatoid arthritis (RA) and is an important contributor to the progressive impairment of joint function. It has been largely attributed to enhanced cartilage destruction under inflammatory conditions. In the affected joints, proinflammatory cytokines such as interleukin (IL)1 $\beta$  and tumour necrosis factor (TNF) $\alpha$  activate synovial macrophages and fibroblasts, as well as chondrocytes themselves, to produce aggrecanases, matrix metalloproteinases and cathepsins.<sup>1</sup> These proteases mediate the degradation of the cartilage extracellular matrix (ECM), as shown by the release of specific degradation products from collagen type II and aggrecan.<sup>2</sup> In early stage arthritis, these biomarkers

of cartilage catabolism predict progressive cartilage loss before it reaches the more advanced and presumably irreversible stage of joint space narrowing.<sup>3</sup> Therapeutic interventions that suppress these degradation products are associated with a better long-term radiographic outcome, thereby indicating that cartilage loss can be slowed down or halted by therapeutic modulation of cartilage catabolism.<sup>4</sup> Accordingly, inflammation-related cartilage degradation is considered to be one of the main culprits of cartilage loss in arthritis.

However, cartilage loss is not exclusively determined by matrix degradation but rather by a disturbed balance between ECM catabolism and anabolism. Importantly, ECM production by chondrocytes is not constitutive but strictly regulated by anabolic cytokines such as insulin-like growth factor (IGF)-1 and transforming growth factor (TGF) $\beta$  and suppressive factors such as IL1 $\beta$  and TNF $\alpha$ .<sup>5</sup> Excessive repair in some forms of immune-mediated arthritis such as spondyloarthritis (SpA) on the one hand, and the abundant presence of suppressive cytokines in the RA joint on the other, suggests that the level of chondrocyte anabolism is also regulated in vivo and hence contributes to the structural outcome of joint inflammation. This combined inhibition of matrix synthesis and promotion of degradation by the same arthritis-related proinflammatory cytokines is of particular relevance in the era of targeted blockade of these mediators.

Melanoma inhibitory activity (MIA), also known as cartilage-derived retinoic acid-sensitive protein (CD-RAP), is an 11 kDa protein which, in the absence of malignancies, was shown to be produced and secreted exclusively by growth plate and articular chondrocytes in mice.<sup>6-8</sup> In established chondrocytes, MIA transcription is suppressed by IL1 $\beta$ <sup>9</sup> and TNF $\alpha$ .<sup>10</sup> Functionally, MIA promotes the chondrocytic differentiation of mesenchymal stem cells and the production of ECM by differentiated chondrocytes in vitro.<sup>11</sup> A role for MIA in ECM homeostasis is further supported by the ultrastructural changes in the cartilage of MIA-deficient mice.<sup>12</sup>

The assumed cartilage specificity of MIA has raised interest in the molecule as a potential cartilage biomarker in human arthritis.<sup>13 14</sup> Considering the importance of the balance between matrix degradation and matrix synthesis to maintain healthy and functional joint cartilage, the present study aimed to assess the significance of MIA in the context of human arthritis.

## MATERIALS AND METHODS

### Tissue preparation and chondrocyte culture

Samples of cartilage, bone, synovium, ligament and striated muscle were obtained at the time of knee surgery in a patient with osteoarthritis and immediately snap frozen in liquid nitrogen for tissue expression analysis. For the isolation of chondrocytes for *in vitro* studies, macroscopically normal cartilage from the knee joint was obtained from young cadaveric donors without articular or bone pathology. The cartilage was cut into 1 mm<sup>3</sup> pieces and enzymatically digested to isolate the chondrocytes. The digestion was performed in Dulbecco modified Eagle medium (DMEM, GIBCO-Invitrogen, Grand Island, New York, USA) supplemented with L-glutamine and penicillin/streptomycin/amphotericin (GIBCO-Invitrogen) at 37°C and consisted sequentially of hyaluronidase (Sigma-Aldrich, St Louis, Missouri, USA) for 2 h, Pronase E (Sigma-Aldrich) for 1 h and finally collagenase (Sigma-Aldrich) in DMEM+10% fetal calf serum (FCS, GIBCO-Invitrogen) for 5 h. The resulting chondrocyte suspension was passed through a 70 µm cell strainer and washed in sterile calcium free phosphate-buffered saline (PBS, GIBCO-Invitrogen). To avoid progressive dedifferentiation<sup>15</sup> freshly isolated or first passage chondrocytes were cultured in alginate beads for a period of 6 days prior to stimulation. This was performed by resuspending the cells in calcium and magnesium free double concentrated Hank balanced salt solution (HBSS, GIBCO-Invitrogen) and gently mixing them with an equal volume of 2% alginate (low viscosity, highly purified alginate from *Macrocystis pyrifera*; Sigma-Aldrich) in HBSS. The chondrocyte/alginate suspension (5 million cells/ml) was then slowly dripped through a 23 gauge needle into a 102 mM calcium chloride solution and allowed to polymerise for 10 min at room temperature. Finally, the beads were washed in a 0.9% sodium chloride solution and resuspended in DMEM+10% FCS culture medium. Under these conditions, the chondrocytes maintained their differentiated phenotype as they still avidly produced aggrecan and collagen type 2 mRNA (data not shown).

### In vitro stimulation of chondrocytes

At 24 h prior to stimulation, the chondrocyte containing alginate beads were resuspended in 1.5 ml fresh DMEM+10% FCS medium (normal metabolic condition) or DMEM+1% FCS (starvation condition) in 24-well culture plates (Costar, Bethesda, Maryland, USA) with 10 beads (approximately 250 000 chondrocytes) per well. The next day, the cells were stimulated for 72 h with increasing concentrations of IGF-1, TGFβ3, TNFα, IL1β (all cytokines from R&D Systems, Abingdon, UK), or PBS+1% BSA (Sigma-Aldrich) as control. All experiments were performed at least in duplicate. After 3 days, the supernatant was collected and stored at -80°C until analysis by ELISA. The chondrocytes were recovered from the alginate beads by 55 mM sodium citrate pH 6.8, 0.15 M NaCl buffer, washed in PBS, lysed in 1 ml Trizol (GIBCO-Invitrogen) and stored at -80°C for further analysis by quantitative PCR.

### Immunohistochemistry

Formalin fixed paraffin sections of macroscopically healthy cartilage were immunostained using an IgG1 mouse monoclonal antibody (14A) raised against MIA. The validation of the specificity of the antibody and the immunostaining procedure, have already been extensively described.<sup>16</sup> In brief, the slides were first blocked with 10% normal human serum in PBS and then incubated overnight with 1 µg/ml 14A antibody. Parallel sections

were incubated with an IgG1 isotype control antibody (Dako, Glostrup, Denmark) as a negative control. After quenching endogenous peroxidase with 1% H<sub>2</sub>O<sub>2</sub>, the staining was performed using the LSAB+ kit following the manufacturer's instructions (Dako). Nuclei were counterstained with haematoxylin.

### ELISA

MIA was measured using a commercially available one-step sandwich ELISA kit (Roche Diagnostics, Mannheim, Germany) following the manufacturer's instructions. The range of the assay is 0.49 ng/ml to 50.0 ng/ml, with an intra-assay variance of 4% and an interassay variance of 6.6%. IL6, IL1β, TNFα, IGF-1 and TGFβ3 concentrations were measured with commercially available ELISA kits (Quantikine ELISA, R&D Systems) following the manufacturer's instructions. When required by the range of the assays, the samples were first diluted in PBS.

### Isolation of total RNA and quantitative real-time (RT)-PCR

Total RNA was obtained from homogenised tissue and cells by a routine Trizol extraction procedure.<sup>17</sup> RNA (1 µg) was reverse transcribed into cDNA using iScript cDNA transcription kit (BioRad, Hercules, California, USA). Subsequently, quantitative RT-PCR was performed with the MiniOpticon RT-PCR detection system (BioRad) using SYBR green dye. The reaction mixture consisted of 2.5 µl cDNA template, 5 µl forward and reverse primer and 12.5 µl IQ SYBR Green Supermix (BioRad). Cycling conditions comprised an initial activation step at 95°C for 3 min followed by 40 amplification cycles of 95°C for 15 s (cycling denaturation) and 60°C for 45 s (primer annealing and extension). The specificity of the amplification reaction was checked by melting curve analysis. Primers used in the assay were designed using Beacon design software V.7.20 (Premier Biosoft International, Palo Alto, California, USA) and checked for specificity by NCBI BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Primer sequences were for glyceraldehyde 3-phosphate dehydrogenase (GAPDH): forward primer 5'-GCTCTCTGCTCCTCCTGTTCC-3', reverse primer 5'-CGCCCAATACGACCAATCC-3'; MIA: forward primer 5'-GACTGCCGATTCCCTGACC-3', reverse primer 5'-TCCATAGTAATCTCCCTGAACG-3'; collagen type 2A: forward primer 5'-CTGGTGATGATGGTGAAGC-3', reverse primer 5'-GGATAACCTCTGTGACCTTTG-3'; aggrecan core protein: forward primer 5'-AACAGTGCCATCATTGCC-3' reverse primer 5'-CTCATCCTTGTCTCCATAGC-3'. The amplification efficiency of each primer set was between 95 and 105%. The mRNA expression was calculated with the formula: 2<sup>-ΔΔCt</sup>, where Ct = cycle threshold.

### Patients and samples

Paired serum, synovial fluid (SF) and synovial tissue biopsies were obtained from 37 patients with RA, all fulfilling the American College of Rheumatology (ACR) classification criteria,<sup>18</sup> and 30 patients with SpA fulfilling the European Spondyloarthropathy Study Group (ESSG) classification criteria.<sup>19</sup> All patients had active disease with at least one swollen knee joint which was subjected to needle arthroscopy for sampling of SF and synovial tissue as described in detail previously.<sup>20</sup> Serum was collected at the time of arthroscopy. Serum was also collected from 32 healthy subjects (14 men and 18 women). All patients gave their written informed consent before entry in the study protocol as approved by the local ethics committee. The demographic and clinical characteristics of the cohorts are given in table 1.

## Extended report

**Table 1** Demographics and clinical parameters of the patient cohorts

	RA	SpA	RA, stable DMARD		RA, anti-TNF $\alpha$		RA, IL1ra		SpA anti-TNF $\alpha$	
n	37	30	15	15	7	25				
Male/female	12/25	20/10	3/12	2/13	6/1	16/9				
Age, years	54 (15)	37 (16)	61 (13)	50 (12)	45 (10)	51 (9)				
Disease duration, years	2.9 (3.4)	6.5 (9.2)	7.9 (7.8)	10.7 (7.0)	3.7 (2.4)	11.7 (9.9)				
DMARD	25	11	15	15	0	0				
Biological	0	0	0	15	7	25				
	Baseline	Baseline	Baseline	1 year	Baseline	1 year	Baseline	1 year	Baseline	1 year
SJC	9 (9)	4 (4)	4 (4)	4 (5)	16+/6)	1 (1)**	25 (11)	13 (9)*	9 (8)	2 (3)**
CRP, mg/litre	54 (42)	73 (20)	23 (23)	15 (18)	17 (12)	5 (3)**	62 (46)	45 (30)	22 (21)	6 (7)*
ESR, mm/h	53 (35)	29 (27)	31 (19)	23 (13)	25 (10)	13 (8)**	53 (30)	40 (28)	23 (23)	11 (9)*

Depicted values are mean (SD).

\* $p < 0.05$ . \*\* $p < 0.005$ .

CRP, C-reactive protein; DMARD, disease-modifying antirheumatic drug; ESR, erythrocyte sedimentation rate; IL, interleukin; IL1ra, IL1 receptor antagonist; RA, rheumatoid arthritis; SJC, swollen joint count; SpA, spondyloarthritis; TNF, tumour necrosis factor.

### Serum samples during antirheumatic treatment

Serum was obtained before and after 1 year of stable treatment in a number of additional patient cohorts. Of the aforementioned patients with RA, 15 were on stable disease-modifying antirheumatic drugs (DMARDs) at least 6 weeks before the inclusion and remained on the same therapy for at least 1 year; 15 patients with RA were treated with TNF $\alpha$  blockade (infliximab, 3 mg/kg at 0, 2 and 6 weeks and every 8 weeks thereafter with concomitant methotrexate, and 7 patients with RA were treated with IL1 blockade.<sup>21</sup> For comparison, we also included 25 patients with SpA treated with TNF $\alpha$  blockade (infliximab, 5 mg/kg at 0, 2 and 6 weeks and every 8 weeks thereafter). All patients gave their written informed consent before entry in the study protocol as approved by the local Ethics Committee. The demographic and clinical features are shown in table 1.

### Assessment of synovial inflammation

Eight formalin-fixed and paraffin-embedded synovial membrane biopsies were cut into 5  $\mu$ m sections. Following haematoxylin and eosin staining, the sections were blinded for diagnosis and scored by two experienced observers (DB and BVD). A score for cellular inflammatory infiltration was given, using a semiquantitative four-point scale which was extensively validated previously: zero represented the lowest and three the highest degree of infiltration.<sup>22</sup> In case of discordant scores between both observers, which never differed by more than one point, the mean of the two scores was used.

### Statistics

All analysis were performed using GraphPad Prism software V.5 (GraphPad, La Jolla, California, USA). Non-parametric data were analysed by Mann-Whitney U test for comparisons between unpaired data or Wilcoxon signed rank test for comparisons between paired data sets. Parametric data were analysed with the Student t test. Correlations were calculated with Spearman rank or Pearson correlation coefficient test as appropriate.

## RESULTS

### MIA is primarily expressed by cartilage in the human joint

The expression of MIA is cartilage-specific in mice.<sup>6-8</sup> To evaluate if MIA is also cartilage-specific in humans, MIA mRNA was measured by quantitative PCR in the different tissues of the joint. As in mice, MIA was abundantly expressed

in human cartilage (fig 1A), whereas the expression was completely absent from ligament and muscle and very low in synovium and bone (35-fold and 105-fold lower than in the cartilage, respectively). In the cartilage, MIA protein was primarily expressed by chondrocytes in the superficial zone (fig 1B,C). When chondrocytes were released from their matrix and subsequently cultured in alginate beads, they secreted large amounts of MIA in the culture supernatant (mean (SD) 98.48 (34.03) ng/ml by 250 000 chondrocytes over 72 h).

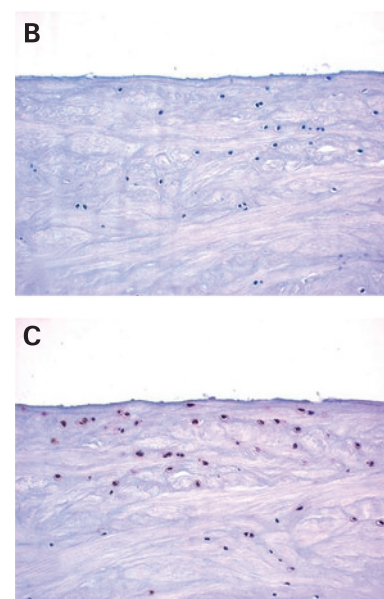
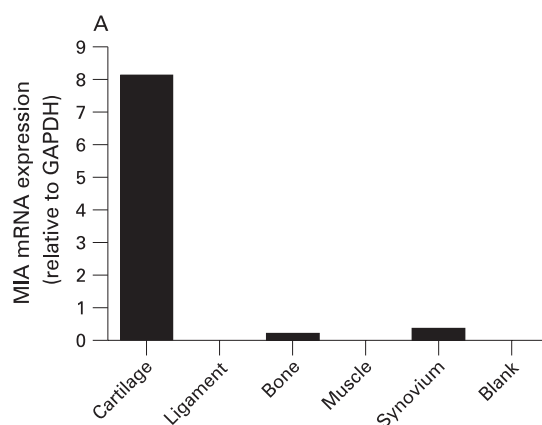
### SF MIA levels are lower in RA than SpA despite similar levels of local inflammation

As MIA is a secreted and cartilage-specific molecule and serum levels were previously proposed to reflect cartilage destruction in RA,<sup>13</sup> we evaluated MIA as a cartilage biomarker in patients with chronic inflammatory arthritis. Levels of MIA were compared between RA, as prototype of destructive arthritis, and SpA, as prototype of a remodelling arthritis,<sup>23</sup> in order to assess potential differences in the regulation of cartilage metabolism between both diseases. Serum levels of MIA were comparable between RA and SpA (9.36 (3.97) ng/ml vs 8.68 (2.81) ng/ml) and were slightly higher than levels in healthy subjects (7.34 (3.41);  $p = 0.042$ ). As the comparison of serum levels is complicated by interfering factors such as the number of swollen joints in both diseases, we next assessed the SF levels. In RA and SpA SF MIA levels were significantly higher in the SF than in the serum ( $p < 0.001$ ), with a trend towards a positive correlation between serum and SF levels (respectively  $r = 0.296$ ,  $p = 0.100$  and  $r = 0.344$ ,  $p = 0.097$ ) (fig 2B,C), thereby supporting the concept that MIA is mainly produced in the joint. In contrast to serum, SF MIA levels were significantly lower in RA (26.5 (15.9) ng/ml) than in SpA (42.3 (29.4) ng/ml;  $p = 0.013$ ) (fig 2A). The degree of infiltration of the synovial membrane by inflammatory cells (score 1.5 (range 0-3) vs 1.75 (0-3);  $p =$  not significant (NS)) as well as SF IL6 levels (1.25 (1.44) ng/ml vs 1.04 (1.64);  $p =$  NS) were comparable between the RA and SpA cohort, indicating that the difference in SF MIA levels could not be explained by differences in the intensity of the local inflammatory process between both diseases.

### MIA levels are inversely correlated with joint inflammation in RA

Subsequently, we investigated which factors could regulate local and systemic MIA levels in patients with arthritis. To this end, SF MIA levels were correlated with parameters of joint inflammation and local cytokine levels. In patients with RA, but

**Figure 1** Comparison of melanoma inhibitory activity (MIA) mRNA expression in different compartments of the synovial joint (A). A paraffin section of articular cartilage from a femur condyl was immunostained with isotype control (B) or anti-MIA monoclonal antibody (C). MIA positive chondrocytes were most prevalent in the superficial zone of the cartilage.

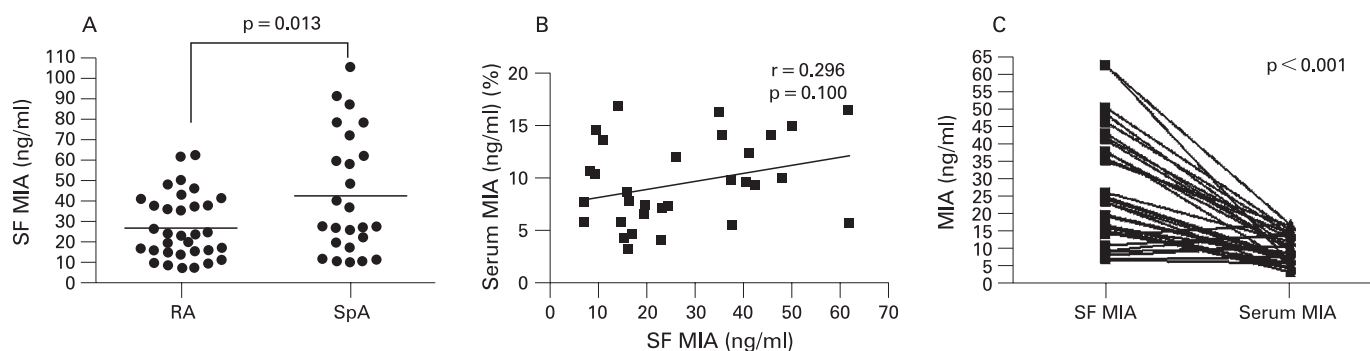


not in patients with SpA, MIA levels correlated inversely with local inflammation as assessed by the degree of infiltration of the synovial membrane by inflammatory cells ( $r = -0.386$ ;  $p = 0.043$ ) and SF IL6 levels ( $r = -0.398$ ;  $p = 0.033$ ) (fig 3A,B). SF levels of the prototypic proinflammatory cytokines IL1 $\beta$  and TNF $\alpha$  did not correlate directly with SF MIA levels, but, interestingly, were significantly higher in RA than in patients with SpA (22.14 (36.35) vs 5.75 (7.9);  $p = 0.035$  and 25.96 (35.92) vs 9.78 (9.35);  $p = 0.036$ , respectively). By contrast, SF levels of potent anabolic factors for chondrocytes were similar (TGF $\beta$ 3: 68.40 (46.89) ng/ml vs 63.25 (42.05) ng/ml) or even slightly elevated in SpA (IGF-1: 51.45 (36.22) vs 70.22 (75.75);  $p = 0.101$ ). Of particular interest with regard to the fact that proinflammatory cytokines such as IL1 $\beta$  can desensitise chondrocytes to the anabolic effects of IGF-1,<sup>24</sup> SF IGF-1 levels correlated with SF MIA levels in SpA ( $r = 0.481$ ;  $p = 0.023$ ) but not in RA. In contrast, SF TGF $\beta$ 3 levels correlated positively with SF MIA levels in RA ( $r = 0.412$ ;  $p = 0.029$ ) but not in SpA. In agreement with the findings in SF, MIA serum levels correlated positively with SF IGF-1 levels in SpA ( $r = 0.686$ ;  $p < 0.001$ ) but not in RA, and with SF TGF $\beta$ 3 levels in RA ( $r = 0.412$ ;  $p = 0.032$ ). Confirming the concept that high local levels of proinflammatory cytokines may play a role in suppressing MIA, MIA serum levels were inversely correlated

with the number of swollen joints in patients with RA ( $r = -0.512$ ;  $p = 0.001$ ) but not in patients with SpA (fig 3C). In contrast, MIA serum levels did not correlate with parameters of systemic disease activity such as C-reactive protein levels, erythrocyte sedimentation rate, or DAS28 result.

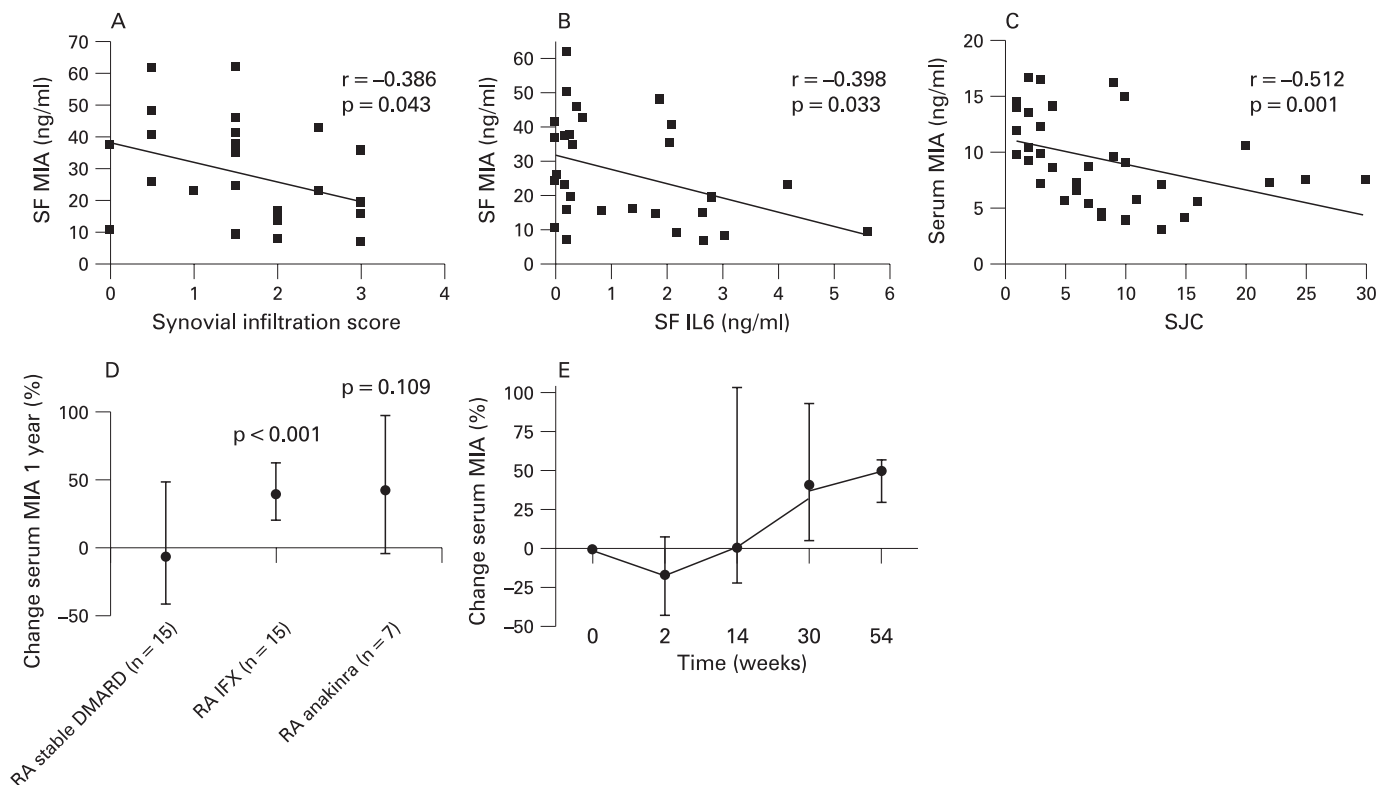
#### IL1 $\beta$ and TNF $\alpha$ suppress MIA production by human chondrocytes cultured in alginate beads

As these in vivo data suggest that high levels of proinflammatory cytokines as observed in the RA joint are associated with lower levels of MIA and as these cytokines have been implicated in the regulation of MIA and cartilage matrix in mice, we next studied the production of MIA, collagen type II and aggrecan by human chondrocytes cultured in alginate beads in the presence of IL1 $\beta$  or TNF $\alpha$ . Each situation was assayed at least in duplicate in three different cartilage donors. IL1 $\beta$  and TNF $\alpha$  consistently induced a dose-dependent decrease of MIA protein production by the chondrocytes (fig 4A,B). In all three donors, the effect of IL1 $\beta$  was more pronounced than that of TNF $\alpha$ . Additionally, at the mRNA level, IL1 $\beta$  and TNF $\alpha$  had a suppressive effect on MIA (fig 4C,D). Additionally, the suppression of MIA mRNA was paralleled by a suppression of collagen type 2A mRNA, a major matrix protein (fig 4C,D). Similar data were obtained for aggrecan (data not shown).



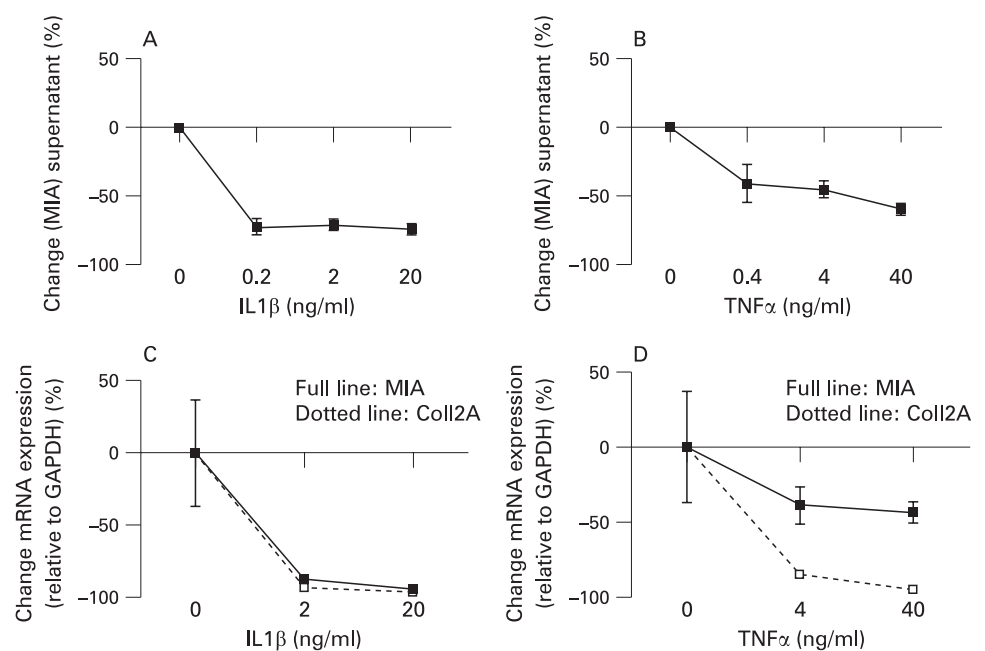
**Figure 2** Melanoma inhibitory activity (MIA) concentrations were significantly lower in the SF of patients with rheumatoid arthritis (RA) in comparison with patients with spondyloarthritis (SpA) (A). Synovial fluid (SF) MIA levels tended to correlate positively with serum MIA levels in patients with RA (B). SF MIA levels were significantly higher in the SF than in the serum in patients with RA (C).

## Extended report



**Figure 3** Local and systemic melanoma inhibitory activity (MIA) levels are modulated by inflammation in patients with rheumatoid arthritis. Synovial fluid (SF) MIA was inversely correlated with the degree of inflammatory cell infiltration of the synovial membrane in patients with rheumatoid arthritis (RA) (A). SF MIA levels were inversely correlated with SF interleukin (IL)6 levels in patients with RA (B). Serum MIA levels were inversely correlated with swollen joint count (SJC) in patients with RA (C). Serum MIA levels in patients with RA were compared before and after 1 year treatment with stable disease-modifying antirheumatic drugs (DMARDs), tumour necrosis factor (TNF) blockade with infliximab, or IL1 blockade with anakinra (D). Serum MIA levels were measured in patients with RA (n = 7) before and at indicated time points after initiation of treatment with infliximab (E). A statistically significant ( $p < 0.05$ ) increase in MIA levels was seen after 30 and 54 weeks of treatment. The data in (D) and (E) are represented as median (interquartile range).

**Figure 4** Analysis of melanoma inhibitory activity (MIA) protein in the culture supernatant and MIA mRNA in human chondrocytes cultured in alginate beads and stimulated for 72 h with indicated cytokines. The concentration of MIA protein in the supernatant decreased after stimulation with interleukin (IL)1 $\beta$  (A) or tumour necrosis factor (TNF) $\alpha$  (B). Additionally, MIA mRNA decreased after stimulation with IL1 $\beta$  (C) or TNF $\alpha$  (D), and this decrease paralleled the decrease in collagen type 2A mRNA. Each cytokine was tested at least in duplicate in three different cartilage donors. A representative experiment is shown.



## MIA levels rise after TNF $\alpha$ or IL1 blockade in patients with arthritis

As these data indicate that IL1 $\beta$  and TNF $\alpha$  suppress MIA production and cartilage anabolism, we aimed to validate this mechanism *in vivo*. Therefore, MIA was measured in the serum of patients with arthritis before and after treatment with proinflammatory cytokine blockade (fig 3D,E). Although serum MIA levels fluctuated in individual patients with RA, the median level did not change ( $-7\%$ , from 5.56 ng/ml to 5.19 ng/ml;  $p = 0.524$ ) over a 1-year follow-up period in patients with RA treated with stable conventional DMARDs. In contrast, 1 year of treatment of patients with RA with the TNF $\alpha$  blocker infliximab induced a consistent and highly significant increase of serum MIA levels with 39% (from 5.4 ng/ml to 7.51 ng/ml;  $p < 0.001$ ). Baseline MIA levels in this selected RA cohort with very active disease (table 1) were significantly lower than in the healthy subjects ( $p = 0.01$ ) or the cohort of consecutive patients with RA ( $p < 0.001$ ). When looking at the kinetics of this effect in a cohort of seven patients with RA, the increase in serum MIA levels became significant at week 30 after initiation of TNF $\alpha$  blockade. Confirming the relationship between MIA levels and joint inflammation in the cross-sectional analysis, the increase of serum MIA levels during TNF $\alpha$  blockade in RA was significantly correlated with the decrease of the swollen joint count ( $r = -0.480$ ;  $p = 0.010$ ) but not of C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), or 28-joint Disease Activity Score (DAS28).

Based on the strong suppression of MIA by IL1 $\beta$  *in vitro*, we additionally investigated a small cohort of patients with RA ( $n = 7$ ) treated with the IL1 receptor antagonist, anakinra. Although this drug had only a modest effect on clinical disease activity in RA (table 1), inhibition of IL1 led to a similar increase of serum MIA levels after 1 year of treatment as TNF blockade (42%; from 4.13 ng/ml to 6.04 ng/ml;  $p = 0.109$ ). Additionally, in this cohort, baseline MIA levels were significantly lower than in healthy subjects ( $p = 0.003$ ) or the consecutive RA cohort ( $p < 0.001$ ). Finally, in agreement with the smaller impact of joint inflammation on MIA in SpA than in RA in our cross-sectional analysis of SF, the size of the increase in serum MIA levels over a 1-year infliximab treatment period was smaller in SpA (20%; from 4.4 ng/ml to 5.27 ng/ml;  $p = 0.021$ ) than in RA. Like in the RA cohorts, in this selected SpA cohort with high swollen joint counts (table 1), baseline MIA levels were also lower than in healthy controls ( $p = 0.002$ ) or the consecutive SpA cohort ( $p < 0.001$ ).

## DISCUSSION

Based on the cartilage-specific expression pattern, the inverse correlation between MIA levels and the degree of joint inflammation in RA and the increase of MIA levels after resolution of joint inflammation, we propose that MIA is a cartilage biomarker that is reversibly suppressed by inflammation in chronic arthritis. These *in vivo* findings as well as the differences in SF levels of MIA, TNF $\alpha$  and IL1 $\beta$  between RA and SpA are remarkably in line with our own *in vitro* data as well as with previous studies indicating that IL1 $\beta$ <sup>9</sup> and TNF $\alpha$ ,<sup>10</sup> two key proinflammatory cytokines in RA, suppress MIA production and secretion by cultured chondrocytes. As the regulation of MIA by these proinflammatory factors parallels the suppression of collagen type II and aggrecan, the two major matrix components of cartilage, these data additionally show that the *in vivo* modulation of MIA may reflect a change in cartilage anabolism.

Our findings contrast with the previous report suggesting that MIA levels reflected cartilage degradation in RA based on the slightly elevated serum levels in destructive versus non-destructive arthritis.<sup>13</sup> Although the interpretation of this report is complicated by its cross-sectional design, the definition of destruction based on bone erosions rather than cartilage damage and the use of a dichotomous rather than continuous analysis, we also found a slightly increased concentration of serum MIA in an unselected cohort of patients with RA with active knee synovitis versus healthy controls. More strikingly, however, the serum values covered a very wide range and were inversely correlated with the number of swollen joints, whereas at the local level the SF titres were inversely correlated with the degree of synovial inflammation. Accordingly, the baseline serum MIA levels in our infliximab-treated and anakinra-treated patients, who needed to have very active disease to be eligible for these treatments were significantly lower than in healthy controls. Although it is not clear if the difference in serum MIA levels between patients with erosive and non-erosive RA as reported by Muller-Ladner and colleagues<sup>13</sup> was independent of the number of actively inflamed joints at the time of blood sampling, our data consistently indicate that MIA levels are suppressed during active joint inflammation in RA. The observed suppression of MIA production in the RA joint is also consistent with a previous report comparing RA with osteoarthritis, a mainly degenerative joint disease with less pronounced inflammation.<sup>25</sup>

Analysing how joint inflammation in RA may lead to the suppression of MIA, an important clue was given by the comparison with a remodelling form of arthritis: SpA. We found suppressed MIA levels in the joints of patients with RA compared to patients with SpA despite a similar degree of histological joint inflammation. However, the levels of the proinflammatory cytokines IL1 $\beta$  and TNF $\alpha$  were significantly lower in SpA than RA. Although there was no direct correlation between MIA and these cytokines, several arguments favour a role for these mediators in the suppression of MIA. Firstly, it is known that factors such as IL1 $\beta$  can desensitise chondrocytes to the effect of IGF-1, the major anabolic factor.<sup>24</sup> We found a clear correlation between IGF-1 and MIA in SpA, but this was lost in RA. Secondly, a CAAT/enhancer binding protein motif is present in the MIA promoter. Binding of c/enhancer binding protein (EBP) $\beta$  or  $\delta$  to this motif mediates repression of MIA under the influence of IL1 $\beta$ .<sup>9</sup> Thirdly, in line with previous animal data we demonstrated *in vitro* that IL1 $\beta$  and TNF $\alpha$  suppress MIA at the mRNA and protein level. Finally, and most importantly, we demonstrated that targeted blockade of these cytokines *in vivo* in patients with RA and patients with SpA increases the serum MIA levels. Taken together, these data provide a mechanistic explanation for the decreased MIA levels in the inflamed RA joint.<sup>25 26</sup>

Similar observations as we report here for MIA, have been reported for serum levels of C-propeptide of type 2 procollagen (CPII) or N-propeptide of type 2 collagen. These peptides are cleaved from the C-terminus or N-terminus of collagen type 2A propeptide during collagen synthesis, and are generally considered to be biomarker of a cartilage matrix neosynthesis.<sup>2</sup> Like MIA, slightly elevated levels of these markers have been reported in the serum of patients with RA in one cohort<sup>27</sup> and decreased levels have been found in another cohort.<sup>28</sup> Levels in SF are decreased compared to osteoarthritis (OA) SF.<sup>29</sup> Moreover, serum levels of CPII increase after 24 months of treatment in patients with arthritis with TNF blockade.<sup>30</sup> These similarities between MIA and cartilage neosynthesis markers

## Extended report

suggest that MIA is a biomarker of chondrocyte anabolism. In agreement with this concept, our *in vitro* experiments with cultured chondrocytes showed a parallel suppression of MIA and matrix molecules such as collagen type II and aggrecan. This parallel regulation can be explained by the fact that the CAAT/EBP motif is not only present in the MIA promoter but also in the collagen type 2 promoter. By contrast, we found a positive correlation between MIA levels and growth factors for cartilage anabolism such as IGF-1 in SpA and TGF $\beta$ 3 in RA. Preliminary *in vitro* experiments seem to confirm this concept as stimulation of chondrocytes with these factors stimulated MIA production (unpublished data). The exact effect of IGF-1 on MIA and cartilage anabolism is currently under investigation *in vitro* and *in vivo*.

Collectively, the present study identifies MIA as a novel and interesting cartilage biomarker in inflammatory arthritis. The coregulation of MIA and the major cartilage ECM molecules under influence of proinflammatory cytokines indicate that joint inflammation in RA not only drives accelerated cartilage degradation, but also suppress formation of new cartilage. An implication of these findings is that cartilage damage is possibly not per definition irreversible as an increase in MIA levels upon effective treatment with TNF or IL1 blockade could be associated with new cartilage matrix formation. This biological concept would fit with recent radiological data demonstrating genuine structural repair after effective treatment of patients with RA.<sup>31</sup> Ongoing studies will attempt to validate this concept by prospective comparison of MIA, other cartilage biomarkers and radiological scores for cartilage damage in patients with RA during effective treatment.

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