

# Alarmin S100A9 Induces Proinflammatory and Catabolic Effects Predominantly in the M1 Macrophages of Human Osteoarthritic Synovium

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**ABSTRACT. Objective.** The alarmins S100A8 and S100A9 have been shown to regulate synovial activation, cartilage damage, and osteophyte formation in osteoarthritis (OA). Here we investigated the effect of S100A9 on the production of proinflammatory cytokines and matrix metalloprotease (MMP) in OA synovium, granulocyte macrophage colony-stimulating factor (GM-CSF)-differentiated/macrophage colony-stimulating factor (M-CSF)-differentiated macrophages, and OA fibroblasts.

**Methods.** We determined which cell types in the synovium produced S100A8 and S100A9. Further, the production of proinflammatory cytokines and MMP, and the activation of canonical Wnt signaling, was determined in human OA synovium, OA fibroblasts, and monocyte-derived macrophages following stimulation with S100A9.

**Results.** We observed that S100A8 and S100A9 were mainly produced by GM-CSF-differentiated macrophages present in the synovium, and to a lesser extent by M-CSF-differentiated macrophages, but not by fibroblasts. S100A9 stimulation of OA synovial tissue increased the production of the proinflammatory cytokines interleukin (IL) 1 $\beta$ , IL-6, IL-8, and tumor necrosis factor- $\alpha$ . Additionally, various MMP were upregulated after S100A9 stimulation. Experiments to determine which cell type was responsible for these effects revealed that mainly stimulation of GM-CSF-differentiated macrophages and to a lesser extent M-CSF-differentiated macrophages with S100A9 increased the expression of these proinflammatory cytokines and MMP. In contrast, stimulation of fibroblasts with S100A9 did not affect their expression. Finally, stimulation of GM-CSF-differentiated, but not M-CSF-differentiated macrophages with S100A9 activated canonical Wnt signaling, whereas incubation of OA synovium with the S100A9 inhibitor paquinimod reduced the activation of canonical Wnt signaling.

**Conclusion.** Predominantly mediated by M1-like macrophages, the alarmin S100A9 stimulates the production of proinflammatory and catabolic mediators and activates canonical Wnt signaling in OA synovium. (First Release August 1 2016; J Rheumatol 2016;43:1874–84; doi:10.3899/jrheum.160270)

*Key Indexing Terms:*

ALARMIN  
SYNOVITIS

S100A8/A9

OSTEOARTHRITIS  
M1/M2 MACROPHAGES

Synovial inflammation is observed in a large subgroup of patients with osteoarthritis (OA) and it is believed to contribute to OA pathology<sup>1,2</sup>. The synovial intima layer consists of mainly 2 cell types: the macrophage-like (type A) and fibroblast-like synoviocytes (type B). Although fewer

synovial macrophages are present in OA compared with rheumatoid arthritis (RA), they are crucial for the production of proinflammatory cytokines, such as interleukin (IL) 6 and IL-8, and cartilage matrix-degrading enzymes, matrix metalloproteases (MMP) 1, MMP-3, and MMP-9<sup>3</sup>. Previous

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studies showed that selective depletion of synovial macrophages during experimental OA largely reduces cartilage damage and osteophyte formation, 2 major hallmarks of OA<sup>4,5</sup>. Multiple types of macrophages can be distinguished, where the distinction into classically activated M1 and alternatively activated M2 macrophages is often used<sup>6,7</sup>. M1-like macrophages can produce numerous proinflammatory cytokines, such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and IL-1 $\beta$ , and express MHC class II and CD86 receptors, whereas M2-like macrophages are characterized by the production of antiinflammatory cytokines, such as IL-10 and IL-1Ra, and have been shown to express scavenger receptor CD163 and mannose receptor (CD206)<sup>8,9</sup>.

Activation of the synovium during OA induces the release of large amounts of the alarmins S100A8 and S100A9, which can easily be measured in synovial fluid as well as in serum<sup>10,11</sup>. S100A8 and S100A9 proteins are proinflammatory mediators produced by myeloid cells, such as monocytes and activated macrophages, and have been shown to stimulate cells through Toll-like receptor 4 (TLR4)<sup>12</sup>. They are implicated in multiple rheumatic diseases<sup>13,14</sup>. Previously, our group mainly studied the involvement of synovial S100A8 and S100A9 production in the development of OA. Using *in vivo* models of OA, we observed that S100A8 and/or S100A9 promote synovial activation, cartilage degradation, and osteophyte formation<sup>11,15</sup>. Treatment with the quinoline-3-carboxamide paquinimod (ABR-215757), which can inhibit the binding of S100A9 to its TLR4 and receptor for advanced glycation endproducts (RAGE)<sup>16</sup>, reduced the OA pathology<sup>17</sup>. Finally, we described that the alarmins S100A8 and S100A9 induce canonical Wnt signaling<sup>18</sup>, which has been shown to be detrimental for cartilage<sup>19</sup>.

However, although in these studies synovial production of S100A8 and S100A9 was found to be detrimental for the joint integrity, to date the effects of these proteins on the synovium and the main cell types residing in this tissue remained elusive. Therefore, to deepen our understanding of S100A9 involvement in the OA joint, in our current descriptive study we analyzed the effects of S100A9 on whole synovial tissue. Further, we investigated S100A8 and S100A9 production in fibroblasts and both granulocyte macrophage colony-stimulating factor (GM-CSF)-differentiated/macrophage colony-stimulating factor (M-CSF)-differentiated macrophages, representing the main cell types present in the synovial lining layer. Finally, we zoomed in to the activation of fibroblasts and GM-CSF/M-CSF-differentiated macrophages by S100A9 stimulation.

## MATERIALS AND METHODS

**Human OA synovium for microarray analysis.** Anonymized human synovium was obtained from patients with endstage OA undergoing total knee or hip joint replacement surgery (n = 12). Control synovium from patients with acute knee joint trauma was obtained at the time of arthroscopic examination (n = 6). The study protocol was approved by the local ethics

committee (Commissie Mensgebonden Onderzoek Arnhem - Nijmegen, approval number 2004/009).

**Microarray analysis.** Preparation of complementary DNA and subsequent microarray analysis was done as described previously<sup>20</sup>. Generation of biotinylated complementary RNA, hybridization, and subsequent staining of MOE 430\_2 oligonucleotide arrays (Affymetrix) was performed according to the Affymetrix Expression Analysis Technical Manual for 1-cycle amplification. Arrays were scanned using a laser scanner (GeneChip Scanner; Affymetrix) and analyzed using Affymetrix GeneChip Operating Software, version 1.4. Array normalization, expression value calculation, and clustering analysis were performed using DNA-Chip Analyzer (dChip) software, version 1.3 ([www.dchip.org](http://www.dchip.org)).

**Human endstage OA synovium.** Biopsies were taken from synovium obtained anonymously from patients with endstage OA undergoing arthroplasty and used for stimulation studies. Three randomized biopsies per well were stimulated in quadruple for 24 h with 1  $\mu$ g/ml recombinant human (rh) S100A9. This dose and timepoint were previously shown to be effective<sup>21</sup>. Other biopsies were incubated with the quinoline-3-carboxamide paquinimod (ABR-215757) for 24 h. A third group of biopsies was directly fixed in formalin and afterward embedded in paraffin. Finally, additional biopsies were used for spontaneous outgrowth of fibroblast-like synoviocytes. Nonadherent cells were washed away daily for 7 days. Fibroblast cultures showed high expression of the fibroblast markers vimentin, collagen type I, and proteoglycan 4, as determined by quantitative real-time PCR (qRT-PCR). Moreover, cultures were negative for expression of the endothelial marker CD31 and Fc- $\gamma$  receptor 1, which is expressed in immune cells (data not shown). Isolated fibroblasts were used between passages 6 and 10 and were stimulated for 24 h with 1  $\mu$ g/ml rhS100A9. After stimulation, tissue was lysed in RLT buffer (Qiagen) and cells were lysed in TRI-reagent (Sigma) for RNA isolation. Supernatants were stored at -20°C for Luminex analysis.

**Differentiation and stimulation of human macrophages.** Peripheral blood mononuclear cells were obtained from healthy volunteers after density gradient centrifugation with Ficoll. Monocytes were isolated by magnetic-activated cell sorting with specific CD14+ magnetic beads (Miltenyi Biotec). Monocytes were differentiated into macrophages by adding 50 ng/ml rhGM-CSF or 20 ng/ml rhM-CSF (R&D Systems) for 6 days with changing of the medium halfway. Mature macrophages were stimulated for 24 h with 1  $\mu$ g/ml rhS100A9, after which cells were lysed in TRI-reagent for RNA isolation. Supernatants were collected and stored at -20°C to be measured for protein release by Luminex.

**RNA isolation and qRT-PCR.** RNA was isolated from OA fibroblasts and differentiated macrophages using TRI-reagent, as described previously<sup>22</sup>. OA synovium pieces were first homogenized with the MagNA Lyser (Roche), 5 times for 20 s at 6500 rpm. Afterward, RNA was isolated using the RNeasy-kit (Qiagen) according to the manufacturer's protocol. RNA was reverse transcribed to cDNA and qRT-PCR was performed with specific primers and the SYBR Green Master mix in the StepOnePlus real-time PCR system (Applied Biosystems) as described previously<sup>22</sup>. Expression levels are  $-\Delta$ Ct values, normalized to the reference gene *GAPDH*.

**Multiplex cytokine and MMP protein measurement with Luminex.** To determine protein levels of MMP (MMP-1, MMP-3, and MMP-9) and cytokines/chemokines (IL-6, IL-8, and TNF- $\alpha$ ) in culture medium, Luminex multianalyte technology on the Bio-Plex 100 system (Bio-Rad) was used in combination with multiplex MMP/cytokine kits (Milliplex from Millipore) according to the Milliplex protocol.

**S100A8 and S100A9 proteins and ELISA.** Recombinant human S100A9 was expressed and purified as described previously<sup>23</sup>. The protein was tested to be endotoxin-free (using the limulus amoebocyte cell lysate; BioWhittaker) and recombinant S100A9 protein lost its activity after heat inactivation at 80°C for 30 min, whereas lipopolysaccharide activity has been shown not to be changed at that temperature<sup>12</sup>. S100A8/A9 concentrations were determined in supernatants of CD14+ monocytes and differentiated macrophages by a sandwich ELISA specifically for human S100A8/A9, which has been produced in-house, as described previously<sup>24</sup>.

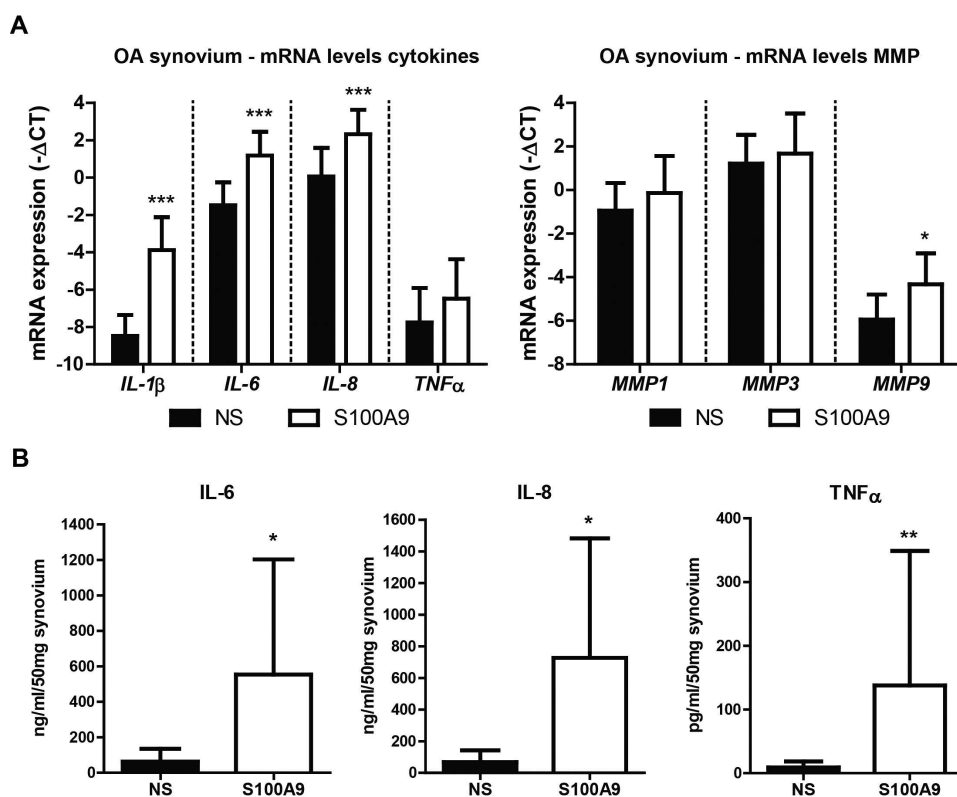
**Immunohistochemistry.** Paraffin-embedded human endstage OA synovial tissue was cut into 7- $\mu$ m sections. Afterward, immunostaining for CD68 (a common macrophage marker), CD86 (a M1-like macrophage marker), and CD163 (a M2-like macrophage marker) was performed. Antigen retrieval was performed using citrate buffer, after which the sections were stained with primary anti-human CD68 (clone KP1, Dako), anti-human CD86 (clone EP1158Y, Abcam), and anti-human CD163 (clone 10D6, Abcam) for 1 h at room temperature. Immunoglobulin G (IgG) isotype controls were included to verify antibody specificity. Afterward, sections were incubated with biotinylated secondary antibodies (Vector Laboratories), followed by an avidin-streptavidin-peroxidase (Vector Laboratories) amplification step. Subsequently, peroxidase binding was visualized using diaminobenzidine and sections were counterstained with hematoxylin.

**Statistics.** Statistical differences were calculated with the Student t test or a 1-way ANOVA with Tukey post-test, using Graph Pad Prism 5 (GraphPad Software).  $P < 0.05$  was considered significant. Statistical analysis for array-based gene expression was performed using dChip software. The t statistic was computed as  $(\text{mean1} - \text{mean2}) / \sqrt{(\text{SE}(\text{mean1})^2 + \text{SE}(\text{mean2})^2)}$ ; its value is calculated based on the t distribution, and the degree of freedom is set according to Welch-modified 2-sample t test<sup>25</sup>.

## RESULTS

*Proinflammatory and catabolic effects after stimulation of total OA synovium with S100A9.* Previously, we described

that S100A8 and S100A9 mRNA were increased in OA synovium compared with control synovium from patients in which acute joint trauma was suspected (11- and 10-fold compared with control synovium<sup>11</sup>). Here, we determined the effects of S100A9 on the production of proinflammatory and catabolic mediators in OA synovial tissue. Therefore, we stimulated human endstage OA synovial biopsies, which mainly consist of fibroblasts and macrophages, with recombinant S100A9 for 24 h. Because it has been shown that S100A8 and S100A9 give comparable results, we only used S100A9 in our current study because that is the most potent stimulator of human cells<sup>26</sup>. S100A9 significantly induced mRNA expression of the proinflammatory cytokines IL-1 $\beta$ , IL-6, and IL-8 (24.3-, 6.4-, and 6.5-fold increases compared with nonstimulated samples, calculated as  $2^{\Delta\Delta\text{CT}}$ ), whereas TNF- $\alpha$  mRNA expression was not significantly increased. Further, S100A9 stimulation increased the expression of the catabolic MMP-9 (3.1-fold increase compared to nonstimulated samples, calculated as  $2^{\Delta\Delta\text{CT}}$ ), but not of MMP-1 and MMP-3 (Figure 1A). Luminex analysis showed that the protein release of IL-6, IL-8, and TNF- $\alpha$  was upregulated by



**Figure 1.** Increased production of proinflammatory mediators and proteases in OA synovium upon stimulation with S100A9. Biopsies from synovial tissue, obtained from patients with endstage OA undergoing arthroplasty of hip or knee, were stimulated *ex vivo* with 1  $\mu$ g/ml S100A9 ( $n = 6$ ). After 24 h, significantly increased expression of the proinflammatory cytokines IL-1 $\beta$ , IL-6, and IL-8, but not of TNF- $\alpha$  after stimulation with S100A9, was observed, as determined by qualitative real-time PCR. Additionally, expression of the protease MMP-9, but not MMP-1 and MMP-3, was significantly increased (A). The protein release of IL-6, IL-8, and TNF- $\alpha$  was upregulated by S100A9, whereas IL-1 $\beta$  protein could not be detected (B). Bars show the mean  $\pm$  SD values. \*  $p < 0.05$ , \*\*  $p < 0.01$ , and \*\*\*  $p < 0.001$  versus NS as measured by Student t test. OA: osteoarthritis; IL: interleukin; TNF- $\alpha$ : tumor necrosis factor- $\alpha$ ; MMP: matrix metalloproteinase; NS: nonstimulated samples.

S100A9 (9-, 14-, and 22-fold increases compared with nonstimulated samples), whereas IL-1 $\beta$  protein could not be detected (Figure 1B).

*Presence of M1-like and M2-like macrophages in OA synovium.* Macrophages account for a substantial part of the cells in the synovial lining layer and have an activated phenotype in OA synovial tissue. However, little is known about their exact activation status. Hence, we investigated gene expression of M1-like and M2-like markers in human endstage OA synovial tissue and healthy control synovium using microarray analysis. We observed increased expression of the M1-like macrophage markers CD86, CCL3, and CCL5 in the OA synovial tissue (1.8-, 2.52-, and 1.97-fold compared with healthy synovium), whereas the M1-like macrophage-associated transcription factor interferon regulatory factors 5 showed a trend toward increased expression. Additionally, we observed increased expression of the M2-like macrophage markers CD206, IL-10, and IL-1Ra (2.18-, 2.32-, and 2.1-fold vs healthy synovium), whereas the expression of CD163 was present, but not significantly altered in OA synovium. These results indicate elevated presence of both M1-like and M2-like macrophages in OA synovium. Next, we confirmed our microarray results with immunohistochemistry on paraffin-embedded sections of human endstage OA synovium. Staining of consecutive sections for the classic macrophage markers CD68 (common macrophage marker), CD86 (M1-like macrophages), and CD163 (M2-like macrophage marker) indicate that, likely, a mixture of M1-like and M2-like macrophages is present in the lining layer of OA synovium (Figure 2A). In the same areas where these macrophage markers were present, we observed expression of S100A8 and S100A9 (Figure 2B).

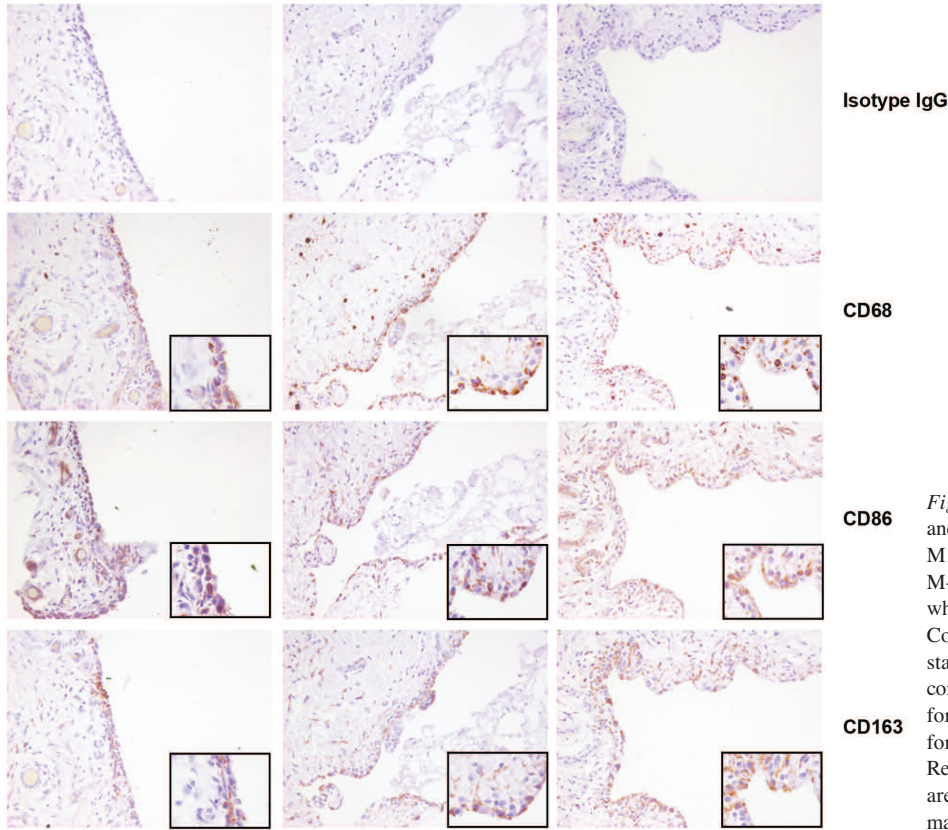
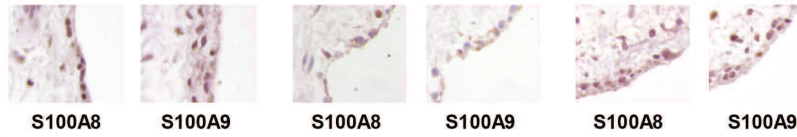
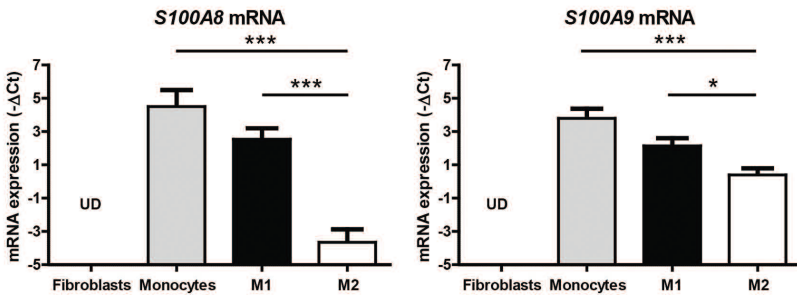
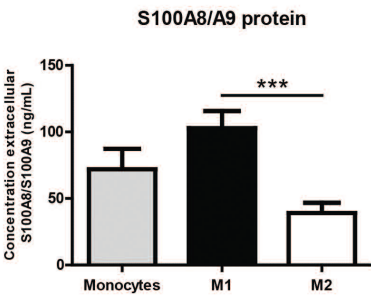
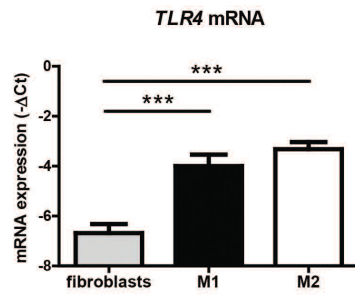
*Expression and secretion of S100A8 and S100A9 in high amounts by GM-CSF-differentiated macrophages and to a lesser extent by M-CSF-differentiated macrophages.* Our next step was to elucidate which cells in the synovium were the main producers of S100A8 and S100A9. The synovial lining layer mainly consists of fibroblasts and both M1-like and M2-like macrophages. To investigate the relative involvement of these various cell types in the production of S100A8 and S100A9, we isolated fibroblasts from OA synovial tissue and differentiated human CD14+ monocytes into mature macrophages using GM-CSF or M-CSF. Gene expression of both S100A8 and S100A9 was not detectable in fibroblasts (Figure 2C). Further, S100A8 and S100A9 mRNA expression was significantly higher in GM-CSF-differentiated macrophages compared with macrophages differentiated with M-CSF (73.2- and 3.4-fold compared with M-CSF-differentiated macrophages). In line, GM-CSF-differentiated macrophages produced significantly higher amounts of S100A8/A9 protein compared with M-CSF-differentiated cells (2.6-fold increase compared with M-CSF-differentiated macrophages), and the production of S100A8/A9 protein was comparable to monocytes (Figure

2D). It has been described that TLR4 is the main ligand for S100A8 and S100A9 proteins in a variety of cell types<sup>12,21,26</sup>. Therefore, we determined the TLR4 expression levels on both fibroblasts and macrophages. TLR4 mRNA production was not significantly different between GM-CSF- and M-CSF-differentiated macrophages, but was significantly lower in fibroblasts (Figure 2E). Next, we proceeded with evaluating the relative contribution of fibroblasts and both GM-CSF- and M-CSF-differentiated macrophages to S100A9-induced upregulation of proinflammatory and catabolic factors in OA synovial tissue.

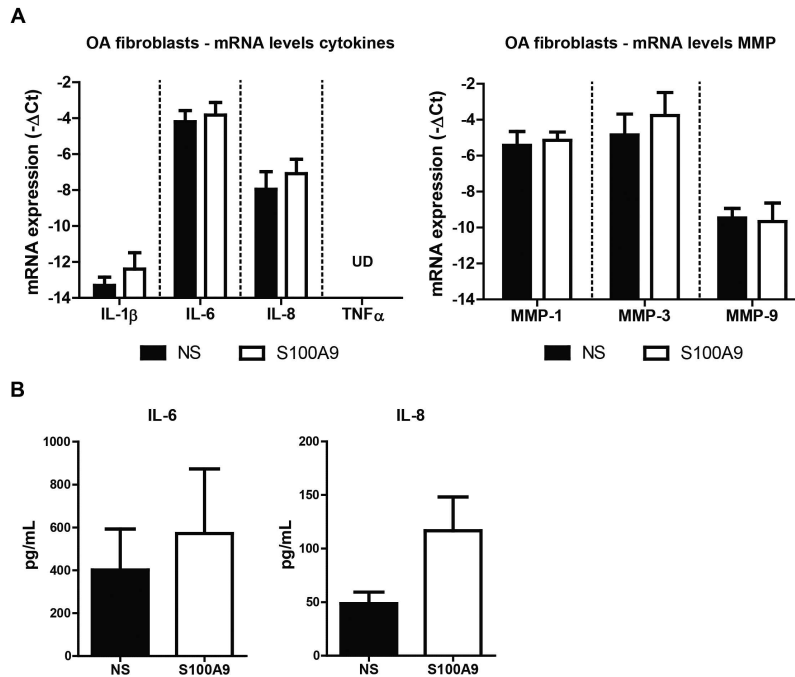
*No effects on fibroblasts after stimulation with the alarmin S100A9.* First, we isolated fibroblasts from endstage OA synovial tissue and subsequently stimulated them with S100A9 protein for 24 h. Analysis by qRT-PCR showed no effect of S100A9 on mRNA expression of IL-1 $\beta$ , IL-6, and IL-8 or on MMP-1, MMP-3, and MMP-9 (Figure 3A). TNF- $\alpha$  expression could not be detected in OA synovial fibroblasts. On the protein level, S100A9 slightly upregulated IL-8 protein release, yet not significantly (2.4-fold increase compared with nonstimulated samples), but not IL-6 (1.2-fold increase compared with nonstimulated samples), whereas TNF- $\alpha$  and IL-1 $\beta$  could not be detected (Figure 3B).

*Proinflammatory and catabolic effects after stimulation of GM-CSF-differentiated macrophages with S100A9.* In the next set of experiments, we sought to determine whether S100A9 stimulation of macrophages, the other main cell type in the synovium, could account for the effects observed in synovial biopsies. Therefore, we differentiated human CD14+ monocytes into macrophages using GM-CSF or M-CSF and stimulated them with S100A9 for 24 h. First, we determined whether our polarization by GM-CSF or M-CSF was successful. Figure 4A shows that, indeed, *INHBA* and MMP-12 as markers of GM-CSF-differentiated M1-like macrophages were higher in GM-CSF-differentiated cells compared with M-CSF-differentiated cells. In contrast, the antiinflammatory marker IL-10 was significantly higher expressed in M-CSF-differentiated macrophages compared with cells that were differentiated with GM-CSF. S100A9 stimulation of GM-CSF-differentiated macrophages significantly upregulated mRNA levels of the proinflammatory cytokines IL-1 $\beta$ , IL-6, and IL-8 (27.7-, 12.8-, and 16.8-fold increases compared with nonstimulated samples, calculated as  $2^{\Delta\Delta Ct}$ ), whereas the mRNA expression of TNF- $\alpha$  was not significantly different. Moreover, MMP-1, MMP-3, and MMP-9 mRNA were also significantly upregulated (8.1-, 6.1-, and 2.9-fold increases compared with nonstimulated samples, calculated as  $2^{\Delta\Delta Ct}$ ; Figure 4B). On the protein level, IL-6, IL-8, and TNF- $\alpha$  were highly upregulated by S100A9 (44.8-, 7.9-, and 106.4-fold increases compared with nonstimulated samples), whereas IL-1 $\beta$  could not be detected on the protein level (Figure 4C).

*Proinflammatory effects after stimulation of M-CSF-differentiated macrophages with S100A9 are less profound*

**A****B****C****D****E**

**Figure 2.** S100A8 and S100A9 are expressed and released mainly by GM-CSF–differentiated M1-like macrophages and to a lesser extent by M-CSF–differentiated M2-like macrophages, which are both present in OA synovial tissue. Consecutive sections of OA synovia were stained with antibodies against CD68 (a common macrophage marker), CD86 (as marker for M1-like macrophages), CD163 (as marker for M2-like macrophages), or isotype IgG. Representative photomicrographs from 3 donors are presented. (A) Clear expression of all markers was present in the synovial lining layer, suggesting the presence of both M1-like and M2-like macrophages in human OA synovium (original magnification  $\times 200$ , inset original magnification  $\times 1000$ ). (B) In the same areas, we observed expression of both S100A8 and S100A9 (original magnification  $\times 1000$ ). (C) Expression of S100A8 and S100A9 was undetectable in fibroblasts ( $n = 5$ ), whereas monocytes and GM-CSF–differentiated macrophages, and to a lesser extent M-CSF–differentiated macrophages, expressed both alarmins, determined by qRT-PCR ( $n = 11$ ). (D) Further, S100A8/A9 proteins levels were significantly higher in GM-CSF–differentiated macrophages compared with M-CSF–differentiated cells. (E) Expression of TLR4, the main receptor for S100A8 and S100A9 in multiple cell types, was lower in fibroblasts compared with macrophages, whereas no significantly different expression was observed between GM-CSF– and M-CSF–differentiated macrophages, as determined by qRT-PCR. Bars show the mean  $\pm$  SEM values. \*  $p < 0.05$  and \*\*\*  $p < 0.001$  versus nonstimulated samples as measured by 1-way ANOVA with Tukey post-test. CSF: colony-stimulating factor; GM-CSF: granulocyte macrophage CSF; M-CSF: macrophage CSF; M1: M1-like macrophages; M2: M2-like macrophages; OA: osteoarthritis; IgG: immunoglobulin G; qRT-PCR: quantitative real-time PCR; TLR4: Toll-like receptor 4; SEM: standard error of the mean.



**Figure 3.** No effects on proinflammatory cytokine and MMP expression in fibroblasts after stimulation with S100A9. OA fibroblasts were isolated from synovial biopsies of patients with endstage OA undergoing arthroplasty of hip or knee and stimulated with 1  $\mu$ g/ml S100A9. S100A9 did not change expression of cytokines IL-1 $\beta$ , IL-6, and IL-8 or of MMP-1 and MMP-3, as determined by quantitative real-time PCR. (A) Expression of TNF- $\alpha$  and MMP-9 was below detection level (n = 5). (B) Determination of protein levels by Luminex multiplex analysis did not show increased release of the cytokines IL-6 and IL-8 by OA fibroblasts upon S100A9 stimulation, whereas IL-1 $\beta$  and TNF- $\alpha$  could not be detected. Bars show the mean  $\pm$  SEM values. No significant differences between S100A9 and NS were found as determined by Student t test. MMP: matrix metalloprotease; OA: osteoarthritis; IL: interleukin; TNF- $\alpha$ : tumor necrosis factor- $\alpha$ ; SEM: standard error of the mean; NS: nonstimulated sample.

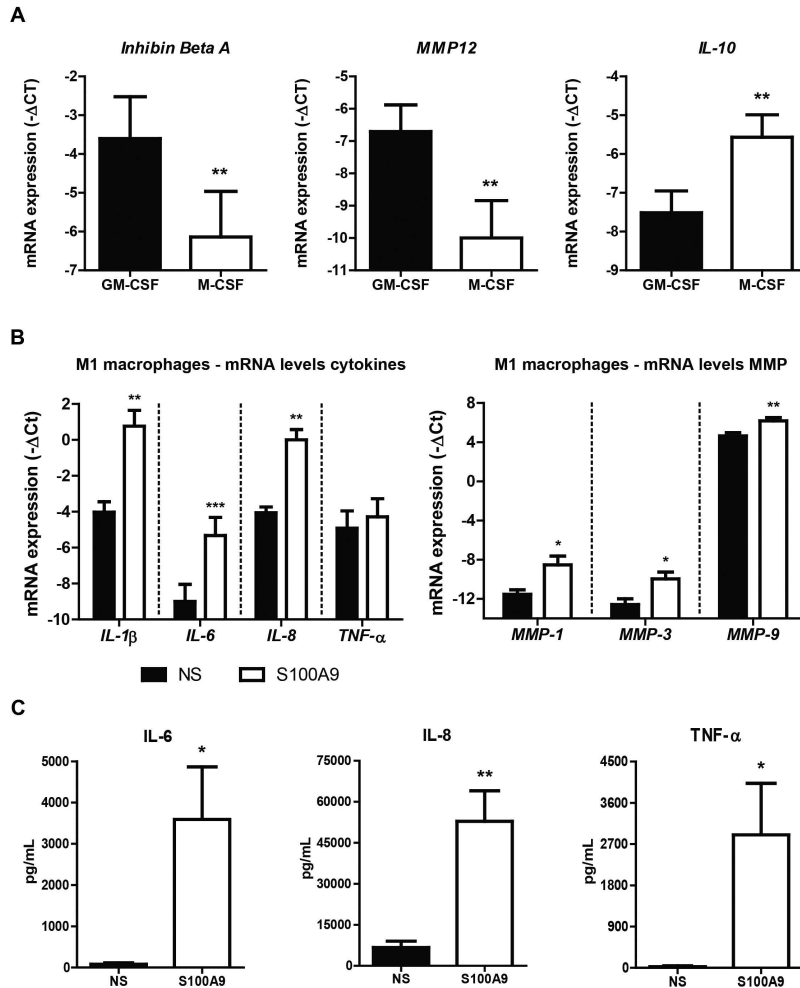
compared with GM-CSF-differentiated macrophages. Interestingly, S100A9 stimulation of the reparative M-CSF-differentiated macrophage also significantly increased mRNA levels of the proinflammatory cytokines IL-1 $\beta$  and IL-8 (15.2- and 4.6-fold increases compared with nonstimulated samples, calculated as  $2^{\Delta\Delta C_t}$ ), but not IL-6 and TNF- $\alpha$ . In contrast to GM-CSF-differentiated macrophages, MMP-1, MMP-3, and MMP-9 mRNA expression levels were not increased in cells differentiated with M-CSF after stimulation with S100A9 (Figure 5A). On protein level, S100A9 also elevated the release of proinflammatory cytokines IL-6, IL-8, and TNF- $\alpha$  (15.3-, 12.9-, and 262.9-fold increases compared with nonstimulated samples). However, absolute protein concentrations were considerably lower for IL-6 and TNF- $\alpha$ , but not IL-8, in M-CSF- compared with GM-CSF-differentiated cells (Figure 5B).

*S100A9 results in enhanced Wnt signaling in GM-CSF-differentiated, but not M-CSF-differentiated macrophages.* In a previous study we showed that S100A8 and S100A9 can increase canonical Wnt signaling in human macrophages, but not fibroblasts. Here, we tested whether the increased Wnt

signaling was the result of S100A9 stimulation of GM-CSF- or M-CSF-differentiated macrophages. S100A9 stimulation of GM-CSF-differentiated cells for 24 h resulted in significantly increased expression of WISP1, a telltale sign of active canonical Wnt signaling, whereas WISP1 expression was either not detectable in nonstimulated and S100A9-stimulated M-CSF-differentiated macrophages, or was not increased after incubation with S100A9 (Figure 6A). Next, we tested our hypothesis that OA synovium has high production of S100A8 and S100A9, thus resulting in Wnt signaling, by incubating OA synovial biopsies with paquinimod, thereby inhibiting the action of S100A9. This resulted in a significantly decreased expression of WISP1, indicating a reduction in canonical Wnt signaling as the result of inhibition of S100A9 (Figure 6B).

## DISCUSSION

In our current study, we show that the alarmins S100A8 and S100A9 are mainly produced by the proinflammatory GM-CSF-differentiated macrophages. Further, we show that stimulation of human OA synovium with S100A9 induces

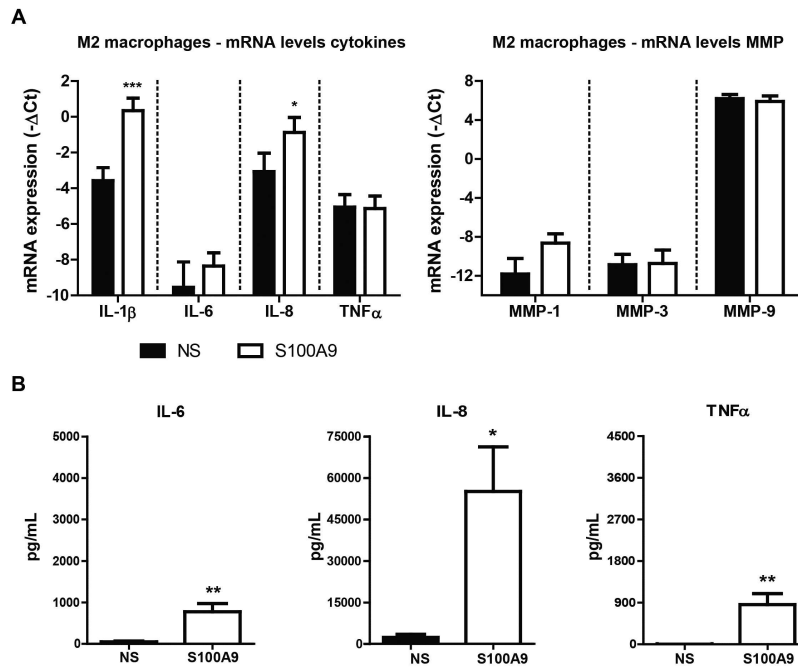


**Figure 4.** Increased expression of proinflammatory cytokines and MMP in GM-CSF–differentiated macrophages after stimulation with S100A9. Human CD14<sup>+</sup> monocytes were differentiated into macrophages with GM-CSF or M-CSF for 6 days and stimulated with 1  $\mu$ g/ml S100A9 for 24 h (n = 6). (A) Differentiation with GM-CSF resulted in significantly increased expression of *INHBA* and *MMP-12* compared with differentiation with M-CSF. In contrast, *IL-10* was significantly higher expressed in M-CSF–differentiated macrophages compared with cells that were differentiated with GM-CSF, confirming a successful polarization of our cells *in vitro*. (B) S100A9 stimulation significantly increased the expression of the proinflammatory cytokines *IL-1 $\beta$* , *IL-6*, and *IL-8*, as determined by quantitative real-time PCR, whereas mRNA expression of *TNF- $\alpha$*  was not increased. In addition, the expression of *MMP-1*, *MMP-3*, and *MMP-9* was upregulated. (C) Further, protein levels of *IL-6*, *IL-8*, and *TNF- $\alpha$*  were significantly increased after S100A9 stimulation. Bars show the mean  $\pm$  SEM values. \*  $p < 0.05$ , \*\*  $p < 0.01$ , and \*\*\*  $p < 0.001$  versus (A) GM-CSF–differentiated macrophages or (B and C) NS as measured by Student t test. MMP: matrix metalloproteinase; CSF: colony-stimulating factor; GM-CSF: granulocyte macrophage CSF; M-CSF: macrophage CSF; IL: interleukin; *TNF- $\alpha$* : tumor necrosis factor- $\alpha$ ; SEM: standard error of the mean; NS: nonstimulated sample; M1: M1-like macrophages; M2: M2-like macrophages.

the production of both proinflammatory and catabolic mediators, in addition to activation of canonical Wnt signaling. Further experiments showed that mainly GM-CSF–differentiated macrophages and to a lesser extent macrophages differentiated with M-CSF but not fibroblasts account for this increased production in the synovial tissue.

The data in our study indicate the presence of both M1-like and M2-like macrophages in OA synovium.

However, the macrophage markers CD86 and CD206 can also be expressed by other immune cells such as dendritic cells and B cells. Nonetheless, clear presence of dendritic cells and B cells in the synovial lining and involvement in the pathology of OA has never been clearly demonstrated. Moreover, Fahy, *et al* showed combined presence of CD86 (M1-like macrophage marker) and CD206 (M2-like macrophage marker) in OA synovium by histology<sup>27</sup>. In



**Figure 5.** Increased expression of proinflammatory cytokines and MMP in M-CSF–differentiated macrophages, though to a lesser extent than in GM-CSF–differentiated macrophages, upon S100A9 stimulation. Human CD14+ monocytes were differentiated into macrophages with M-CSF for 6 days and stimulated with 1 μg/ml S100A9 for 24 h (n = 6). (A) The expression of IL-1β, IL-6, and IL-8 was upregulated after S100A9 stimulation, as determined by quantitative real-time PCR, whereas TNF-α was not increased. In contrast, mRNA expression of MMP-1, MMP-3, and MMP-9 were not upregulated by S100A9 in M-CSF–differentiated macrophages. (B) On protein level, S100A9 significantly upregulated cytokine release of IL-6, IL-8, and TNF-α by macrophages that were differentiated with M-CSF, although the absolute levels of IL-6 and TNF-α were lower as by GM-CSF–differentiated macrophages. Bars show the mean ± SEM values. \* p < 0.05, \*\* p < 0.01, and \*\*\* p < 0.001 versus NS as measured by Student t test. MMP: matrix metalloproteinase; CSF: colony-stimulating factor; M-CSF: macrophage CSF; GM-CSF: granulocyte macrophage CSF; IL: interleukin; TNF-α: tumor necrosis factor-α; SEM: standard error of the mean; NS: nonstimulated sample; M2: M2-like macrophages.

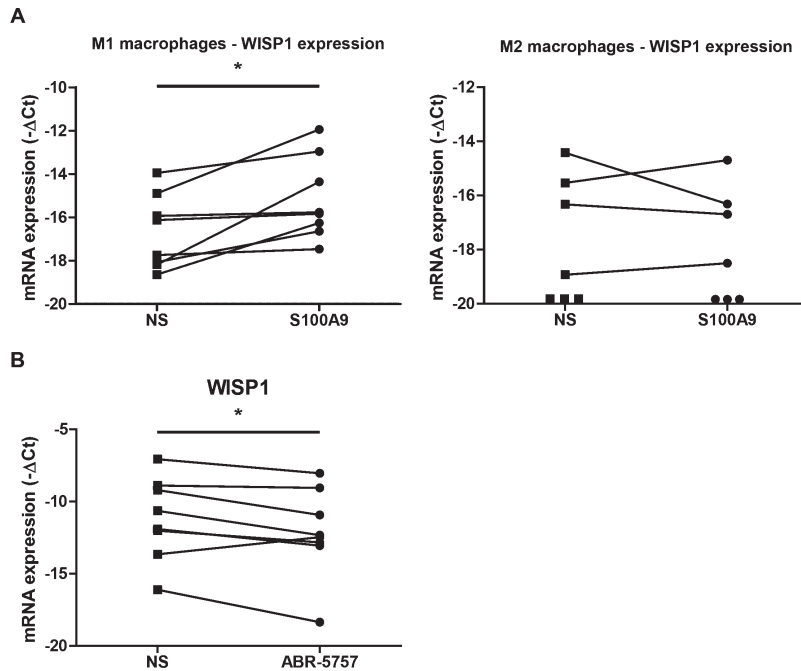
spondyloarthritis and RA, both types are present as well<sup>28</sup>. Together with our data, this suggests the presence of both M1-like and M2-like macrophages in arthritic synovium.

From several studies, it seems clear that macrophages in OA exhibit an activated phenotype, producing among others IL-6, IL-8, and vascular endothelial growth factor. Moreover, OA synovial cell cultures depleted of macrophages no longer produce IL-1β and TNF-α<sup>3,29</sup>, suggesting that the macrophage is the major cell type regulating the release of proinflammatory factors in OA synovial activation. This is in line with our finding that mainly stimulation of macrophages with S100A9 results in increased expression of various proinflammatory and catabolic factors, which have been implicated in OA development. In contrast to the increased protein levels of TNF-α, we did not find increased mRNA expression after 24 h of stimulation. However, it has been described that TNF-α is an early-response protein and mRNA might be increased at earlier timepoints<sup>30</sup>. IL-1β was clearly upregulated on the mRNA level, whereas we could

not detect IL-1β protein in the culture supernatant. This, however, might be the result of our *in vitro* approach. It has been shown that secretion of IL-1β by processing in the inflammasome is based on a 2-hit system. The first signal can be provided by TLR4 signaling, which increases the production of IL-1β mRNA and pro-IL-1β protein and additionally primes the inflammasome. A second signal that activates the inflammasome, such as crystals or ATP, is required to activate the inflammasome to process the pro-form of IL-1β into the mature form and excrete this protein<sup>31,32</sup>.

Additionally, MMP protein levels were too low to detect with Luminex analysis in the majority of synovium donors and in the fibroblast and macrophages cultures, probably because of the low number of cells. However, we previously described that inhibition of S100A9 with the quinoxaline-3-carboxamide paquinimod (ABR-215757) resulted in a significant decrease of the S100A9-induced production of various MMP<sup>17</sup>.





**Figure 6.** Activation of canonical Wnt signaling by S100A9. GM-CSF-differentiated (n = 8) and M-CSF-differentiated macrophage (n = 7) were stimulated with 1  $\mu$ g/ml S100A9 for 24 h. To determine activation of canonical Wnt signaling, the expression of WISP1, a canonical Wnt signaling-induced gene, was determined. (A) Stimulation of macrophages differentiated with GM-CSF with S100A9 significantly upregulated WISP1 expression, as determined by quantitative real-time PCR. In contrast, WISP1 expression was not detectable or not affected by S100A9 stimulation in M-CSF-differentiated macrophages. (B) Further, S100A9 was inhibited in synovial biopsies from patients with end-stage OA (n = 8) for 24 h using 350  $\mu$ M paquinimod (ABR-5757). WISP1 expression was significantly decreased in the paquinimod-treated samples. Bars show the mean  $\pm$  SEM values. \*  $p < 0.05$  versus NS as measured by Student t test. CSF: colony-stimulating factor; GM-CSF: granulocyte macrophage CSF; M-CSF: macrophage CSF; OA: osteoarthritis; SEM: standard error of the mean; NS: nonstimulated sample; M1: M1-like macrophages; M2: M2-like macrophages.

In our study, we show that S100A8 and S100A9 are preferentially expressed and produced by GM-CSF-differentiated macrophages, even as high as monocytes, known to be potent producers of S100A8 and S100A9 proteins<sup>33</sup>. In line with our results, a recent mouse study by Dessing, *et al* also showed that murine bone marrow-derived M1-like macrophages expressed more S100A8 and S100A9 mRNA than M2-like cells. Moreover, they showed that S100A9  $-/-$  bone marrow cells preferentially differentiate into arginase 1-positive M2-type macrophages, suggesting that the absence of S100 proteins skews macrophages to an M2-like phenotype<sup>34</sup>. Moreover, we did not observe S100A8 and S100A9 expression in fibroblasts. This is in contrast with a previous study that showed S100A8 and S100A9 mRNA expression and S100A8/A9 protein expression by isolated OA and RA fibroblasts<sup>35</sup>. However, isolated cells from human synovial tissue were used between passages 3 and 9, which could imply that other cell types (e.g., fibrocytes) could be responsible for the low S100A8/A9 expression. Therefore, in our study, fibroblasts isolated from human OA synovium were

not used before passage 6. Moreover, the protein levels that were produced were very low (500-fold lower compared with our GM-CSF-differentiated macrophages).

We also show that S100A9 acts most potently on GM-CSF-differentiated macrophages, but also stimulates the expression of proinflammatory cytokine in macrophages differentiated with M-CSF, yet to a lesser extent. Proinflammatory M1 and reparative M2 macrophages represent the 2 extremes of the spectrum of macrophage activation, with a lot of intermediate activation states. It is generally accepted that M1 macrophages are involved in proinflammatory responses. It has been shown that mainly M1 macrophages have critical roles in host defense against infection, but these cells are also implicated in many inflammatory diseases, such as atherosclerosis, obesity, and RA<sup>36,37,38</sup>. In contrast to macrophages, we did not observe significant effects of S100A9 on synovial fibroblasts. This difference in potency between macrophages and fibroblasts might be due to the different expression levels of TLR4, as we show by qRT-PCR. However, this does not explain the differences

observed between GM-CSF- and M-CSF-differentiated macrophages. It was previously shown that S100A8 and S100A9 effects were mainly mediated by TLR4 signaling<sup>12,21</sup>. Some studies report the interaction of S100A8 and/or S100A9 with RAGE<sup>39</sup>, yet signaling through RAGE was only reported for the S100 family members S100A11 and S100A4<sup>40,41</sup>. TLR4 expression in synovial fibroblasts, however, is not completely absent, and S100A8 and S100A9 consequently could still have some effects. Therefore, the differences in response between macrophages and fibroblasts as reported here might derive from the various signaling pathways that can be activated by binding of a ligand to TLR4. Previously, we described that TLR4 signaling in fibroblasts was specifically independent of transforming growth factor- $\beta$ -activated kinase (TAK1)<sup>42</sup>, whereas TAK1 was a key regulator of TLR-mediated activation of nuclear factor- $\kappa$ B (NF- $\kappa$ B) and mitogen-activated protein kinase pathways in macrophages. This could explain the divergent effects we observed between fibroblasts and macrophages upon stimulation with S100A9 in our study. Moreover, S100A8 and S100A9 have been shown to act through the TAK1-NF- $\kappa$ B pathway<sup>43</sup>. Possibly, TLR4 signaling through the MyD88-TAK1-dependent pathway might be less dominant or even ablated in fibroblasts. Additionally, differences in co-receptors, such as CD14 and MD2, might affect the TLR4 signaling in the different cell types.

Our study indicates that S100A8 and S100A9 can induce a proinflammatory environment in OA synovium through stimulation of GM-CSF-differentiated macrophages, and to a lesser extent, M-CSF-differentiated macrophages, which possibly contributes to the joint destruction that is found during OA. Considering the longtime expression of S100A8 and S100A9 in the synovium during OA, targeting these proteins could be an interesting option for future OA therapies.

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