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Original Article

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Basic science

S100A8/A9 drives monocytes towards M2-like macrophage differentiation and associates with M2-like macrophages in osteoarthritic synovium

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Abstract

Objectives: Macrophages are key orchestrators of the osteoarthritis (OA)-associated inflammatory response. Macrophage phenotype is dependent on environmental cues like the inflammatory factor S100A8/A9. Here, we investigated how S100A9 exposure during monocyte-to-macrophage differentiation affects macrophage phenotype and function.

Methods: OA synovium cellular composition was determined using flow cytometry and multiplex immunohistochemistry. Healthy donor monocytes were differentiated towards M1- and M2-like macrophages in the presence of S100A9. Macrophage markers were measured using flow cytometry, and phagocytic activity was determined using pHrodo Red Zymosan A BioParticles. Gene expression was determined using qPCR. Protein secretion was measured using Luminex multianalyte analysis and ELISA.

Results: Macrophages were the dominant leucocyte subpopulation in OA synovium. They mainly presented with an M2-like phenotype, although the majority also expressed M1-like macrophage markers. Long-term exposure to S100A9 during monocyte-to-macrophage differentiation increased M2-like macrophage markers CD163 and CD206 in M1-like and M2-like differentiated cells. In addition, M1-like macrophage markers were increased in M1-like, but decreased in M2-like differentiated macrophages. In agreement with this mixed phenotype, S100A9 stimulation modestly increased expression and secretion of pro-inflammatory markers and catabolic enzymes, but also increased expression and secretion of anti-inflammatory/anabolic markers. In accordance with the upregulation of M2-like macrophage markers, S100A9 increased phagocytic activity. Finally, we indeed observed a strong association between *S100A8* and *S100A9* expression and the M2-like/M1-like macrophage ratio in end-stage OA synovium.

Conclusion: Chronic S100A8/A9 exposure during monocyte-to-macrophage differentiation favours differentiation towards an M2-like macrophage phenotype. The properties of these cells could help explain the catabolic/anabolic dualism in established OA joints with low-grade inflammation.

Keywords: osteoarthritis, inflammation, macrophage differentiation, S100A8/A9, synovium.

Rheumatology key messages

- OA synovial macrophages have a mixed phenotype and express both M1-like and M2-like macrophage markers.
- Exposure to S100A9 during monocyte-to-macrophage differentiation promotes an M2-like macrophage phenotype and activation.
- M2-like/M1-like macrophage ratio in end-stage OA synovium positively associates with synovial *S100A8* and *S100A9* expression.

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Introduction

Osteoarthritis (OA) is a chronic heterogeneous joint disease that is highly prevalent worldwide [1]. OA patients suffer from pain, stiffness and impaired mobility, leading to an overt decrease in their quality of life. Although it is now generally considered that inflammation is involved in disease progression in many OA patients, the exact nature of this inflammation and how it contributes to disease onset and progression is not yet clear [2].

In contrast to the clear involvement of lymphocytes in rheumatoid arthritis (RA), mainly cells of the innate immune system are relevant in OA-related inflammation [3, 4]. A key role in the orchestration of the OA-associated inflammatory response has been attributed to macrophages [4, 5]. These cells present a broad spectrum of phenotypes, ranging from pro-inflammatory (M1-like) to anti-inflammatory (M2-like) macrophages. In accordance with their pro-inflammatory nature, M1-like macrophages produce high amounts of pro-inflammatory mediators, including IL-1 and IL-6 and have high expression of HLA-DR and CD86 [5, 6]. In contrast, M2-like macrophages show a strong phagocytic capacity, produce immunoregulatory factors including IL-10 and TGF-β and are characterized by expression of cell surface scavenger receptor CD163 and mannose receptor CD206 [6, 7]. The balance between these phenotypes likely contributes to catabolic processes, like cartilage degradation, and more anabolic processes, including fibrosis and ectopic bone formation, which characterize the OA environment.

The macrophage phenotype is strongly influenced by environmental cues including growth factors and inflammatory factors [5, 8, 9]. An inflammatory factor that is present in high quantities throughout the course of OA is the heterodimeric protein S100A8/A9 [10, 11]. With respect to cell types present in the joint, previous studies in our lab showed that acute S100A8/A9 stimulation of chondrocytes, macrophages or osteoclasts strongly increased the production of pro-inflammatory and catabolic mediators [12–14]. Interestingly, next to decreased catabolic processes, *S100a9*$^{-/-}$ mice showed decreased OA-associated anabolic processes such as osteophyte formation in an inflammatory experimental OA model with high S100A8/A9 expression, indicating a more complex function of S100A8/A9 in synovial inflammation during OA [15].

Up until now, in the context of OA, most S100A8/A9 research was focused on acute effects on mature cell types like macrophages. However, the effects of long-term S100A8/A9 exposure during monocyte-to-macrophage differentiation, which takes places in the osteoarthritic joint, remain to be elucidated. Here, we investigated how exposure to S100A8/A9 during monocyte-to-macrophage differentiation affects macrophage phenotype and function and how this relates to the macrophage phenotype that is observed in established OA synovial tissue.

Methods

Cellular composition, including the presence of macrophage subtypes, of synovium from end-stage OA patients was determined using flow cytometry and multiplex immunohistochemistry. Monocytes were isolated from buffy-coats obtained from healthy donors using peripheral blood mononuclear cell isolation followed by CD14$^+$ magnetic-activated cell sorting. The obtained cells were differentiated during 6 days to either M1- or M2-like macrophages (Mf) using 50 ng/ml GM-CSF ($Mf_{(GM\text{-}CSF)}$) or 20 ng/ml M-CSF ($Mf_{(M\text{-}CSF)}$), respectively, in the presence of 1 μg/ml S100A9 homodimer during the entire course of differentiation. On day 5 of culture, part of the samples was activated by adding 20 ng/ml IFN-γ ($Mf_{(IFN\text{-}\gamma)}$) or 10 ng/ml IL-4 ($Mf_{(IL\text{-}4)}$) for the GM-CSF and M-CSF differentiated cells, respectively.

In this study, we used a S100A9 homodimer that does not tetramerize in culture medium as a model for the S100A8/A9 heterodimer *in vivo*. *In vivo*, S100A8 and S100A9 monomers form intracellularly the S100A8/A9 heterodimer, which can be secreted in response to cell stress. Due to high Ca^{2+} concentrations outside the cell and in cell culture medium, the S100A8/A9 heterodimer tetramerizes to form the $[S100A8/A9]_2$ tetramer. The $[S100A8/A9]_2$ tetramer has its TLR4 binding site folded inside, making it unable to bind to TLR4. *In vivo*, S100A8/A9 is continuously secreted, making it possible to locally exert its effects before tetramerizing. To study TLR4 receptor dependence of the S100A9-induced effects, we used the TLR4 small molecule inhibitor TAK242 and the S100A8/A9 heterodimer, which is known not to function via TLR4.

Macrophage marker expression was measured using flow cytometry and their phagocytic activity was determined using pHrodo Red Zymosan A BioParticles (Thermo Fisher Scientific, Waltham, MA, USA). Expression of inflammatory, catabolic and anabolic factors known to be involved in OA development were determined using qPCR after reverse transcription of full RNA into cDNA, and secretion was determined with a multianalyte Luminex multianalyte analysis (Bio-Rad, Hercules, CA, USA) and an ELISA. Details about the antibody clones and Opal fluorophore combinations used for the multiplex immunohistochemistry are shown in Supplementary Table S1, available at *Rheumatology* online. Details about the used antibody clones and fluorochromes for flow cytometry are shown in Supplementary Table S2, available at *Rheumatology* online.

A full description of the methods can be found in Supplementary Data S1, available at *Rheumatology* online.

This study complies with the Declaration of Helsinki. The locally appointed ethics committees, METC Oost-Nederland and the local ethics committee of the Radboud University Medical Center, approved the research protocol (study numbers 2019-5617 and 2018-4319, respectively). Patients gave written informed consent or had the opportunity to object to anonymized tissue donation.

Results

Macrophages in osteoarthritic synovium present with a mixed phenotype

First, we used flow cytometry to identify the immune cell types that were present in synovial tissue of end-stage OA patients. As shown in Fig. 1A, macrophages were the most abundant immune cell type, followed by mast cells and lymphoid cells, whereas hardly any neutrophils could be observed in these synovial tissues. When looking in more detail at specific macrophage subsets, there were relatively few M1-like cells, defined as CD45$^+$CD68$^+$CD163$^-$HLA-DR$^+$CD86$^+$ (Fig. 1B), but we observed an abundance of M2-like macrophages, identified as CD45$^+$CD68$^+$CD163$^+$CD206$^+$.

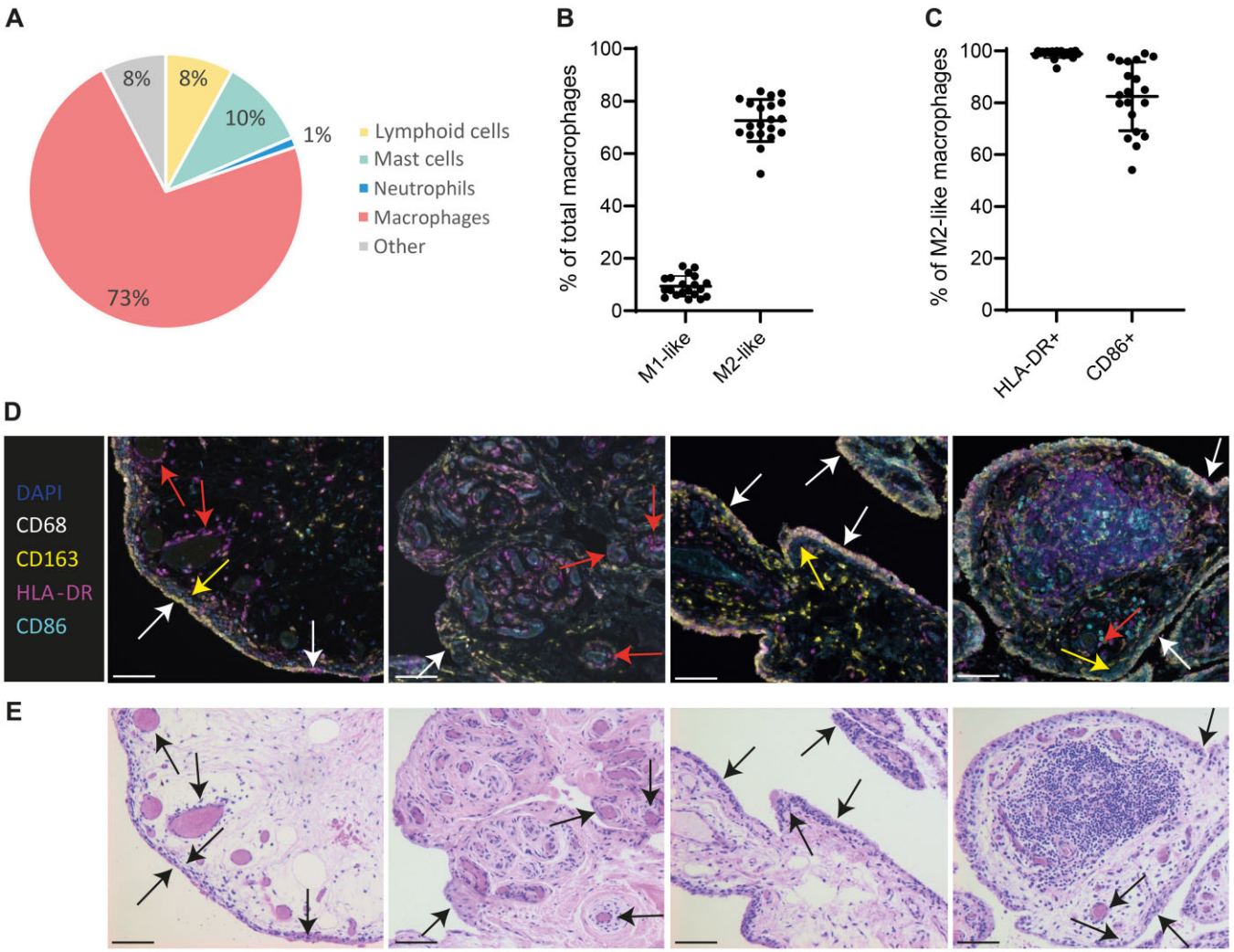

Figure 1. Macrophages are the most abundant immune cells in OA synovium and have a mixed phenotype. (**A**) End-stage OA synovium immune cell composition determined with flow cytometry showed predominantly macrophages ($CD45^+CD3^-CD19^-CD56^-CD117^-CD68^+$), followed by mast cells, lymphoid cells ($CD45^+CD3^+/CD19^+/CD56^+/CD117^+$) and neutrophils ($CD45^+CD15^+$). (**B**) Graph showing an abundance of M2-like ($CD163^+CD206^+$) compared with M1-like ($CD163^-CD86^+HLA\text{-}DR^+$) macrophages. (**C**) High percentages of $HLA\text{-}DR^+$ and $CD86^+$ M2-like macrophages. (**D**, **E**) Multiplex immunohistochemistry (**D**) and haematoxylin and eosin staining (**E**) showed mostly $CD163^+$ macrophages in the synovial lining (white arrows), $HLA\text{-}DR^+$ macrophages, often co-expressing CD86 surrounding blood vessels (red arrows), and $CD86^+HLA\text{-}DR^-$ macrophages adjacent to the lining layer (yellow arrows). Scale bar: 100 μm. Flow cytometry: $n = 20$; immunohistochemistry: $n = 4$. In (**B**, **C**), horizontal and vertical bars represent mean and standard deviation, respectively

Interestingly, however, many of these M2-like macrophages were also positive for the M1-like macrophage markers HLA-DR and CD86 (Fig. 1C). Cells that were $CD45^+CD68^+CD163^-$ but did not express significant levels of HLA-DR and CD86 were classified as intermediate and not shown here. The gating strategy is shown in Supplementary Fig. S1, available at *Rheumatology* online. To determine whether different macrophage subtypes could be found in different areas of the synovial tissue, e.g. in the lining or sublining layer or surrounding blood vessels, we conducted multiplex immunohistochemistry. Here, we observed that the lining layer mainly consisted of $CD163^+$ macrophages of which a part was $HLA\text{-}DR^+$, but with a variable expression of CD86 between patients, underlining our flow cytometry data (white arrows). Interestingly, in four out of seven patients we observed a $CD86^+$ macrophage population that was negative for HLA-DR adjacent to the lining layer (yellow arrows). Surrounding the blood vessels, most macrophages had a high HLA-DR expression with often co-expression of CD86 (red arrows) (Fig. 1D). Additionally, we performed haematoxylin and eosin staining to better distinguish the various tissue areas (Fig. 1E). Single markers and merged images for all patients are shown in Supplementary Fig. S2, available at *Rheumatology* online.

S100A9 exposure during monocyte-to-macrophage differentiation promotes the expression of M2-like macrophage markers

Since the macrophage phenotype is strongly influenced by inflammatory factors, we next determined whether the S100A8/A9 that is present at high levels in the synovium during OA and is known to affect the phenotype of myeloid cells could induce the M2-like macrophage differentiation as observed in our OA patients. Hereto, cells were exposed to S100A9 for 6 days during monocyte-to-macrophage differentiation and the macrophage phenotype was evaluated using flow

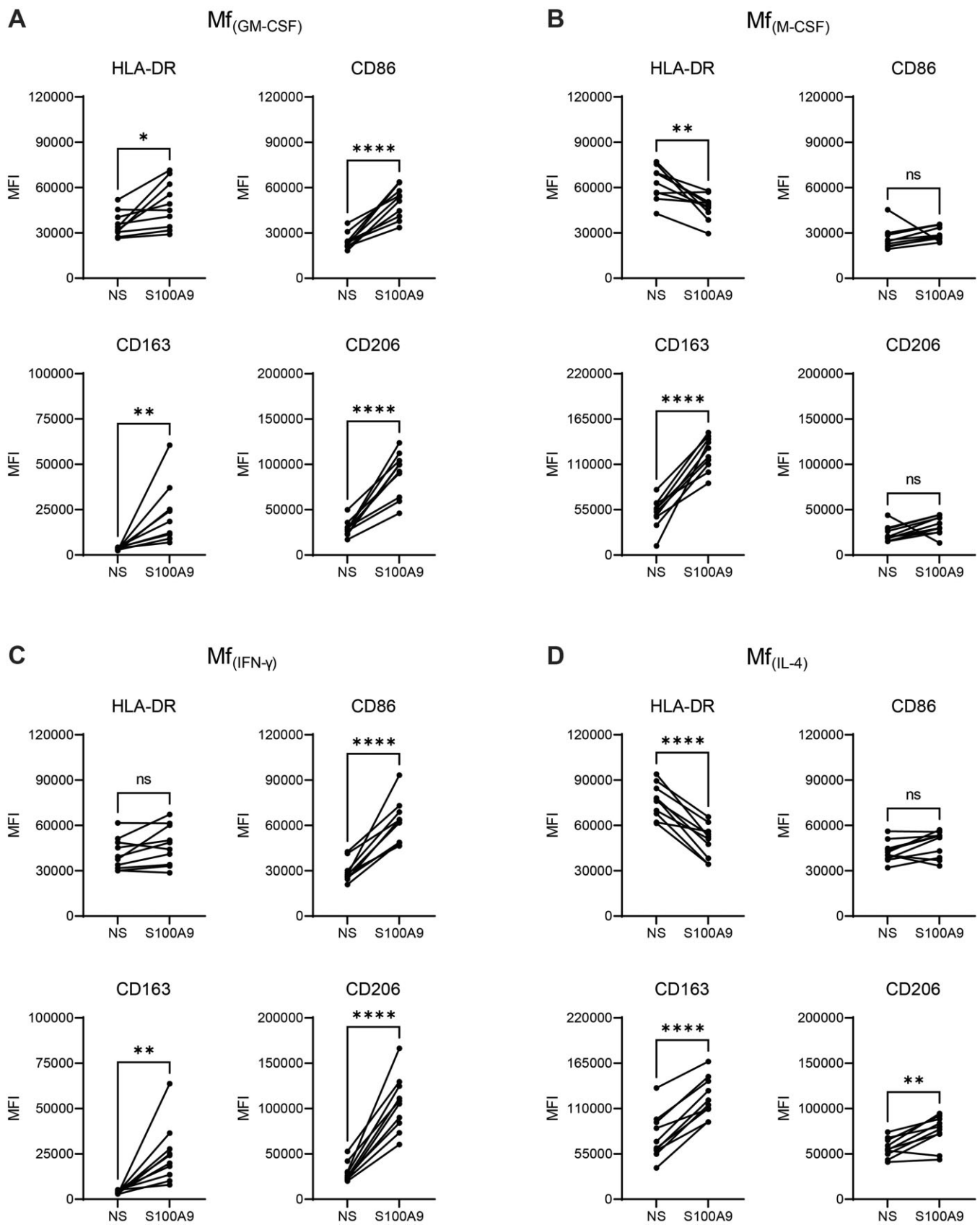

Figure 2. Exposure to S100A9 during monocyte-to-macrophage differentiation upregulates M2-like macrophage marker expression. Changes in expression of M1-like macrophage markers HLA-DR and CD86 and M2-like macrophage markers CD163 and CD206 upon exposure to S100A9 in $Mf_{(GM\text{-}CSF)}$ (**A**), $Mf_{(M\text{-}CSF)}$ (**B**), $Mf_{(IFN\text{-}\gamma)}$ (**C**) and $Mf_{(IL\text{-}4)}$ (**D**) measured with flow cytometry. Both M1-like and M2-like macrophage marker expression was increased in $Mf_{(GM\text{-}CSF)}$ and $Mf_{(IFN\text{-}\gamma)}$, while in $Mf_{(M\text{-}CSF)}$ and $Mf_{(IL\text{-}4)}$ M1-like macrophage marker expression was decreased and M2-like macrophage marker expression was increased. $n = 10$ donors. * $P_{FDR} < 0.05$, ** $P_{FDR} < 0.01$, **** $P_{FDR} < 0.0001$, ns: not significant. FDR: false discovery rate; $Mf_{(GM\text{-}CSF/M\text{-}CSF/IFN\text{-}\gamma/IL\text{-}4)}$: macrophages differentiated using GM-CSF/M-CSF/GM-CSF+IFN-γ/M-CSF+IL-4; MFI: mean fluorescence intensity; NS: non-stimulated

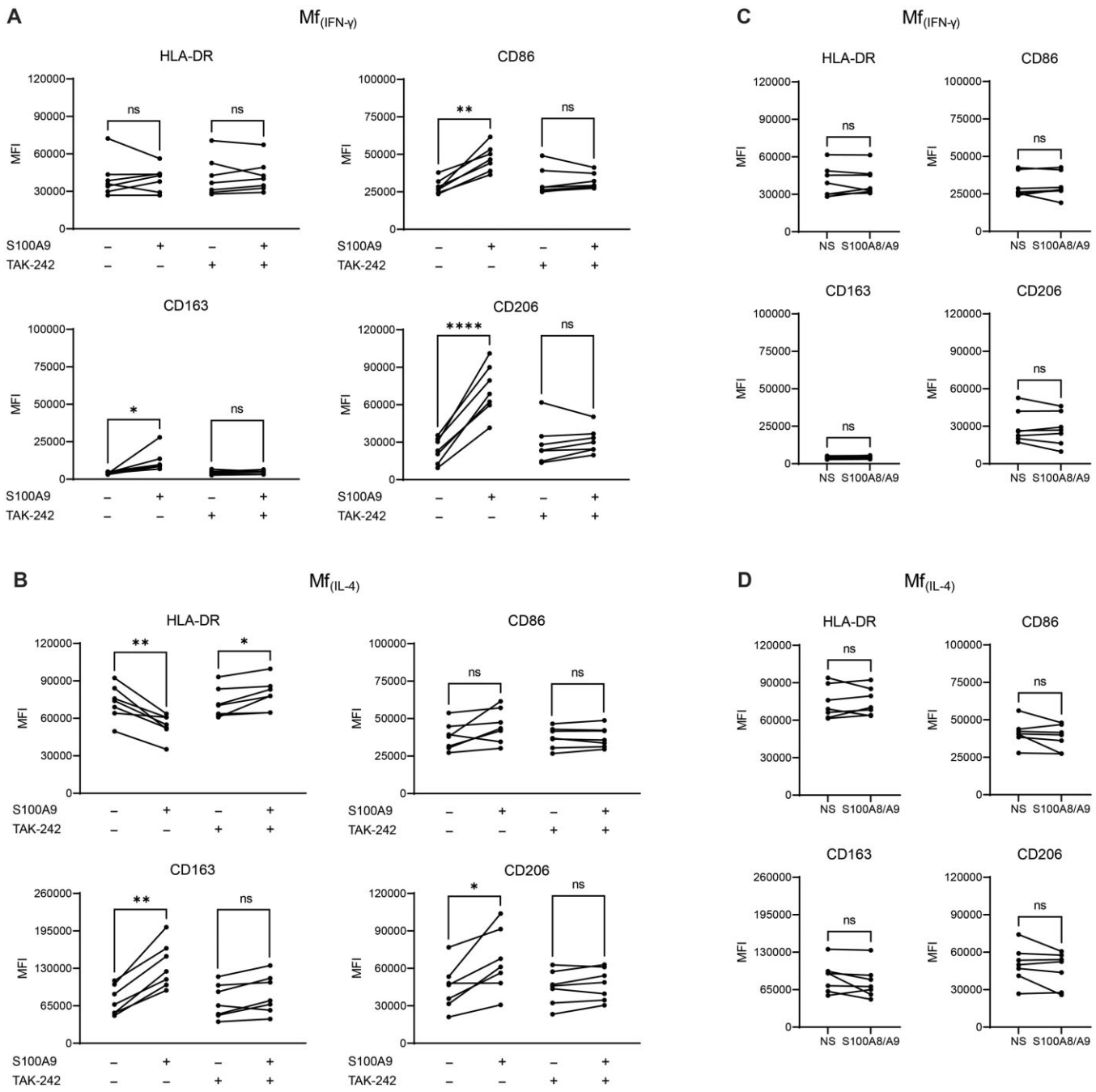

Figure 3. The effects of S100A9 exposure during monocyte-to-macrophage differentiation run via TLR4. (**A**, **B**) TAK-242, a small molecule TLR4 inhibitor, diminished the effects of S100A9 exposure observed in the controls in $Mf_{(IFN\text{-}\gamma)}$ (**A**) and $Mf_{(IL\text{-}4)}$ (**B**). (**C**, **D**) No changed expression of M1-like macrophage markers HLA-DR and CD86 and M2-like macrophage markers CD163 and CD206 upon exposure to the wild-type S100A8/A9 heterodimer, which is known not to function via TLR4, during monocyte-to-macrophage differentiation in $Mf_{(IFN\text{-}\gamma)}$ (**C**) and $Mf_{(IL\text{-}4)}$ (**D**). Expression levels were measured with flow cytometry. $n = 7$ donors. $*P_{FDR} < 0.05$, $**P_{FDR} < 0.01$, $****P_{FDR} < 0.0001$, ns: not significant. FDR: false discovery rate; $Mf_{(GM\text{-}CSF/M\text{-}CSF/IFN\text{-}\gamma/IL\text{-}4)}$: macrophages differentiated using GM-CSF/M-CSF/GM-CSF+IFN-γ/M-CSF+IL-4; MFI: mean fluorescence intensity; NS: non-stimulated

cytometry. This showed that exposure to S100A9 significantly increased the expression of M2-like macrophage markers CD206 in $Mf_{(GM\text{-}CSF)}$ and CD163 in both $Mf_{(GM\text{-}CSF)}$ and $Mf_{(M\text{-}CSF)}$ (Fig. 2A and B). Interestingly, S100A9 stimulation of $Mf_{(GM\text{-}CSF)}$ resulted in a robust expression of HLA-DR and CD86 as we previously observed in the synovia of OA patients (Fig. 2A). In contrast, $Mf_{(M\text{-}CSF)}$ showed a significant decrease in HLA-DR expression upon S100A9 stimulation, indicating a more M2-polarized phenotype (Fig. 2B). Comparable results were found after IFN-γ activation of $Mf_{(GM\text{-}CSF)}$, giving rise to $Mf_{(IFN\text{-}\gamma)}$ (Fig. 2C), and after IL-4 activation of $Mf_{(M\text{-}CSF)}$, giving rise to $Mf_{(IL\text{-}4)}$ (Fig. 2D), to further push their polarization states, although HLA-DR was not significantly increased any more in GM-CSF-differentiated cells after S100A9 stimulation. As expected, in line with the pro-inflammatory effects observed after stimulation of mature macrophages with S100A9, this decreased the expression of the M2-like macrophage markers CD163 and

CD206 (Supplementary Fig. S3, available at *Rheumatology* online).

Effects of S100A9 on monocyte-to-macrophage differentiation are toll-like receptor 4 dependent

To study whether these effects of S100A9 exposure on macrophage differentiation were dependent on TLR4 signalling, TLR4 activity in $Mf_{(IFN\text{-}\gamma)}$ and $Mf_{(IL\text{-}4)}$ was inhibited using the small-molecule TLR4 inhibitor TAK242. Importantly, samples with dimethyl sulfoxide solvent showed similar effects of S100A9 to samples without dimethyl sulfoxide, which are shown in Fig. 2. Inhibition of TLR4 signalling abrogated the S100A9 effects, indicating that the effects of S100A9 during monocyte-to-macrophage differentiation are mediated through TLR4 signalling (Fig. 3A and B). To substantiate the TLR4 dependency, we used the wild-type S100A8/A9 heterodimer that quickly tetramerizes in *in vitro* cultures and which is known not to function via TLR4. This did not change the expression of any of the tested macrophage markers (Fig. 3C and D).

Macrophages exposed to S100A9 during monocyte-to-macrophage differentiation upregulate factors associated with pro-inflammatory/catabolic but also anti-inflammatory/anabolic processes

Next, we investigated whether the expression profile of various factors that are relevant for the pathogenesis of OA was in line with the mixed macrophage phenotype we observed in our flow cytometry experiments. Whereas it is known from previous studies that S100A9 strongly induced the expression of various pro-inflammatory factors like *IL1B*, *IL6* and *IL8* in mature cell types, exposure during monocyte-to-macrophage differentiation did not significantly alter the *IL1B* expression, but increased *IL6* and *IL8* expression in $Mf_{(IFN\text{-}\gamma)}$ and $Mf_{(IL\text{-}4)}$ cells, respectively. Additionally, we observed a significant increase in chemokine *CCL2* expression in both cell types (Fig. 4A). On the other hand, S100A9 also significantly increased expression of the anti-inflammatory factor *IL10* in both $Mf_{(IFN\text{-}\gamma)}$ and $Mf_{(IL\text{-}4)}$ and *SOCS3*, *TGFB1* and *VEGFA* in $Mf_{(IL\text{-}4)}$, underlining the induction of both pro-inflammatory and anti-inflammatory processes (Fig. 4B). In addition, S100A9 increased the expression of the catabolic enzymes *MMP2*, *MMP9* and *MMP14* (Fig. 4C). Interestingly, we could validate these gene expression patterns at the protein level, as we found an increased secretion of IL-6, IL-8, IL-10, MCP-1 and MMP9 in both $Mf_{(IFN\text{-}\gamma)}$ and $Mf_{(IL\text{-}4)}$ upon exposure to S100A9. In contrast to our findings on the mRNA level, IL-1β protein levels were increased in $Mf_{(IFN\text{-}\gamma)}$ upon S100A9 exposure, although the observed levels were very low overall (Fig. 4D and E).

Macrophages show an increased phagocytic activity after exposure to S100A9 during monocyte-to-macrophage differentiation

Next, we set out to determine whether the cells obtained after exposure to S100A9 during monocyte-to-macrophage differentiation showed increased M2-like macrophage activity. Therefore, we determined the phagocytic activity using pHrodo Red Zymosan A BioParticles. Stimulation with S100A9 increased the uptake of extracellular particles in $Mf_{(GM\text{-}CSF)}$, whereas $Mf_{(M\text{-}CSF)}$ already showed high uptake in the absence of S100A9 (Fig. 5A). Quantification of multiple donors confirmed that both $Mf_{(GM\text{-}CSF)}$ and $Mf_{(IFN\text{-}\gamma)}$ had a lower phagocytic activity compared with $Mf_{(M\text{-}CSF)}$ and $Mf_{(IL\text{-}4)}$ in the absence of S100A9. However, they strongly increased their phagocytic activity upon exposure to S100A9 during monocyte-to-macrophage differentiation (Fig. 5B). Expression of *AXL* and *GAS6*, both involved in efferocytosis, was not increased by S100A9 (Fig. 5C). No further increase in phagocytosis was observed in $Mf_{(M\text{-}CSF)}$ and $Mf_{(IL\text{-}4)}$. This suggests that on a functional level, cells obtain more M2-like characteristics after exposure to S100A9 during differentiation.

Synovial *S100A8* and *S100A9* expression positively associate with the M2-like over M1-like macrophage ratio in OA synovial tissue

Finally, we determined whether in OA patients indeed *S100A8* and *S100A9* associated with the number of M2-like macrophages. In agreement with our other data, both *S100A8* and *S100A9* expression positively correlated with the M2-like over M1-like macrophage ratio in synovial tissue ($P = 0.0019$ and $P = 0.0174$, respectively) (Fig. 6).

Discussion

Inflammation of the synovium is present in many OA patients and is associated with disease progression. A particularly important role in the orchestration of this synovial inflammation has been attributed to macrophages. Here, we show that macrophages in the OA synovium have a mixed phenotype and express both M1-like and M2-like macrophage-associated markers. Moreover, we show that the presence of the alarmin S100A8/A9 during monocyte-to-macrophage differentiation simultaneously promotes M2-like macrophage marker expression and activity and some of the M1-like macrophage characteristics. Finally, we show that indeed *S100A8* and *S100A9* expression is associated with an increased M2-like over M1-like macrophage ratio.

The alarmin S100A8/A9 is present at high levels in the synovial fluid of OA patients [16]. A previous study from our lab showed that S100A8/A9 was predominantly secreted by M1-like macrophages [12]. Stimulation of both macrophages and chondrocytes with S100A9 for 24 h resulted in the production of a plethora of pro-inflammatory mediators, including IL-1β, IL-6, IL-8 and TNFα, and catabolic mediators, including various MMPs [12, 14]. In agreement, mice deficient in *S100a9*, which also lack S100A8 protein expression in the periphery, showed strongly decreased cartilage degeneration compared with wild-type mice in an induced preclinical OA model. Moreover, early symptomatic OA patients with high S100A8/A9 serum levels at the time of inclusion showed stronger disease progression compared with those with low levels [17]. Together, this indicates that S100A8/A9 is involved in the destructive processes that are characteristic for OA development. Interestingly, however, in the preclinical OA model using *S100a9* deficient mice, we also observed decreased osteophyte formation [15]. This indicates that the involvement of S100A8/A9 in OA is more complex than just stimulating inflammation and the resulting catabolic activity. S100A8/A9 also seems to be involved in the anabolic processes that are observed along the development of OA, like ectopic bone formation and fibrosis. This is underlined by studies in other diseases showing that S100A8/A9 pretreatment of human mesenchymal stem cells with S100A8/A9

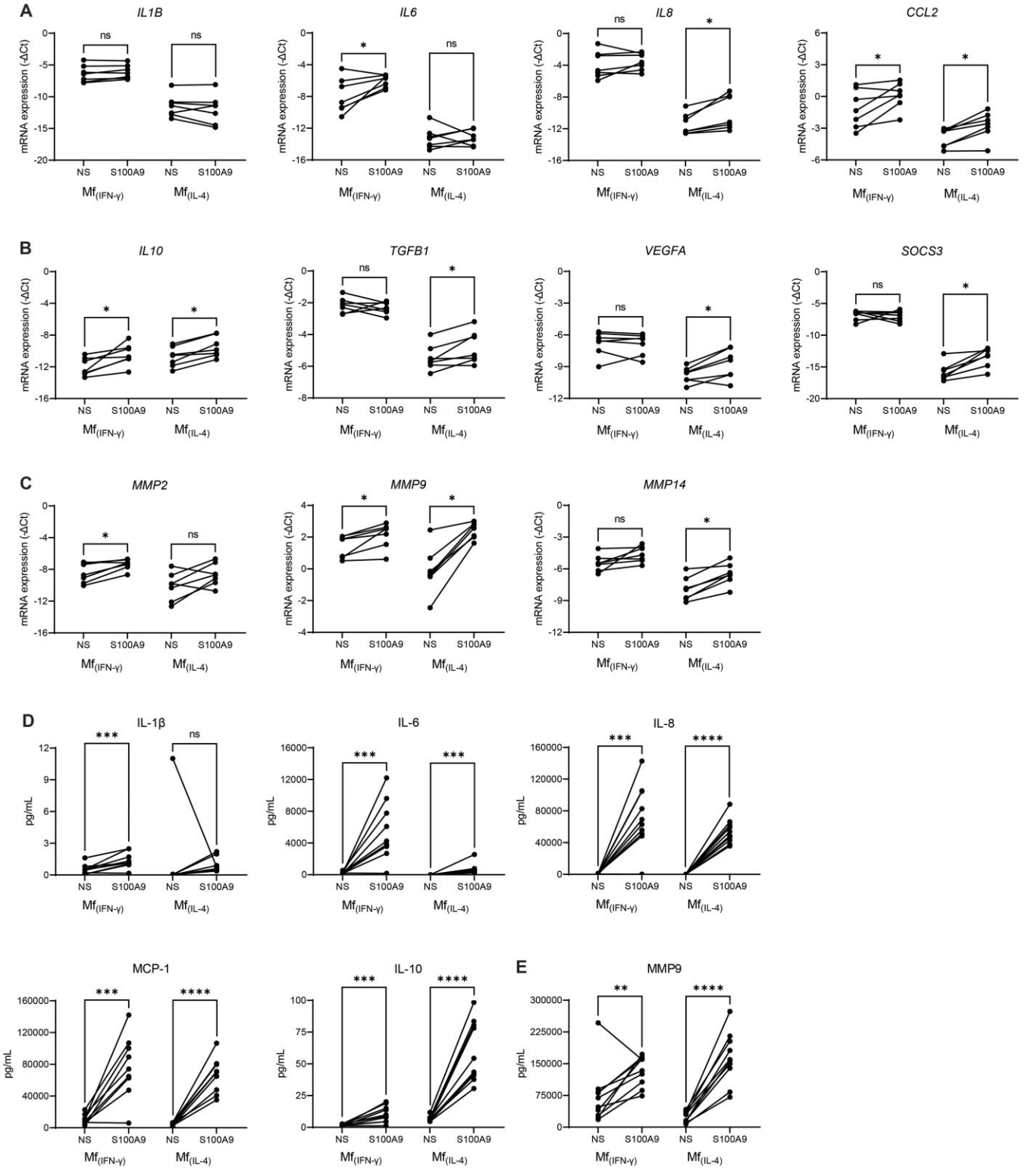

Figure 4. Factors associated with both pro-inflammatory/catabolic and anti-inflammatory/anabolic processes are upregulated upon S100A9 exposure. (**A**) Upon S100A9 exposure, $Mf_{(IFN\text{-}\gamma)}$ increased *IL6* expression, $Mf_{(IL\text{-}4)}$ increased *IL8* expression and both increased *CCL2*, but not *IL1B* expression. (**B**) S100A9 exposure induced *TGFB1*, *VEGFA* and *SOCS3* expression in $Mf_{(IL\text{-}4)}$, while increasing *IL10* expression in $Mf_{(IFN\text{-}\gamma)}$ and $Mf_{(IL\text{-}4)}$. (**C**) S100A9 exposure increased *MMP2* expression in $Mf_{(IFN\text{-}\gamma)}$, *MMP14* expression in $Mf_{(IL\text{-}4)}$ and *MMP9* expression in both. (**D**) IL-6, IL-8, IL-10 and MCP-1 were increased in $Mf_{(IFN\text{-}\gamma)}$ and $Mf_{(IL\text{-}4)}$ and IL-1β in $Mf_{(IFN\text{-}\gamma)}$ upon S100A9 exposure. (**E**) S100A9 exposure increased MMP9 secretion in $Mf_{(IFN\text{-}\gamma)}$ and $Mf_{(IL\text{-}4)}$. $n = 7$ for RNA analyses, $n = 10$ for protein measurements. * $P_{FDR} < 0.05$, ** $P_{FDR} < 0.01$, *** $P_{FDR} < 0.01$, **** $P_{FDR} < 0.0001$, ns: not significant. C_t: cycle threshold; FDR: false discovery rate; $Mf_{(GM\text{-}CSF/M\text{-}CSF/IFN\text{-}\gamma/IL\text{-}4)}$: macrophages differentiated using GM-CSF/M-CSF/GM-CSF+IFN-γ/M-CSF+IL-4; NS: non-stimulated

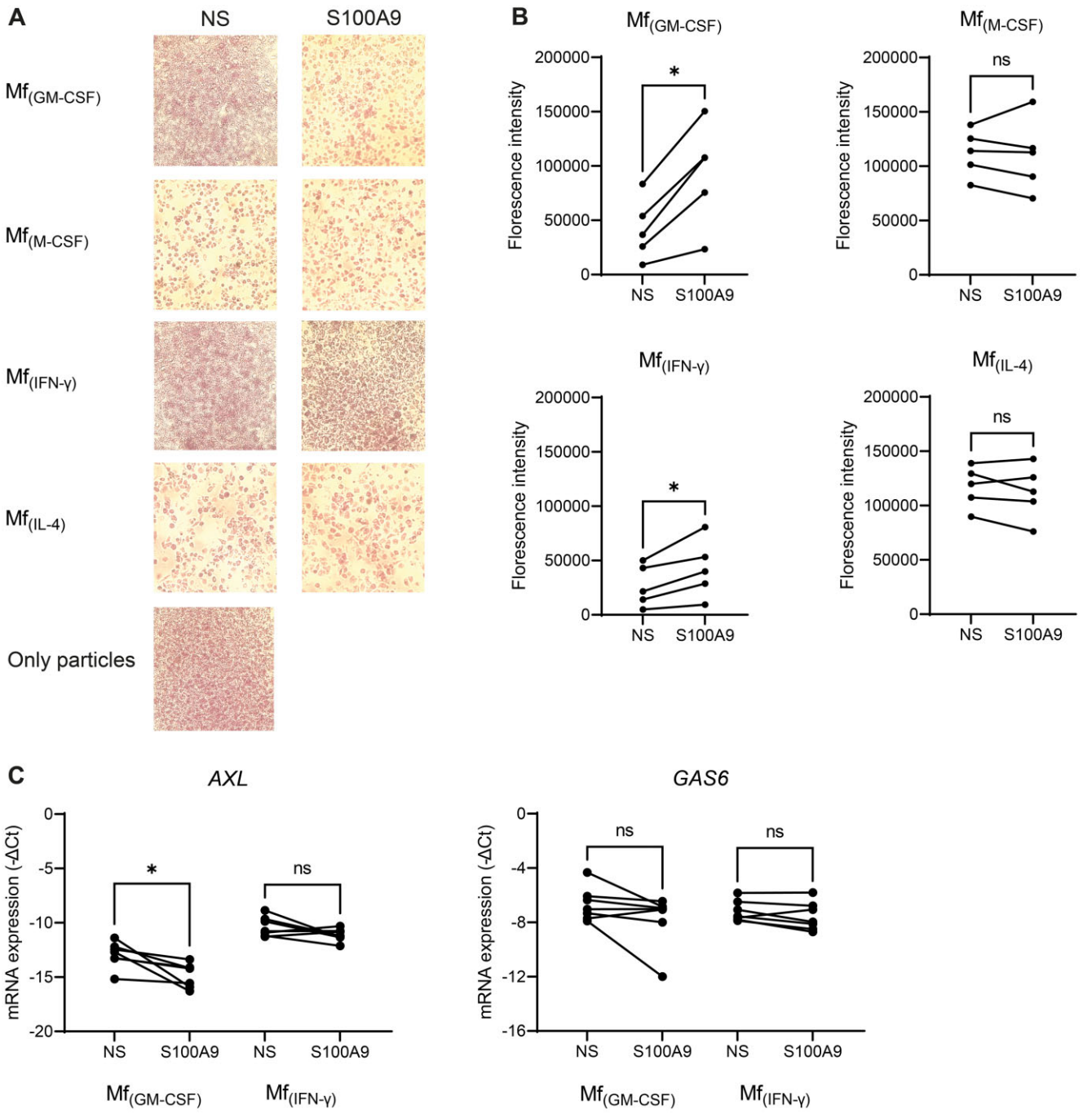

Figure 5. The phagocytic activity of $Mf_{(GM\text{-}CSF)}$ and $Mf_{(IFN\text{-}\gamma)}$ is increased upon S100A9 exposure during monocyte-to-macrophage differentiation. (**A**, **B**) Visualization (**A**) and quantification (**B**) showed an increased uptake of pHrodo Red Zymosan A BioParticles upon exposure to S100A9 in $Mf_{(GM\text{-}CSF)}$ and $Mf_{(IFN\text{-}\gamma)}$ but not in $Mf_{(M\text{-}CSF)}$ and $Mf_{(IL\text{-}4)}$. (**C**) Upon S100A9 exposure *AXL* expression was decreased in $Mf_{(GM\text{-}CSF)}$ but not in $Mf_{(IFN\text{-}\gamma)}$. *GAS6* expression did not change. Phagocytosis assay: $n = 5$ donors; gene expression: $n = 7$ donors. * $P_{FDR} < 0.05$, ns: not significant. C_t: cycle threshold; FDR: false discovery rate; $Mf_{(GM\text{-}CSF/M\text{-}CSF/IFN\text{-}\gamma/IL\text{-}4)}$: macrophages differentiated using GM-CSF/M-CSF/GM-CSF+IFN-γ/M-CSF+IL-4; NS: non-stimulated

enhances both anti-inflammatory and anti-fibrotic effects of these cells in murine myocardial ischaemia [18] and that S100A8/A9 has anti-inflammatory properties in rat models of inflammatory disorders [19].

In agreement with previous studies, we observed that macrophages were the most dominant immune cell type, followed by mast cells, lymphoid cells and a small percentage of neutrophils [20]. Whereas part of these macrophages most likely consists of tissue-resident macrophages, monocyte infiltration in the OA synovium can result in monocyte-to-macrophage differentiation in the inflamed OA synovium. Therefore, in this study we set out to study the effects of S100A8/A9 on macrophage polarization and activation in detail.

In a healthy joint, the synovial lining layer generally is only a few cell layers thick and next to the fibroblast-like synoviocytes consists of tissue-resident macrophages. These macrophages express CD163 and CX3CR1 and are important for maintaining tissue homeostasis with their anti-inflammatory

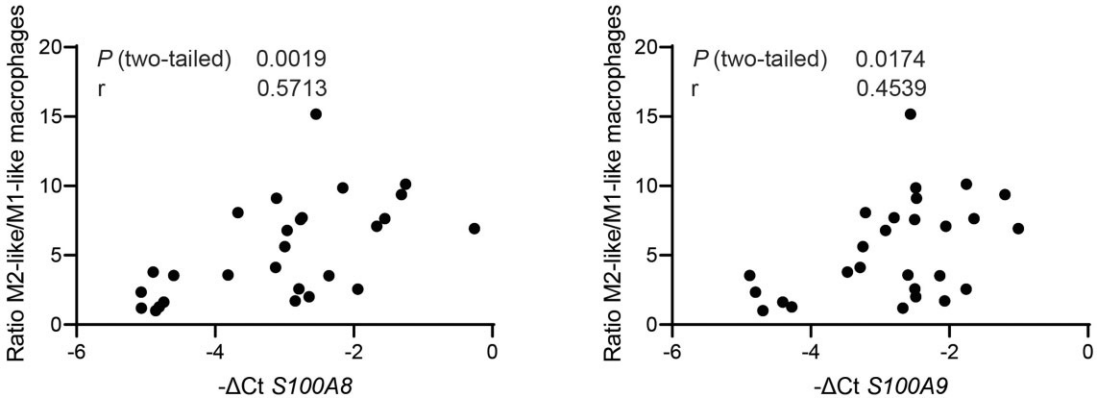

Figure 6. *S100A8* and *S100A9* expression correlate with the M2-like/M1-like macrophage ratio in end-stage OA synovium. Positive correlation between the M2-like/M1-like macrophage ratio measured with flow cytometry and both *S100A8* and *S100A9* expression in end-stage OA synovium. $n = 22$ patients. C_t: cycle threshold

character [6, 21]. During inflammation, the tissue resident macrophages can become activated and monocytes are recruited to the joint where they will differentiate towards macrophages, causing inflammation and thickening of the synovium. Monocyte-derived macrophages are generally considered to have a more pro-inflammatory phenotype [21].

Therefore, we studied the macrophage subpopulation present in established OA synovium in more detail. As CD163 is a well-described M2-like macrophage marker and has been shown to be absent on M1-like macrophages [22], we used CD163 as the major discriminator to separate M1-like and M2-like macrophages and further identified subtypes based on CD206, HLA-DR and CD86 expression levels. Using this strategy, we observed a relatively small population of M1-like macrophages that were negative for CD163 and positive for both the M1-like macrophage markers CD86 and HLA-DR. Interestingly, we observed a large population of macrophages with a mixed phenotype, characterized by the expression of CD163 and also relatively high levels of HLA-DR and CD86. This is in contrast with many previous papers that state that M1-like cells are the predominant macrophage phenotype found in OA synovium [23]. In line with our findings, a previous study using transcriptomic data has shown a dominance of M2-like macrophages over M1-like macrophages [24]. Moreover, in agreement with our own findings, other groups found co-expression of M1-like and M2-like macrophage markers in OA synovium [25]. These studies, combined with ours, show the importance of an extended flow cytometry panel to identify macrophage phenotype beyond the M1/M2 dichotomy [26]. Moreover, we conducted multiplex immunohistochemistry to investigate the localization of the macrophage populations we observed with flow cytometry. This largely underlined our flow cytometry results and predominantly showed expression of the M2-like macrophage marker CD163 in the lining, with co-expression of HLA-DR and CD86. In contrast, a more M1-like type of macrophage was observed in the sublining around blood vessels and lymphocyte clusters, characterized by the expression of HLA-DR and CD86, but with no or only very weak staining of CD163. This suggests that the infiltrating cells have a more pro-inflammatory phenotype whereas the cells residing in the lining have a more immunoregulatory function. However, whereas in our flow cytometry analysis almost all $CD163^+$ cells were $HLA\text{-}DR^+$ and the vast majority was also $CD86^+$, here these markers were only present on a subset of $CD163^+$ cells, which were mainly observed in the lining layer. This discrepancy might be caused by activation of the cells as the result of the enzymatic digestion that is needed to prepare a single cell suspension for flow cytometry or by differences in detection threshold between the techniques. However, it should be noted that the macrophage phenotype may vary along the development of OA as the result of disease cause, state and incidence of flares.

To mimic this variation of macrophage phenotypes in the osteoarthritic joint, we differentiated monocytes in the presence of either GM-CSF or M-CSF, which are growth factors present in the synovial fluid of OA patients that are widely used to model M1-like and M2-like macrophages *in vitro* [27]. We observed that upon chronic S100A9 exposure during the monocyte-to-macrophage differentiation that occurs in the OA joint, characterized by a chronic inflammatory response, the $Mf_{(GM\text{-}CSF)}$ and $Mf_{(IFN\text{-}\gamma)}$ increased both CD163 and CD206 but also CD86 and HLA-DR expression in most donors. In addition, these cells also showed an upregulation of *IL6* and *IL10* expression upon stimulation with S100A9, together indicating a macrophage phenotype that might represent M2b macrophage characteristics [28]. Interestingly, these cells closely resemble the predominant macrophage subset that we observed in the synovial tissue of OA patients, showing a mixed phenotype with M2-like macrophage markers such as CD163 and CD206, but also high expression of CD86 and HLA-DR. A previous study showed that GM-CSF levels in OA synovial fluid are higher compared with M-CSF levels [29]. Therefore, it is tempting to speculate that the environment rich in GM-CSF and S100A8/A9 that is present in the OA joint might give rise to the macrophage population with a mixed phenotype that we observed in OA synovium.

Upon stimulation with S100A9 the $Mf_{(M\text{-}CSF)}$ and $Mf_{(IL\text{-}4)}$ upregulated CD163, downregulated HLA-DR and at the same time showed an upregulation of anti-inflammatory/anabolic factors *IL10* and *VEGFA*, but also pro-inflammatory factor *IL8* and catabolic enzymes *MMP9* and *MMP14*. Therefore, phenotypically, these cells seem to represent an even more polarized M2-like macrophage subtype, while their gene expression profile still shows upregulation of pro-inflammatory/catabolic factors. This expression profile might be representative of locally proliferating tissue-resident macrophages in the inflamed OA synovium.

However, it should be noted that we used *in vitro* models with cells from donors that were not necessarily diagnosed with osteoarthritis to assess how S100A8/A9 affects the phenotype and function of resting and activated M1-like and M2-like macrophages. Although OA is not a typical systemic disease and therefore it is not expected that monocytes from OA patients would react differently from those from healthy individuals, it is important to consider that in a subset of OA patients systemic changes can be found as a result of, for example, the metabolic syndrome, which is prevalent in OA patients. This may result in prior training of monocytes to inflammatory stimuli and can cause them to react differently on S100A8/A9 exposure upon entering the joint.

OA is characterized by a chronic, but relatively low-grade inflammatory reaction, which is accompanied by coinciding catabolic processes like cartilage degeneration, but also by anabolic processes like neovascularization, ectopic bone formation and fibrosis. The findings from this study are in agreement with these characteristics. We observed that chronic stimulation with S100A9 did not increase *IL1B* expression and only slightly increased IL-1β secretion in $Mf_{(GM\text{-}CSF)}$, with very low overall levels. Recent literature indicates that the involvement of this cytokine in OA pathogenesis might be limited. Absence or inhibition of IL-1β did not alleviate end-stage pathology in a pre-clinical model for OA with clear synovial inflammation [30]. Moreover, several clinical trials using IL-1 as a treatment target have failed to show clinical efficacy [31, 32]. In contrast, we observed increased *IL6* and *IL8* expression and strongly increased IL-6 and IL-8 secretion upon S100A9 exposure. Previous research on these cytokines in the context of OA shows elevated IL-6 and IL-8 levels in serum and synovial fluid of a subset of patients [33]. Although a clinical trial with IL-6RA tocilizumab showed no effects on pain relief in hand OA patients [34], IL-6 was shown to be a predictor of radiographic knee OA [33] and modulation of the IL-6/signal transducer and activator of transcription 3 signalling pathway protected against cartilage damage in pre-clinical OA models [35]. In addition to these pro-inflammatory cytokines, anti-inflammatory cytokines like IL-4 and IL-10 appear relevant in OA pathogenesis by showing chondroprotective effects and restraining the joint inflammation [36, 37]. We indeed found an increased expression of anti-inflammatory factors like *IL10* and *TGFB* and an increased IL-10 production.

One of the main hallmarks of OA is the breakdown of articular cartilage that is caused by catabolic enzymes like MMPs. Interestingly, we observed a consistent increase in *MMP9*, but also *MMP2* and *MMP14* expression, which are relevant mediators of cartilage breakdown in OA [38–40]. Additionally, we found a strong increase of MMP9 secretion on protein level, indicative of the presence of catabolic processes as observed in OA.

Cartilage fragments that are released as the result of microtraumas and the active breakdown of articular cartilage by proteolytic enzymes are cleaned up by phagocytic cells, which in the synovial tissue are mainly represented by macrophages [41, 42]. Interestingly, our findings showed that S100A9 enhanced this clearance function by increasing the phagocytic activity of $Mf_{(GM\text{-}CSF)}$ and $Mf_{(IFN\gamma)}$ towards levels observed in M2-like macrophages. However, whereas macrophages are involved in the uptake of apoptotic cells, we observed no upregulation of *GAS6* and *AXL*, which are genes involved in efferocytosis [43]. This implies that S100A9 may be a regulator of phagocytic mechanisms that are involved in cleaning matrix debris rather than in apoptotic cell clearance. Together, this might represent the vicious circle that is thought to be present in OA, with an increased cartilage breakdown caused by an increased production of matrix degrading enzymes like MMP9, leading to an increased inflammation, which again can promote cartilage breakdown and phagocytosis of the matrix debris.

Apart from pro-inflammatory and catabolic processes, OA is characterized by more anabolic processes like angiogenesis and fibrosis. Inflammation-induced macrophage activation and production of proangiogenic factors stimulates synovial angiogenesis via, for example, the production of VEGF that we found increased upon S100A9 exposure [44]. TGF-β has been shown to be a key factor in the induction of synovial fibrosis as extensively reviewed before [45]. Of note, we observed that S100A9 increased the expression of *TGFB1*, but also *MMP14*, which has been described in the activation of TGF-β [46]. Together, these findings link S100A8/A9 also to the more anabolic processes that occur along OA development.

Taken together, we have shown that macrophages in OA synovium predominantly present with a mixed M2/M1-like phenotype and that S100A8/A9 induces a macrophage phenotype that resembles these cells. This implies that chronic exposure to the alarmin S100A8/A9 underlies the dual-faced macrophage phenotype as observed in OA synovium, which could explain the combined catabolic and anabolic character of the disease.

Supplementary material

Supplementary material is available at *Rheumatology* online.

Data availability

Data are available upon request.

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