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Collagen morphology influences macrophage shape and marker expression *in vitro*

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ABSTRACT

Fibrosis is a process in which an accumulation of extracellular matrix (ECM) leads to an impaired function of the affected organ. Pulmonary fibrosis is the end-stage of several lung diseases, characterized by scarring of the lungs. Although macrophages are known to be important players in ECM homeostasis, their ability to respond to fibrosis-related morphological and mechanical changes of the ECM is relatively unexplored. In this study we aimed to elucidate the effect of ECM stiffness and morphology on macrophage polarization, by using a collagen type I-based *in vitro* system. Collagen morphology, but not stiffness, affected the relative expression of CD206 (the mannose receptor) and Ym1 (a murine marker of pro-healing M2 macrophages). Higher expression of Ym1 was found when macrophages were cultured on fibrous collagen. Globular collagen led to higher expression of CD206, a marker known to be upregulated on alveolar macrophages in idiopathic pulmonary fibrosis. Moreover, macrophages exhibited distinct differences in shape with actin-rich protrusions on fibrous collagen and more filopodia on globular collagen. In addition to these cytoskeletal changes, transmigration was higher when macrophages were cultured on fibrous collagen. Together these findings indicate that macrophages are sensitive to collagen morphology, responding with subtle changes in marker expression, shape and behavior rather than a complete polarization switch. This study emphasizes the complex interaction between macrophages and their surroundings, and the need for further exploration of both mechanical and morphological aspects.

1. Introduction

Fibrosis is a complex disease with a diverse etiology that can affect different organs. Characteristics and development of the disease vary depending on both the cause and the affected organ. In general, an imbalance in the production and degradation of extracellular matrix (ECM) proteins results in altered composition and stiffness of the ECM.[1–3] Pulmonary fibrosis is the end-stage of several lung diseases and is characterized by scarring of the lungs and subsequent loss of function.[4,5] Patients with pulmonary fibrosis increasingly suffer from shortness of breath and dry cough, and this progressive disease has an average survival rate of around 2–3 years after diagnosis.[6–9] Only recently, in 2014, the first two antifibrotic agents were approved for the treatment of idiopathic pulmonary fibrosis: pirfenidone and nintedanib.[10] Although these new agents can reduce the progression of the disease, they do not improve mortality.[10] The exact mechanisms underlying the development of

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pulmonary fibrosis, as well as many other forms of fibrosis, are still unknown but macrophages are known to play an important role in both stimulating the production and the degradation of ECM proteins. Therefore, dysregulation of macrophage function may contribute to fibrosis.[11,12]

Activation of macrophages can result in polarization towards different phenotypes, depending on the stimulus. Lipopolysaccharide (LPS), interferon-gamma (IFN-γ) and tumor necrosis factor (TNF) result in polarization towards a pro-inflammatory phenotype (also known as M1).[13] This activation state is important in fighting infections and in inflammatory responses.[11] Interleukin-4 (IL-4) and interleukin-13 (IL-13) induce a pro-healing phenotype in macrophages (also known as M2) that assist in tissue repair.[11] Interleukin-10 (IL-10) and tumor growth factor-β (TGF-β) induce a macrophage phenotype mainly involved in dampening of immune responses (also known as M2-like, anti-inflammatory, or M2c).[11] The last two phenotypes are highly associated with fibrosis.[11] However, their exact roles and the mechanisms by which macrophage functions are regulated are unclear and this is partly caused by the lack of knowledge on the interplay between macrophage function and the altered tissue architecture observed in fibrosis.

Both the stiffness and morphology of tissue have shown to be dissimilar in healthy and fibrotic lungs.[14–17] Booth et al. reported significantly higher stiffness of fibrotic lungs compared to healthy lungs.[17] Therefore, several studies have assessed the effect of substrate rigidity on macrophages. Most differences were observed in the shape of macrophages, as rigid substrates induced a larger, more flattened shape and the formation of filopodia.[18–21] In addition to alterations in ECM stiffness and composition, changes in the morphology of ECM have been observed in fibrosis. Diseased lung tissue was shown to have collagen type I with a more disorganized and immature morphology compared to collagen type I in healthy control lungs.[14–16] However, the morphology of ECM has not been studied yet as a modulator of macrophage function in addition to tissue stiffness.

In our studies we used a collagen type I-based system offering control over both matrix stiffness and morphology, to study effects of both variables on macrophage function. Alveolar-like macrophages were cultured on matrices consisting of collagen-coated glass without further chemical treatment or crosslinking, whilst using pH, temperature and concentration as parameters to control and tune stiffness and morphology. The effects of these collagen type I layers on macrophage polarization, marker expression, shape and migration were investigated. Knowledge obtained from these results will yield more insight in the interactions between macrophages and the morphologically and mechanically altered ECM.

2. Materials and methods

2.1. Materials

Rat tail collagen type 1 was purchased from Ibidi (Martinsried, Germany). 400-mesh copper grids were purchased at Agar Scientific (Stansted, United Kingdom). RPMI and gentamycin were supplied by Gibco Laboratories (Grand Island, USA). GM-CSF was provided by Peprotech (Rocky Hill, USA). Fetal calf serum (FCS, heat-inactived) was purchased from Biowest (Nuaillè, France). EDTA was supplied by Merck (Darmstadt, Germany). Lidocaine HCl, Bovine Serum Albumin (BSA) and phalloidin-FITC were bought from Sigma-Aldrich (Zwijndrecht, The Netherlands). The antibodies anti-MHCII APC/Cy7 and anti-CD206 AlexaFluor647 were purchased from Biolegend (Fell, Germany), anti-Ym1 from R&D Systems (Oxon, UK). DAPI was bought from Boehringer (Mannheim, Germany). Donkey-anti-goat AlexaFluor 555 was purchased from Invitrogen (Carlsbad, US). Transwell cell culture inserts with a pore size of 8 μm were bought from Corning Life Sciences (#3464, Amsterdam, The Netherlands).

2.2. Collagen-coated substrates

Glass slides were cleaned by air plasma surface treatment for 10 min at 120 mTorr (Plasma Activate Flecto 10 USB, Plasma Technology, Rottenburg, Germany) and sterilized at 180 °C for 4 h. The slides were then coated under sterile conditions with 75 μl/cm^2 collagen type 1, rat tail at a concentration of 2.8 mg/mL, 0.28 mg/mL or 0.028 mg/mL, by diluting the collagen in either 17.5 mM CH_3COOH (final pH = 3) or 17.5 mM NaOH (final pH = 7) at 4 °C or 37 °C. After 1 h of incubation, slides were washed twice with sterile water and dried overnight at 37 °C. Prior to use for cell culture, the collagen layers were washed with cell culture medium and pH measurements verified no influence of the collagen coating conditions on the pH of the cell culture medium. For transmission electron microscopy, mesh copper grids covered with a carbon film were coated with 0.0028 mg/mL collagen at an acidic or neutral pH. For the transmigration assay, the transwells were coated with 2.8 mg/mL collagen at an acidic or neutral pH.

2.3. Characterization of collagen layers

Collagen layers were characterized in terms of mechanical properties and morphology. The combined stiffness (Young's modulus) of the collagen layer and glass base was measured using an atomic force microscope (AFM) model Catalyst (Bruker, MA, USA) and Nanoscope V software. Measurements were performed in QMN (quantum-mechanical nano mapping) mode using cantilevers made from silicon nitride with silicon made tips (Bruker model DNP-10, 0.06 N/m). The tips were calibrated prior to every experiment using glass as non-deformable surface. For topography measurements a Dimension 3100 Nanoscope V (Veeco, NY, USA) was used in contact mode and wet state with 0.24 N/m tips. All data were processed using Nanoscope Analysis (Veeco, Version 1.70). Additionally, collagen layers were visualized with bright field microscopy (Olympus IX50, Tokyo, Japan) and transmission electron microscopy (TEM). For TEM, samples on the grid were stained with 2% uranyl acetate for 1 min and imaged with a CM120 cryo-electron microscope (Philips, Eindhoven, The Netherlands) at 120 kV. Images were captured by a slow scan CCD camera. Morphology of the collagen was determined to be fibrous (collagen fibers visible) or globular (globular collagen structures visible, non-fibrous) based on images from atomic force microscopy, bright field microscopy and TEM.

2.4. Cell culture

Max Planck Institute (MPI) macrophages (alveolar-like macrophages as described by Fejer et al.,[22] a kind gift by Dr. G. Fejer) were cultured in RPMI containing 10% FCS and 0.4 mg/mL gentamycin, supplemented with 20 μg/mL murine GM-CSF. Cells were reseeded once a week at a density of 1×10^5 cells/mL, after detaching with 1 mM EDTA. Fresh medium was applied after 4 days. For experiments, cells were seeded at a density of 1.7×10^4 cells/cm^2 between passage number 5 and 13. The cells were incubated at 37 °C and 5% CO_2.

2.5. Macrophage response towards collagen-coated substrates

Macrophages were cultured on the collagen layers or on non-coated glass (control) for 72 h, after which they were either collected for flow cytometry, or fixed on the collagen layer for fluorescent staining. Cell culture medium was collected and stored at −80 °C until further analysis.

Cells were collected for flow cytometric analysis by using PBS with 10 mM EDTA and 4 mg/mL lidocaine. Frequencies of macrophage subsets were examined based on the expression of MHCII (major histocompatibility complex II) and CD206 (mannose receptor). M1 macrophages were defined as MHCII(hi)-CD206(lo), M2 macrophages as MHCII(hi)CD206(hi) and anti-inflammatory macrophages (also known as M2-like) as MHCII(lo)-CD206(hi).[13,23] Macrophages were incubated with an antibody mix containing anti-MHCII labeled with APC/Cy7 and anti-CD206 labeled with AlexaFluor 647 and 5% normal mouse serum in PBS containing 2% FCS 5 mM EDTA (PFE), on ice in the dark for 30 min. Finally, the cells were washed twice and resuspended in PFE and kept in the dark at 4 °C until flow cytometric analysis (FACS Array, BD Biosciences, Breda, The Netherlands). Flow cytometry data were analyzed using FlowJo Software (Tree Star, Ashland, USA). A fluorescence minus one (FMO) control was used for proper gating. An example of the gating strategy is shown in Supplementary Fig. 1.

For confocal microscopy and TissueFAXS analysis, cells were washed in PBS and fixed in 3.7% paraformaldehyde in PBS for 20 min, followed by two additional washes and storing in PBS at 4 °C. Prior to fluorescent staining, the samples were permeabilized with 0.5% Triton X-100 in PBS for three minutes. Nonspecific binding was blocked by incubating with 5% BSA in PBS for 30 min. Macrophages were stained with a primary antibody for Ym1 for 1 h. After incubation, the cells were washed three times in 1% BSA in PBS for five minutes. DAPI and Phalloidin-FITC were then added together with the secondary antibody for Ym1 (donkey-anti-goat AlexaFluor 555) and incubated for 1 h. Subsequently the cells were washed twice in 1% BSA in PBS for five minutes and once in PBS for five minutes. The samples were kept in PBS at 4 °C until further analysis. Analysis was done using a LEICA SP2 confocal microscope, with a HCX Apo L 63 × / 0.9 water objective. The number of cells with filopodia was quantified manually in three separate experiments. Within each experiment, three fields of view with in total at least 40 cells per condition were analyzed. Additionally, Ym1 expression in the whole sample was imaged using TissueFAXS cytometry (TissueGnostics, Vienna, Austria) and quantified using TissueQuest software (TissueGnostics).

Secretions of Ym1 (Chitinase 3-like 3 or ECF-L) and TNF-α in cell culture medium were determined with ELISA kits (both from R&D Systems), according to the instructions of the manufacturer.

2.6. Transmigration assay

Macrophages were cultured in the upper chamber of non-coated, fibrous collagen-coated and globular collagen-coated transwells. After 72 h, the transmigrated macrophages in the lower chamber were collected, washed in PFE and counted with a flow cytometer (CytoFLEX, Beckman Coulter, Woerden, The Netherlands). Flow cytometry data were analyzed using FlowJo Software.

2.7. Statistical analysis

A one-way ANOVA was used to detect significant differences ($p < 0.05$), followed by a Bonferroni post-hoc test to compare the groups. Statistical analyses were performed with use of GraphPad Prism 7.0 (GraphPad Software, La Jolla, USA).

3. Results

3.1. Properties of collagen-coated surfaces

Collagen layers were characterized with respect to stiffness (Young's modulus) and morphology using AFM (Fig. 1). Collagen coating conditions affected the morphology and stiffness of the layers (Supplementary Fig. 2). Morphology of the collagen layer was determined to be fibrous or globular (non-fibrous). Neutral conditions resulted in fibrous collagen layers (Fig. 1A–C and G-I), whereas an acidic environment resulted in globular collagen layers (Fig. 1D–F). These differences in morphology were visible with bright field microscopy as well. Transmission electron microscopy confirmed the absence of fiber formation on the globular collagen layers (Fig. 2). Eight conditions were selected for cell experiments, reflecting stiffness in a comparable range (48–170 kPa) but with different morphology (fibrous or globular). Therefore, the substrate with a stiffness of 225 kPa with a fibrous structure (Fig. 1A) was excluded, as it did not have a globular counterpart.

3.2. Collagen-induced polarization in macrophages

Cellular experiments were continued with the eight selected conditions as mentioned above. MPI alveolar-like macrophages were cultured on collagen layers for 72 h, followed by flow cytometric analysis for the three different macrophage phenotypes, M1, M2 and M2-like (Fig. 3A). Culturing of macrophages on collagen-coated substrates led to significantly lower percentages of MHCII(hi)-CD206(lo) M1 macrophages (around 20%) and concomitantly significantly higher percentages of MHCII(lo)-CD206(hi) M2-like macrophages (approximately 50%) compared to control macrophages cultured on uncoated glass (around respectively 40% and 20%). Stiffness and morphology of the collagen layers did not have an additional modulating effect on macrophage polarization (Supplementary Fig. 3).

As macrophage polarization did not seem to be affected by substrate stiffness or collagen morphology, we assessed whether these variables influenced the quantitative expression of MHCII or CD206 (mannose receptor) on MHCII(hi) or CD206(hi) macrophages, respectively. No effect of total substrate stiffness or collagen morphology on MHCII expression was observed (data not shown). However, in the case of CD206, we did find that macrophages cultured on substrates coated with globular collagen expressed 1.5 times more CD206 than cells cultured on glass (Fig. 3B).

3.3. Collagen morphology influences macrophage shape

To assess the shape of macrophages, cells were stained for the cytoskeletal component F-actin (Fig. 4A–I). Differences in localization of F-actin were observed (Fig. 4A–I and Supplementary Fig. 4 for enlarged pictures). Macrophages on fibrous collagen layers (Fig. 4B–F) exhibited dense localization of F-actin at the cell membrane, with actin-rich protrusions (inset Fig. 4B). In comparison, macrophages grown on globular collagen layers had a more equal spreading of F-actin throughout the cell (Fig. 4G–I) and significantly more macrophages showed filopodia (inset Fig. 4H) when cultured on globular collagen layers (>20%), compared to the fibrous collagen layers (<10%) (Fig. 4J). All these observations were independent of substrate stiffness.

3.4. Collagen morphology affects Ym1 expression and secretion by macrophages

In order to quantify the activity of the pro-healing M2 macrophages that are associated with fibrosis, we quantified the expression and secretion of the marker Ym1 with a fluorescent staining and ELISA analysis (Fig. 4K and L). Ym1 is widely used as a murine marker of alternatively activated, pro-healing M2 macrophages.[11,24,25] Macrophages grown on fibrous collagen had significantly higher Ym1 expression (Fig. 4B–F and K) than macrophages grown on glass (Fig. 4A and K) or globular collagen (Fig. 4G–I and K), independent of substrate stiffness. Furthermore, we observed a red background staining when macrophages were grown on fibrous collagen layers, most pronounced on the softest collagen

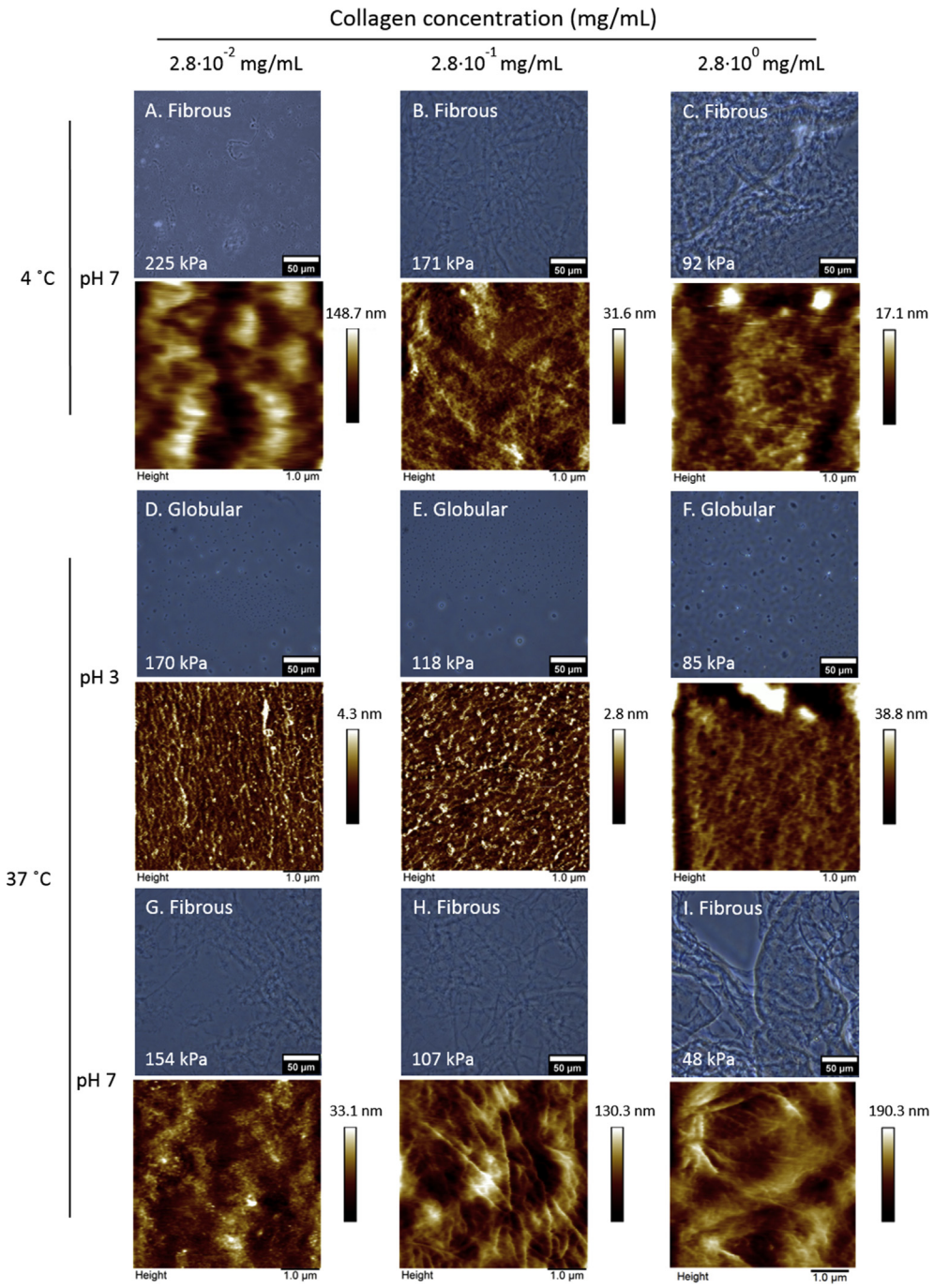

Fig. 1. Characterization of collagen-coated surfaces. Collagen layers formed under different conditions (pH, temperature and concentration), as visualized by bright field microscopy (top pictures, scale bars 50 μm) and atomic force microscopy (bottom pictures, scale bars 1.0 μm). The stiffness of the collagen layers (in kPa) was measured by atomic force microscopy (N = 6).

layers (Fig. 4E–F). This background staining was not observed when macrophages were grown on globular collagen. When quantifying secreted Ym1, we found that the levels of Ym1 did not change when macrophages were grown on fibrous collagen as compared to macrophages grown on glass (Fig. 3L) but macrophages grown on globular collagen secreted significantly less Ym1 (approximately 50% less) than macrophages on glass or fibrous collagen.

The observed changes in Ym1 expression and secretion by M2 macrophages led us to investigate whether the collagen morphology also has an effect on the activity of pro-inflammatory M1 macrophages, by measuring the secretion of M1 marker TNF-α. No significant differences in TNF-α secretion were measured, but we did see a trend ($p = 0.08$) towards lower secretion of TNF-α by macrophages grown on globular collagen as compared to cells grown on glass (Supplementary Fig. 5).

3.5. Fibrous collagen increases transmigration of macrophages

A transmigration assay was performed to assess whether the observed cytoskeletal changes are accompanied by functional changes in migratory behavior of the macrophages. We observed that, after 72 h, significantly more macrophages transmigrated over

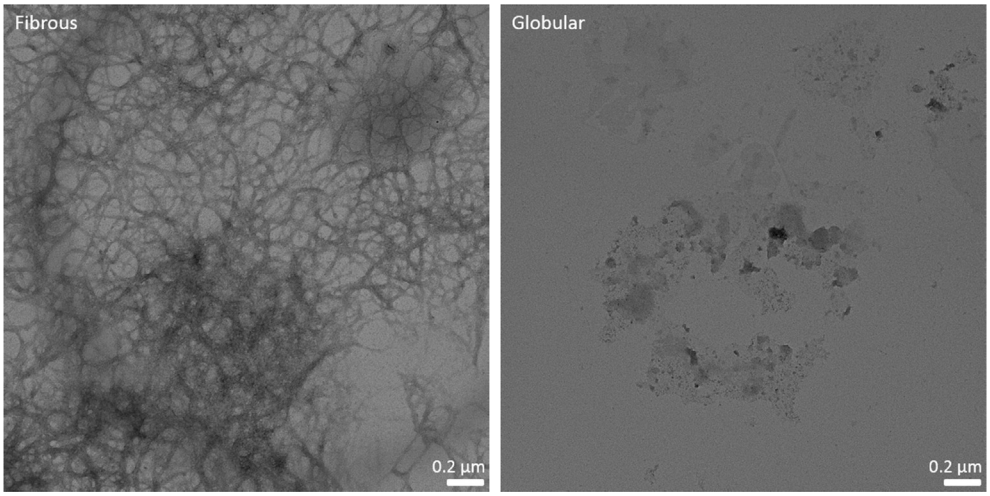

Fig. 2. Analysis of collagen morphology with transmission electron microscopy (TEM). Grids were coated with 2.8 µg/mL collagen type I in either neutral or acidic conditions, resulting in respectively fibrous and globular collagen layers.

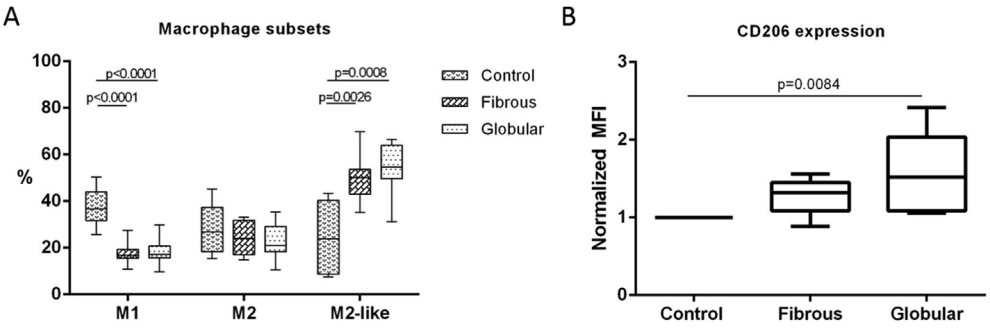

Fig. 3. The effect of collagen coating on macrophage subsets and marker expression. Glass surfaces were coated with collagen layers of different stiffness and morphologies. MPI alveolar-like macrophages were incubated on these collagen layers or glass for 72 h, after which the expression of MHCII and CD206 was assessed by flow cytometry. **A.** Macrophage subsets were defined as MHCII(hi)-CD206(lo) M1 macrophages, MHCII(hi)-CD206(hi) M2 macrophages or MHCII(lo)-CD206(hi) M2-like macrophages and represented as percentages of the total population. **B.** The mean fluorescent intensity (MFI) of CD206 expression on CD206(hi) macrophages. The results of the different collagen layers were pooled based on their morphology, as stiffness did not have an effect on CD206 expression. Data displayed as box-and-whiskers plots with 2.5 and 97.5 percentiles, statistically tested with one-way ANOVA followed by Bonferroni post-hoc test (N = 7).

a transwell membrane coated with fibrous collagen compared to an uncoated transwell (Fig. 5). Coating the transwell membrane with globular collagen did not exert this effect.

4. Discussion

In this study, we showed that the structure of collagen type I has an effect on marker expression, shape and migratory behavior of macrophages. The morphology of the collagen (globular or fibrous) did not have a direct effect on macrophage polarization, but did influence the macrophages in a more subtle fashion by differentially influencing cell shape, migration and the expression of Ym1 and CD206.

In our studies, no significant influence of substrate stiffness on macrophage polarization, shape, or marker expression was observed although several previous studies did find effects of substrate stiffness.[18–21] Wide ranges of stiffness were used in these studies, some comprising stiffness values of different orders of magnitude (Pa, kPa and MPa). The absence of significant stiffness effects in our experiments may be caused by the relatively small range of Young's moduli we used (48–171 kPa). We chose to limit ourselves to this range as stiffer substrates are not likely to be encountered by macrophages in organs *in vivo* and therefore any effects found would not be physiologically relevant. Additionally, the absence of a pronounced stiffness effect enabled us to obtain insights into the effects of ECM morphology more clearly.

We found that macrophages grown on collagen had lower expression of MHCII and higher expression of CD206, suggesting a phenotype switch from an M1 to M2-like phenotype compared to macrophages grown on glass. This may imply that the presence of collagen type I pushes the macrophages from a pro-inflammatory phenotype towards a more regulatory, anti-inflammatory phenotype. We also found that culturing macrophages on globular collagen led to higher expression of macrophage mannose receptor CD206. CD206 can play a role in both the attachment of cells to collagen and degradation of collagen. Previous studies have shown that CD206 is able to bind to collagen through its fibronectin type II domain, thereby facilitating adhesion to ECM and that deletion of CD206 increased migration.[26,27] Higher CD206 expression in response to the globular collagen may therefore suggest that macrophages bind more strongly to globular collagen. This is in accordance with a previous observation that RAW 264.7 macrophages adhere stronger to monomeric collagen type I, than to fibrillary collagen.[28] In addition, Madsen et al. described the importance of mannose receptors for collagen uptake, as expression of these receptors led to efficient internalization and degradation of collagen.[29] Combining these

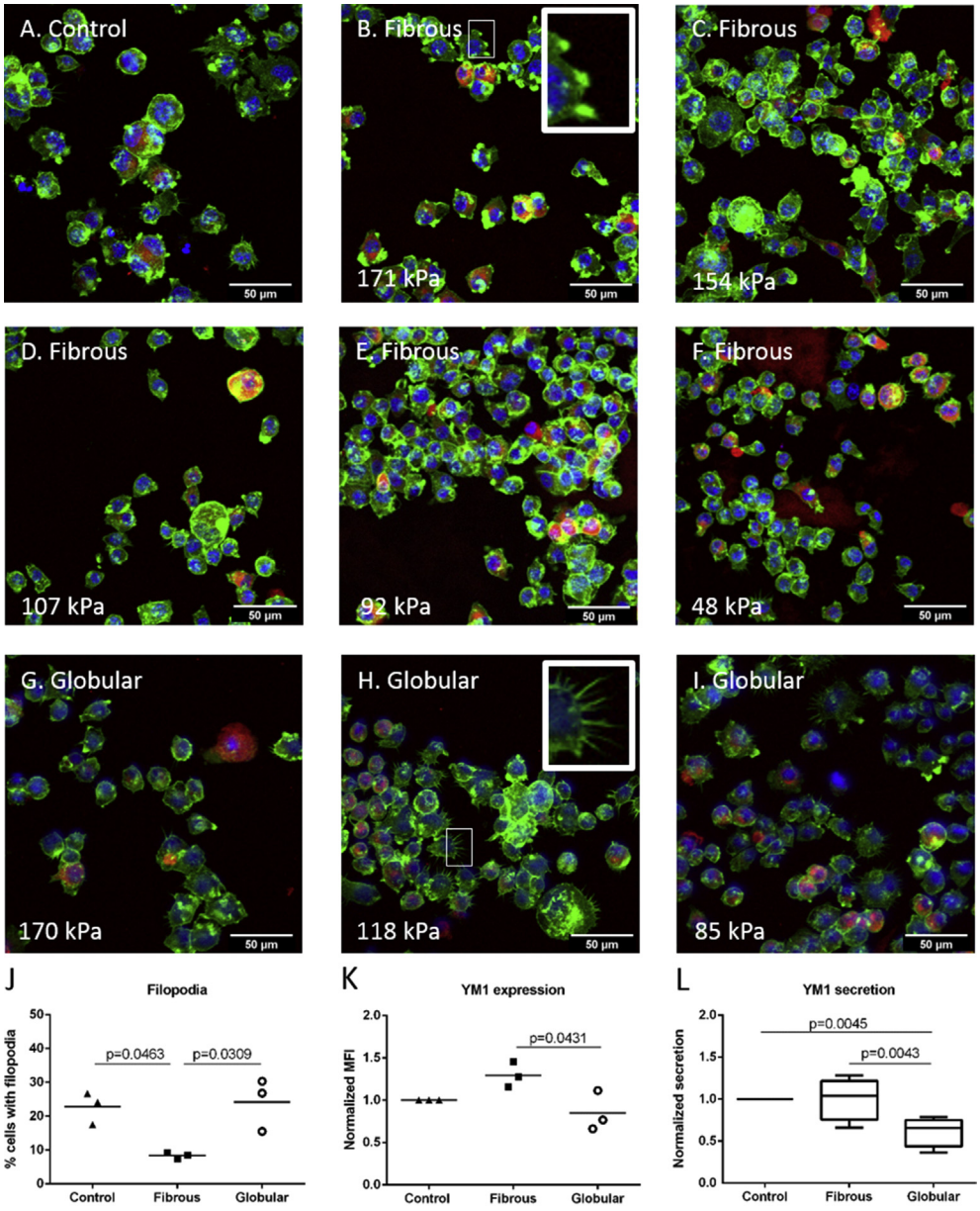

Fig. 4. Collagen morphology affects the morphology of macrophages and the expression of Ym1. Staining of fixed macrophages on the various collagen layers for F-actin (green), Ym1 (red) and DAPI (blue), visualized by confocal microscopy. **A.** Macrophages grown on glass (control). **B**–**F.** Macrophages grown on fibrous collagen. **G-I**. Macrophages grown on globular collagen. **J.** Percentage of cells displaying filopodia. **K.** Intracellular Ym1 expression (marker of M2 macrophages) quantified by TissueFAXS cytometry, normalized to control (N = 3). **L.** Secretion of Ym1 quantified by ELISA, normalized to control (N = 6). Data statistically tested with one-way ANOVA followed by Bonferroni post-hoc test. Insets showing a magnification of actin-rich protrusions (**B**) and filopodia (**H**). Pictures **B** and **H** can be found enlarged in Supplementary Fig. 4.

observations, when sensing globular collagen, macrophages may induce higher expression of CD206 to strengthen attachment to this type of matrix and subsequently facilitate CD206-mediated internalization and degradation of collagen type I. Interestingly, in patients with idiopathic pulmonary fibrosis, CD206 has shown to be strongly upregulated on alveolar macrophages.[30] This may be a functional consequence of the presence of more disorganized and immature collagen type I in these lungs as compared to healthy control lungs.[14–16]

As a functional read-out, we analyzed the expression and secretion of Ym1, a marker of M2 macrophages that are associated with fibrosis. Even though the exact function of Ym1 is not yet known, Ym1 has been reported to have an effect on other immune cells involved in wound healing such as eosinophils and dendritic cells[31,32] and may therefore play a role in fibrosis through these cells. We observed that macrophages grown on fibrous collagen express more Ym1 than macrophages grown on globular collagen. The red background staining observed when macrophages were grown on fibrous collagen suggests that secreted Ym1 is able to bind to these collagen fibers. According to literature, the lectin Ym1 is indeed capable of binding to some ECM components.[33] Therefore the significant difference in Ym1 secretion we found between fibrous and globular conditions may be even more pronounced than measured in our experiments, because the ELISA cannot detect the ECM-bound Ym1 in the fibrous conditions. The fact that macrophages cultured on globular collagen secreted less Ym1 in comparison to the control may indicate a global phenomenon of reduced secretion by these macrophages. The trend towards lower

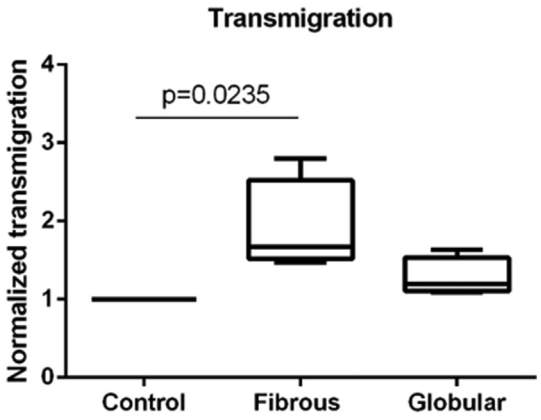

Fig. 5. Fibrous collagen increases transmigration of macrophages. Transmigration of macrophages over fibrous and globular collagen-coated transwell membranes, normalized to the control of transmigration over a non-coated transwell membrane (N = 4). Data statistically tested with one-way ANOVA followed by Bonferroni post-hoc test.

secretion of M1 marker TNF-α on globular collagen supports this notion, although this may also be caused by the lower percentage of M1 macrophages present in collagen-coated conditions. Interestingly, the collagen morphology-induced changes in Ym1 expression and secretion were observed without any evidence of macrophages switching in phenotype by the different morphologies of collagen. This suggests that the morphology of the collagen layer influences functional aspects of macrophages, and not a complete switch in phenotype.

The shape of macrophages clearly changed in response to different morphological properties of collagen. Fibrous collagen induced a round morphology and dense localization of F-actin at the cell membrane of the macrophages, whereas globular collagen induced uniform expression of F-actin throughout the cell with filopodia. These cytoskeletal changes were observed without changes in macrophage subsets, even though several papers described that kind of cytoskeletal changes in macrophage polarization.[34–36] However, literature is ambiguous when linking the presence of filopodia to polarization status. Combined with our observations, this may suggest that having a particular phenotype does not necessarily restrict macrophages to a certain shape. The observed cytoskeletal changes did indicate an effect of collagen morphology on macrophage behavior. The rounded morphology of the macrophages cultured on fibrous collagen suggested that they are migrating in an amoeboid manner.[37,38] The filopodia-rich morphology, observed with macrophages cultured on globular collagen, may be more characteristic of mesenchymal migration.[38] These notions are supported by the results from our transmigration assay, in which a coating with fibrous collagen resulted in significantly higher transmigration. The more amoeboid morphology observed in fibrous conditions has indeed been associated with a higher migration speed.[38] Mesenchymal migration on the other hand has been associated with the presence of matrix-binding and -degrading surface markers,[38] matching the higher CD206 expression we observed on macrophages cultured on globular collagen. Altogether, these data show that macrophages appear to respond to their surroundings by changing their own shape and migratory behavior, which may have functional consequences in fibrosis.

It is unclear to what extent the morphology of the used collagen layers is translatable to an *in vivo* situation, as the exact morphology of our fibrous and globular collagen layers may deviate from actual *in vivo* morphologies. We used acidic conditions in order to create globular collagen layers and interestingly elevated lactic acid levels have been found in idiopathic pulmonary fibrosis, which could lead to acidic conditions.[39] The metabolite lactic acid induced myofibroblast differentiation and the production of collagen, in a pH-dependent manner as neutralizing the pH antagonized this effect. It is therefore not unlikely that slightly acidic conditions in pulmonary fibrosis result in impaired maturation of collagen fibers, in addition to the effects on myofibroblast differentiation. This may then yield ECM with a more immature and disorganized morphology. Imaging of human tissue has indeed shown that the microstructure of collagen is different in usual interstitial pneumonia (the histopathology underlying pulmonary fibrosis), based on fibril diameter, density and organization.[15] Combining these data with our findings suggests that interactions between macrophages and the altered collagen morphology could play a role in the pathogenesis of fibrosis.

5. Conclusion

We analyzed the effects of collagen morphology and substrate stiffness on macrophage polarization, marker expression, shape, and migration. Globular collagen resulted in higher expression of the mannose receptor CD206, synchronous with lower production of Ym1, a marker of pro-healing M2 macrophages. Fibrous collagen led to higher production of Ym1, without significantly affecting CD206 expression. Moreover, macrophages exhibited distinct differences in shape with actin-rich protrusions on fibrous collagen and more filopodia on globular collagen. In addition to these cytoskeletal changes, transmigration was higher when macrophages were cultured on fibrous collagen. Our findings indicate that macrophages are sensitive to ECM morphology, responding with subtle changes in marker expression, shape and behavior rather than a complete switch in phenotype. This study emphasizes the complexity of interactions between macrophages and their microenvironment and the need for further exploration of possibly combined effects of morphological and mechanical changes on macrophage behavior.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.regen.2018.01.002.

References

1. McKleroy W, Lee TH, Atabai K. Always cleave up your mess: targeting collagen degradation to treat tissue fibrosis. *Am J Physiol Lung Cell Mol Physiol*. 2013;304:L709–L721.
2. Wells RG. Tissue mechanics and fibrosis. *Biochim Biophys Acta*. 2013;1832:884–890.
3. Hinz B. Tissue stiffness, latent TGF-beta1 activation, and mechanical signal transduction: implications for the pathogenesis and treatment of fibrosis. *Curr Rheumatol Rep*. 2009;11:120–126.
4. Todd NW, Luzina IG, Atamas SP. Molecular and cellular mechanisms of pulmonary fibrosis. *Fibrogenesis Tissue Repair*. 2012;5, 11–1536-5-11.
5. Wuyts WA, Agostini C, Antoniou KM, et al. The pathogenesis of pulmonary fibrosis: a moving target. *Eur Respir J*. 2013;41:1207–1218.
6. Spagnolo P, Sverzellati N, Rossi G, et al. Idiopathic pulmonary fibrosis: an

update. *Ann Med*. 2015;47:15–27.
7. Ley B, Collard HR, King Jr TE. Clinical course and prediction of survival in idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med*. 2011;183:431–440.
8. Vancheri C, Failla M, Crimi N, Raghu G. Idiopathic pulmonary fibrosis: a disease with similarities and links to cancer biology. *Eur Respir J*. 2010;35:496–504.
9. Chakraborty S, Chopra P, Ambi SV, Dastidar SG, Ray A. Emerging therapeutic interventions for idiopathic pulmonary fibrosis. *Expet Opin Invest Drugs*. 2014;23:893–910.
10. Sathiyamoorthy G, Sehgal S, Ashton RW. Pirfenidone and nintedanib for treatment of idiopathic pulmonary fibrosis. *South Med J*. 2017;110:393–398.
11. Boorsma CE, Draijer C, Melgert BN. Macrophage heterogeneity in respiratory diseases. *Mediat Inflamm*. 2013;2013:769214.
12. Wynn T, Vannella K. Macrophages in tissue repair, regeneration, and fibrosis. *Immunity*. 2016;44:450–462.
13. Martinez FO, Gordon S. The M1 and M2 paradigm of macrophage activation: time for reassessment. *F1000Prime Rep*. 2014;6, 13–13. eCollection 2014.
14. Tjin G, Xu P, Kable SH, Kable EP, Burgess JK. Quantification of collagen I in airway tissues using second harmonic generation. *J Biomed Optic*. 2014;19, 36005.
15. Kottmann RM, Sharp J, Owens K, et al. Second harmonic generation microscopy reveals altered collagen microstructure in usual interstitial pneumonia versus healthy lung. *Respir Res*. 2015;16, 61–6015-0220-8.
16. Bonnans C, Chou J, Werb Z. Remodelling the extracellular matrix in development and disease. *Nat Rev Mol Cell Biol*. 2014;15:786–801.
17. Booth AJ, Hadley R, Cornett AM, et al. Acellular normal and fibrotic human lung matrices as a culture system for in vitro investigation. *Am J Respir Crit Care Med*. 2012;186:866–876.
18. Fereol S, Fodil R, Labat B, et al. Sensitivity of alveolar macrophages to substrate mechanical and adhesive properties. *Cell Motil Cytoskeleton*. 2006;63:321–340.
19. Patel NR, Bole M, Chen C, et al. Cell elasticity determines macrophage function. *PLoS One*. 2012;7, e41024.
20. Hayenga H, Adlerz Katrina N, Aranda-Espinoza Helim. Substrate stiffness regulates the behavior of human monocyte-derived macrophages. *Biophys J*. 2015;108:306a–307a.
21. Blakney AK, Swartzlander MD, Bryant SJ. The effects of substrate stiffness on the in vitro activation of macrophages and in vivo host response to poly(-ethylene glycol)-based hydrogels. *J Biomed Mater Res A*. 2012;100:1375–1386.
22. Fejer G, Wegner MD, Gyory I, et al. Nontransformed, GM-CSF-dependent macrophage lines are a unique model to study tissue macrophage functions. *Proc Natl Acad Sci USA*. 2013;110:E2191–E2198.
23. Stein M, Keshav S, Harris N, Gordon S. Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation. *J Exp Med*. 1992;176:287–292.
24. Raes G, De Baetselier P, Noel W, Beschin A, Brombacher F, Hassanzadeh Gh G. Differential expression of FIZZ1 and Ym1 in alternatively versus classically activated macrophages. *J Leukoc Biol*. 2002;71:597–602.
25. Roszer T. Understanding the mysterious M2 macrophage through activation markers and effector mechanisms. *Mediat Inflamm*. 2015;2015, 816460.
26. Sturge J, Todd SK, Kogianni G, McCarthy A, Isacke CM. Mannose receptor regulation of macrophage cell migration. *J Leukoc Biol*. 2007;82:585–593.
27. Napper CE, Drickamer K, Taylor ME. Collagen binding by the mannose receptor mediated through the fibronectin type II domain. *Biochem J*. 2006;395:579–586.
28. Gowen BB, Borg TK, Ghaffar A, Mayer EP. Selective adhesion of macrophages to denatured forms of type I collagen is mediated by scavenger receptors. *Matrix Biol*. 2000;19:61–71.
29. Madsen DH, Leonard D, Masedunskas A, et al. M2-like macrophages are responsible for collagen degradation through a mannose receptor-mediated pathway. *J Cell Biol*. 2013;202:951–966.
30. Pechkovsky DV, Prasse A, Kollert F, et al. Alternatively activated alveolar macrophages in pulmonary fibrosis-mediator production and intracellular signal transduction. *Clin Immunol*. 2010;137:89–101.
31. Zhao J, Lv Z, Wang F, et al. Ym1, an eosinophilic chemotactic factor, participates in the brain inflammation induced by Angiostrongylus cantonensis in mice. *Parasitol Res*. 2013;112:2689–2695.
32. Cai Y, Kumar RK, Zhou J, Foster PS, Webb DC. Ym1/2 promotes Th2 cytokine expression by inhibiting 12/15(S)-lipoxygenase: identification of a novel pathway for regulating allergic inflammation. *J Immunol*. 2009;182:5393–5399.
33. Hung SI, Chang AC, Kato I, Chang NC. Transient expression of Ym1, a heparin-binding lectin, during developmental hematopoiesis and inflammation. *J Leukoc Biol*. 2002;72:72–82.
34. Vogel DY, Heijnen PD, Breur M, et al. Macrophages migrate in an activation-dependent manner to chemokines involved in neuroinflammation. *J Neuroinflammation*. 2014;11, 23–2094-11-23.
35. Ploeger DT, Hosper NA, Schipper M, Koerts JA, de Rond S, Bank RA. Cell plasticity in wound healing: paracrine factors of M1/M2 polarized macrophages influence the phenotypical state of dermal fibroblasts. *Cell Commun Signal*. 2013;11, 29–811X-11-29.
36. Li C, Levin M, Kaplan DL. Bioelectric modulation of macrophage polarization. *Sci Rep*. 2016;6, 21044.
37. Van Goethem E, Poincloux R, Gauffre F, Maridonneau-Parini I, Le Cabec V. Matrix architecture dictates three-dimensional migration modes of human macrophages: differential involvement of proteases and podosome-like structures. *J Immunol*. 2010;184:1049–1061.
38. Bear JE, Haugh JM. Directed migration of mesenchymal cells: where signaling and the cytoskeleton meet. *Curr Opin Cell Biol*. 2014;30:74–82.
39. Kottmann RM, Kulkarni AA, Smolnycki KA, et al. Lactic acid is elevated in idiopathic pulmonary fibrosis and induces myofibroblast differentiation via pH-dependent activation of transforming growth factor-beta. *Am J Respir Crit Care Med*. 2012;186:740–751.