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Substrate fiber alignment mediates tendon cell response to inflammatory signaling

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ABSTRACT

Healthy tendon tissue features a highly aligned extracellular matrix that becomes disorganized with disease. Recent evidence suggests that inflammation coexists with early degenerative changes in tendon, and that crosstalk between immune-cells and tendon fibroblasts (TFs) can contribute to poor tissue healing. We hypothesized that a disorganized tissue architecture may predispose tendon cells to degenerative extracellular matrix remodeling pathways, particularly within a pro-inflammatory niche. This hypothesis was tested by analyzing human TFs cultured on electrospun polycaprolactone (PCL) mats with either highly aligned or randomly oriented fiber structures. We confirmed that fibroblast morphology, phenotype, and markers of matrix turnover could be significantly affected by matrix topography. More strikingly, the TF response to paracrine signals from polarized macrophages or by stimulation with pro-inflammatory cytokines featured significant downregulation of signaling related to extracellular synthesis, with significant concomitant upregulation of gene and protein expression of matrix degrading enzymes. Critically, this tendency towards degenerative re-regulation was exacerbated on randomly oriented PCL substrates. These novel findings indicate that highly aligned tendon cell scaffolds not only promote tendon matrix synthesis, but also play a previously unappreciated role in mitigating adverse resident fibroblast response within an inflammatory milieu.

Statement of Significance

Use of biomaterial scaffolds for tendon repair often results in tissue formation characteristic of scar tissue, rather than the highly aligned type-1 collagen matrix of healthy tendons. We hypothesized that non-optimal biomaterial surfaces may play a role in these outcomes, specifically randomly oriented biomaterial surfaces that unintentionally mimic structure of pathological tendon. We observed that disorganized scaffold surfaces do adversely affect early cell attachment and gene expression. We further identified that disorganized fiber surfaces can prime tendon cells toward pro-inflammatory signaling. These findings represent provocative evidence unstructured fiber surfaces may underlie inflammatory responses that drive aberrant collagen matrix turnover. This work could be highly relevant for the design of cell instructive biomaterial therapies that yield positive clinical outcomes.

1. Introduction

Tendons display a unique hierarchical organization of highly aligned type-1 collagen extracellular matrix (ECM) that enables efficient transfer of high mechanical forces from muscle to bone [1]. The physical demands on the tissue can overwhelm its intrinsic ability to self-repair, leading to an accumulation of damage and the onset of the pathological repair mechanisms that drive degenerative tendon disease [2]. Clinically termed tendinopathy, tendon degeneration is often painful, and accounts for approximately 30% of all orthopaedic consulations [3]. Tendinopathy is

Abbreviations: BGN, Biglycan; COL1, Collagen type 1; COL3, Collagen type 3; CCR7, C-C chemokine receptor type 7; DCN, Decorin; ECM, Extracellular matrix; IL1B, Interleukin-1beta; MKX, Mohawk; MMP1, Matrix metalloproteinase 1; MMP2, Matrix metalloproteinase 2; MMP3, Matrix metalloproteinase 3; MMP9, Matrix metalloproteinase 9; MMP13, Matrix metalloproteinase 13; MRC1, Mannose receptor C-type 1; PCL, Polycaprolactone; SCX, Scleraxis; TF, Tendon fibroblast; TGFB1, Transforming growth factor beta-1; TIMP1, tissue inhibitors of matrix metalloproteinase 1; TIMP2, tissue inhibitors of matrix metalloproteinase 2; TNF, Tumor necrosis factor alpha; TNMD, Tenomodulin.

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histologically characterized by structural derangement of the collagen matrix, hypercellularity, and vascular ingrowth.

Tendon fibroblasts (TFs) are the principal cell type in tendons, and often appear to be connected in a head-to-tail fashion along the length of parallel collagen fibers in healthy tissue [4]. TFs coordinate tissue adaptation and repair mechanisms regulated by various biochemical factors in synergy with a well-regulated balance between ECM synthesis and breakdown [5–7]. When this balance is disrupted, the collagen matrix composition becomes more heterogeneous and increasingly disordered [8]. Complex tissue changes follow, including cellular, vascular and neurological alterations [5,9,10]. Tendon injury was originally thought to be primarily associated with accumulation of mechanical damage and associated alterations of the tendon matrix [11,12]. This involves elevated matrix turnover with altered gene expression of the structural matrix proteins including *COL1*, *COL3*, *DCN* and *BGN* and certain proteases (e.g. metalloproteinases) that regulate mechanical and biological tissue homeostasis. The long-held misconception of tendinopathy as a "non-inflammatory" disease has only recently been exposed by evidence that early stage tendinopathy involves infiltration of immune cells, such as macrophages, mast cells, T lymphocytes and Natural killer cells [13–16]. These findings point to the coexistence of inflammation and degenerative changes in non-ruptured tendinopathic tendons, and open questions regarding the potential impact of immune-cells and their secreted factors on TFs. Among immune cells, macrophages are known to play a critical role in the coordination of the healing process in injured and diseased tissues [17], including tendon [15,18]. By extension, successful healing after implantation of a biomaterial graft or scaffold is also likely to require successful resolution of tissue repair mechanisms in an inflammatory milieu. Macrophages can play an active role in ECM turnover by secreting proteases, cytokines and other signaling molecules that may modulate the activity of TFs [19]. Although the spectrum of macrophage phenotypes and activities is broad, for conceptual simplicity, macrophages are often ascribed to one of two polarization profiles, namely a pro-inflammatory (M1-like) or an anti-inflammatory (M2-like) phenotype [19,20]. These two phenotypes are thought to represent the outer ends of a continuum of phenotypes ranging from M1 to M2, and have been subgrouped depending on their stimulation scenarios [20]. Initially, in response to tissue damage under inflammatory conditions, the M1 macrophages predominate and regulate processes such as phagocytosis and apoptosis [21,22]. M1 macrophages are characterized by the secretion of an array of pro-inflammatory cytokines including TNF and IL1B [19,20]. Furthermore, they have the ability to control ECM turnover by regulating the expression of various matrix metalloproteinases (MMPs) and tissue inhibitors of matrix metalloproteinases (TIMPs) [23]. In contrast, M2 macrophages are considered to suppress pro-inflammatory reaction and promote tissue homeostasis [24]. Cytokines-mediated regulation by differently polarized macrophages has been implicated in degenerative tendon disease (tendinopathy), regulating the interplay between TFs, immune cells and the ECM [25]. For example, inflammation-related nuclear translocation of the transcription factor NF-κB in tenocytes [26] has been attributed a critical role in the disease mechanism of tendinopathy [27].

Among many factors in the extracellular niche that regulate tendon cell behavior [28], extracellular matrix structure, particularly matrix fiber alignment, has emerged as a dominant factor in tendon fibroblast and tendon progenitor cell behavior [29–31]. These studies collectively indicate that highly aligned fibrous biomaterials tend to promote tenogenic cell differentiation, expression of tendon markers, and promotion of type-1 collagen synthesis and assembly. However, the converse hypotheses of these studies, that disorganized fibrous structures may promote aberrant matrix repair mechanisms, or even potentially confer sensitivity to pro-inflammatory signaling, have not been rigorously investigated. Such information would not only be relevant to the design of synthetic tendon biomaterial grafts, but also for a basic understanding of the mechanisms that may underlie tendon tissue pathologies.

We thus tested the hypothesis that tendon cells seeded on aligned biomaterial scaffolds would be less susceptible to a catabolic inflammatory stimulus than seeded on biomaterials presenting a more disorganized topography. We first assessed baseline behaviors of tendon cells on these two classes of materials in terms of tendon lineage markers, and markers of ECM turnover. We then probed how substrate driven cell morphology may predispose cell sensitivity to pro-inflammatory signaling. Our results reveal that the alignment of electro-spun polymer surfaces significantly affects tendon cell response to inflammatory signals such as those involved in early stages of tendon repair.

2. Materials and methods

2.1. Tissue collection and isolation of tendon fibroblasts

Fragments of healthy hamstring tendons were collected from male patients with a mean age of 23 ± 7 years, undergoing surgical reconstruction of the anterior cruciate ligament. Tissues were collected in accordance with granted ethical permission of the Canton of Zurich (permission number 2015-0089). Immediately upon collection, tendon tissues were immersed in Dulbecco's minimum essential medium (DMEM/F12 (Sigma)). Tendon fibroblasts (TFs) were then isolated following a previously described protocol [32]. Briefly, samples were stripped from surrounding tissue, cut into small pieces and dissolved in collagenase B (Roche). Next, the tendon fragments were digested for 6 h at 37 °C. The isolated cells were cultured in DMEM/F12 supplemented with 20% fetal bovine serum (FBS, Sigma), 1% penicillin-streptomycin (P/S, Sigma, Switzerland) and 1% amphotericin B (Gibco) for 7–12 days until 80% confluency was reached. At this time point, the cells were cryopreserved at −80 °C in 70% DMEM/F12, 20% FBS and 10% DMSO (Sigma). For all experiments TFs were thawed, expanded and deployed at P3.

2.2. Macrophage differentiation

The human monocytic leukemia THP-1 cell line (American Type Culture Collection) was cultivated in suspension in RPMI culture medium (Sigma-Aldrich Switzerland), supplemented with 10% FBS and 1% P/S at 37 °C in a humidified 5% CO_2 atmosphere and sub-cultured routinely before a cell density of 1×10^6 cells/ml was reached. Monocytes were differentiated towards M0 macrophages by stimulation with 100 nM phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich, Switzerland) for 3 days, followed by 24 h cultivation with PMA-free cell culture medium [33,34]. Subsequently, M0 macrophages were chemically stimulated for 24 h with either 20 ng Interferon-γ (IFN-γ; Miltenyi Biotec) and 100 ng LPS (Lipopolysaccharide; Sigma) to obtain an M1 phenotype, or with 20 ng Interleukin-4 (IL-4; Miltenyi Biotec) to obtain an M2 phenotype. The chemical polarization towards the pro-inflammatory M1-like phenotype or the anti-inflammatory M2-like phenotype was validated using the specific surface markers CCR7 (M1) and MRC1 (M2). For experiments, M1 or M2 macrophages were seeded in Transwell™ Permeable supports (Corning, pore size: 0.4 μm) at a density of 1×10^5 cells/cm^2 and submerged in 2 ml of culture medium.

2.3. Tendon fibroblast seeding and co-culture with macrophages

Primary human tendon fibroblasts (TFs) were seeded on purchased electrospun polycaprolactone (Nanofiber solutions, NanoAligned™, Cat# Z694616-12EA, NanoECM™, Cat# Z694517-12EA) nanofiber mats that structurally mimic the characteristic matrix of healthy (aligned nanofibers) or diseased (randomly organized nanofibers) tendon (Fig. 1). Before seeding, electrospun PCL substrates were washed in PBS and incubated with RPMI medium for 4 h. Next, TFs were seeded at a density of 15,000 cells/cm^2 and cultured in RPMI medium for 24 h. A Transwell™ system was used that allowed the exchange of soluble factors produced by macrophages (cultured on the Transwell™ inserts, upper compartment) and TFs (on the PCL substrates, bottom compartment). Subsequently, the primarily differentiated (4 days) and afterwards chemically polarized (1 day) macrophages (M1- and M2-like) were co-cultured with TFs seeded on both substrates, resulting in six different experimental conditions, while mono-cultured TFs on aligned scaffolds served as controls for the co-culture conditions. After 24 h, the TF response was assessed using quantitative PCR against lineage-specific markers, e.g. tenomodulin, scleraxis and mohawk and phenotypic markers of matrix synthesis including collagen 1 and 3. Analysis of the expression and activity of matrix metalloproteinases (MMP) and their inhibitors (TIMPs) was used to study the matrix remodeling capacity of TFs for all conditions.

2.4. RNA isolation and quantitative real-time PCR

Total RNA was extracted from human TFs and macrophages separately using the RNeasy micro kit (Qiagen, Switzerland). Briefly, the cell culture medium was removed before the cells were washed with PBS and lysed with the RLT/βME buffer. The subsequent RNA isolation was performed according to the manufacturer instructions. The quality and quantity of the isolated RNA were analyzed using a Take 3 spectrophotometer (BioTek, Switzerland). Complementary DNA (cDNA) was synthesized in a volume of 20 μl from 9 ng of total RNA using High Capacity RNA-to-cDNA Kit (Thermo Scientific, Switzerland). Gene expression analysis was performed by qPCR to confirm the TF and macrophage phenotypes and to assess levels of matrix turnover genes after co-culture. qPCR reactions consisted of 2 μl of cDNA and 8 μl of Mastermix (5 μl TaqMan® Universal PCR Master Mix, 0.5 μl of forward and reverse TaqMan primer, 2.5 μl of ultrapure water) in a total volume of 10 μl. Samples were amplified using a StepOne thermocycler (Applied Biosystems) under the following PCR cycling conditions: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 sec and 60 °C for 1 min. All reactions were run in technical duplicates. Quantification was performed using the comparative $2^{-\Delta CT}$ method with glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) as a reference gene. GAPDH was chosen since it was shown to be stable over the different conditions analyzed in this study (Supplementary Fig. S1). Results are presented as relative gene expression levels normalized to TFs seeded on aligned substrates in RPMI medium (control), in the absence of macrophages.

2.5. MMP activity assay

Analysis of matrix metalloproteinase (MMP) activity in the cultured medium of co-culture experiments was performed using gelatin zymography. The gelatinolytic activity of MMP1, -2 and -9 was examined with 10% polyacrylamide gels containing 0.6% gelatin (G1890, Sigma). Following electrophoresis, gels were incubated at 37 °C for 18 h. The gels were then stained with Coomassie brilliant blue (Sigma, Switzerland) for 1 h and subsequently destained for 2 h. The resulting bands were imaged using the Chemi Doc MP gel imaging system (Bio Rad). Densitometric

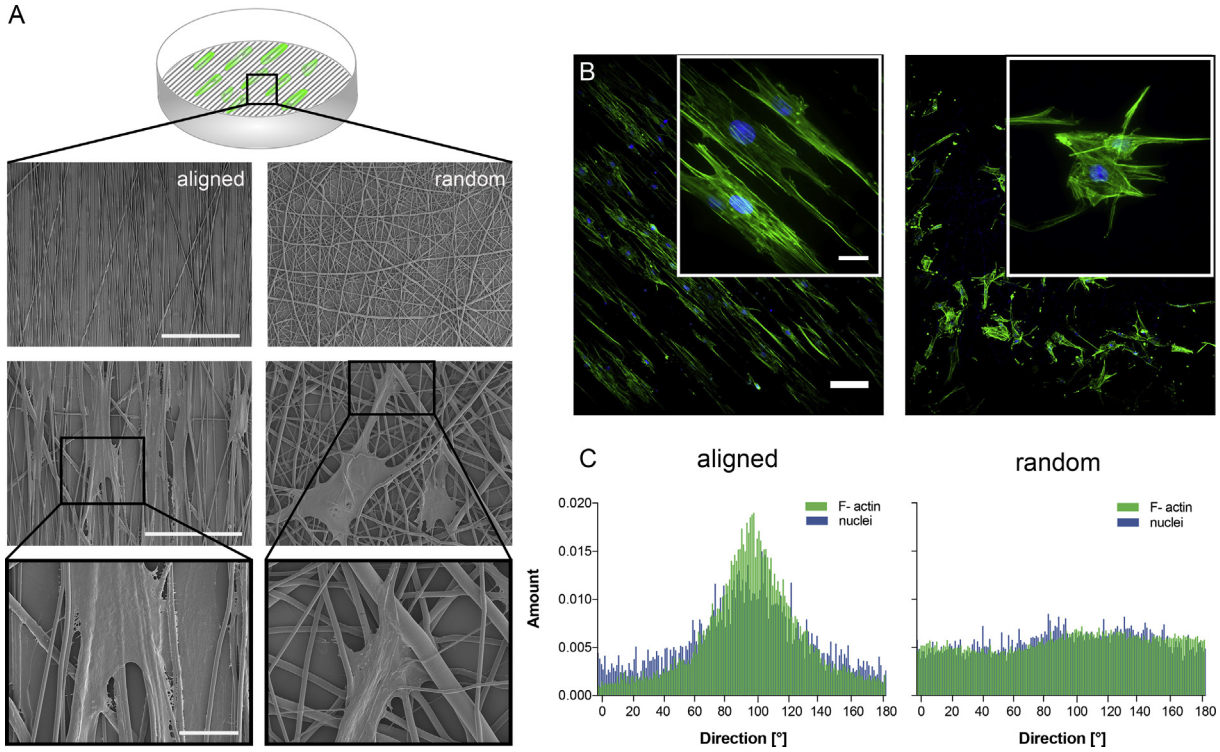

Fig. 1. An in vitro "minimal tendinopathy" model. (A) High magnification SEM micrographs reveal morphological differences between TFs attaching to aligned (left panels) and randomly oriented PCL mats (right panels). (B) Fluorescent images of the cells seeded on the two substrates confirm the aligned and random organization of the actin cytoskeleton (green channel) and of the nuclei (blue channel). (C) Directionality analysis revealed an aligned orientation of the cells and nuclei on the aligned substrates in contrast to a random orientation of the cells seeded on the random scaffolds (n = 4). Scale bar represents (A) 100, 50 and 10 μm from top to bottom and (B) 100 μm with 20 μm (inserts). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

analysis of the bands was performed using ImageJ (version 2.0.0-rc-15). Active proteins within the samples were identified by subtracting the bands of the untreated control (RPMI medium only) from each sample band.

2.6. Scanning electron microscopy (SEM)

TFs cultured on aligned and random oriented PCL nanofiber mats for 24 h and subsequently washed with phosphate buffer saline (PBS) and fixed in 4% formaldehyde (Sigma) for 30 min at room temperature. Subsequently, samples were dehydrated using an ascending series of ethanol (from 50 to 100%), followed by a 5 min exposure to hexamethyldisiloxane (HMDSO, 205389, Sigma). Next, samples were air-dried overnight and sputter coated with 5 nm gold-palladium (high vacuum coater Leica EM ACE 600, Switzerland). Images of the samples with cells on the different substrates were acquired using a Hitachi S-4800 scanning electron microscope (Hitachi High-Technologies Corporation, Japan) at an acceleration voltage of 5 kV and 10 μA current, with a magnification between 5000 and 15,000 times.

2.7. Immunofluorescent staining, image acquisition and analysis

Primary human TFs were cultured on aligned and random oriented PCL substrates for 24 h at a density of 15,000 cells/cm^2. After 24 h, cells were washed with PBS and fixed in 4% formaldehyde (Sigma) for 20 min at room temperature. In a further step, cells were permeabilized with 0.2% Triton-X in PBS for 5 min before incubation with Alexa Fluor™ 488 phalloidin (1:100, a12379, Thermo Fischer, Switzerland) for the actin cytoskeleton and NucBlue™ reagent (1 drop/ml, R37606, Thermo Fischer, Switzerland) to stain the cell nuclei. To stimulate nuclear translocation of NFκB p65, the TFs were exposed to 5 ng/ml IL1B for 3.5, 7.5 15 and 30 min following 24 h of culture. At different time points after stimulation cells were fixed, permeabilized and stained as described above but with the addition of the staining of the p65 subunit of NFκB. For NFκB p65 staining, samples were incubated 1 h with the primary anti-p65 rabbit polyclonal antibody (1:50, sc-372, Santa Cruz Biotechnology, Germany) followed by a 1 h incubation with Alexa Fluor 488-conjugated anti-rabbit IgG (1:200, A21206, Thermo Fisher) secondary antibody together with SiR-actin (1:250, SC001, SpiroChrome). After three washing steps, the samples were mounted in Mowiol mounting medium. Z-stack fluorescent images were obtained using a spinning disc confocal microscope (FEI) equipped with a 10× and a 60× 1.35 N.A. oil-immersion objective. Analysis of the cell alignment and nuclear orientation as well as the cellular location of NFκB p65 was performed using ImageJ software (version 2.0.0-rc-15). To assess the directionality of the cells, three random positions of each sample were imaged with a 10× objective. Briefly, the orientation of the actin cytoskeleton and the cell nuclei was determined with the Directionality plugin of ImageJ, which uses the local gradient method based on intensity gradients [35]. The gradients were quantified in intervals of 1° (0°-179°; 180-element array) in an accumulator scheme (histogram) [36]. Cellular localization of NFκB p65 was quantitatively assessed using confocal Z-stack images of p65. Briefly, the nuclear-to-cytoplasmic p65 ratio was calculated by measuring the intensity of fluorescence in both compartments, and included 10 cells per condition and time point.

2.8. Statistical analysis

A Mann-Whitney-*U* test was performed to analyze the difference between the experimental and the control group of the in vitro model of tendinopathy using GraphPad Prism (version 7.0a). Results are presented as relative expression of TFs seeded on specific substrate normalized to corresponding control by dividing through the median of the control. Differences between co-culturing conditions were evaluated by Friedman test and the Dunn's post hoc multiple comparison method, comparing every treatment and with its corresponding control using GraphPad Prism (version 7.0a). All results are expressed as median ± range of several independent experiments (n represents a new tendon donor and is indicated in the figures). A value of $p \leq 0.05$ was regarded as statistically significant.

3. Results

3.1. Randomly oriented fiber substrates suppress markers of the tenogenic lineage and matrix turnover

We implemented a cell culture system in which primary human TFs are cultured on PCL nanofiber mats that structurally mimic the characteristics of healthy (aligned nanofibers) and degenerated (randomly distributed nanofibers) tendon ECM. Scanning electron images and fluorescent staining of the F-actin cytoskeleton of TFs revealed striking morphological differences between both substrates (Fig. 1A–C). Aligned nanofibers induced TFs to acquire an elongated shape, similar to that found in native tendons. Randomly distributed nanofibers yielded polygonal TFs, morphologically comparable to cells within tendinopathic tissue. Directionality analysis was performed to quantify the orientation of the cells seeded on the two different substrates. The TFs cultured on the aligned scaffolds expressed a clear alignment to the fiber direction (90°), whereas the cells on the random scaffolds did not exhibit a preferred orientation (Fig. 1C).

To determine if these morphological changes were associated with alterations in tenogenic and extracellular matrix gene expression, the transcription of different tendon specific markers and genes related to ECM turnover was analyzed. We observed a tendency towards downregulation of all studied tenogenic markers on randomly oriented PCL, with a significant reduction in mohawk (*MKX*), a key transcriptional regulator of tendon fate (Fig. 2A). Expression levels of matrix proteins and ECM-remodeling enzymes were generally downregulated in cells on random scaffolds (Fig. 2B and C). More precisely, after 24 h in culture on the randomly distributed nanofibers, TFs displayed a significant reduction in the expression levels of collagen type 1 and 3 (*COL1*, *COL3*), biglycan (*BGN*) and decorin (*DCN*) (Fig. 2B) as well as a significant downregulation in the expression of *MMP1* (Fig. 2C). Furthermore, gene expression levels of the tissue inhibitors of metalloproteinases 1 and 2 (*TIMP1* and *TIMP2*) displayed similar trends with lower expression levels in cells seeded on the random substrates (Fig. 2D). A global trend was thus observed for a diminished collagen matrix remodeling potential of TFs on randomly oriented substrates after 24 h in culture, when compared to highly aligned substrates.

3.2. Substrate topography differentially regulates macrophage/TF crosstalk, with M1 polarized macrophages driving TF expression of catabolic markers

To explore potential crosstalk between macrophages and TFs on aligned and random biomaterial surfaces, we cultured M1 and M2 macrophages in the upper chamber of a Transwell™ system (Corning, pore size: 0.4 μm), while the TFs on differently structured substrates were placed in the lower compartment (Fig. 3A). Chemically-induced macrophage polarization was validated by measuring the phenotype-specific markers CCR7 for M1-like and MRC1 for M2-like macrophages (Fig. 3B). After 24 h of co-culture with M1 or M2 macrophages, a significant downregulation of the tendon transcription factors mohawk and scleraxis (*SCX*) was observed in TFs on aligned oriented matrix models with significant

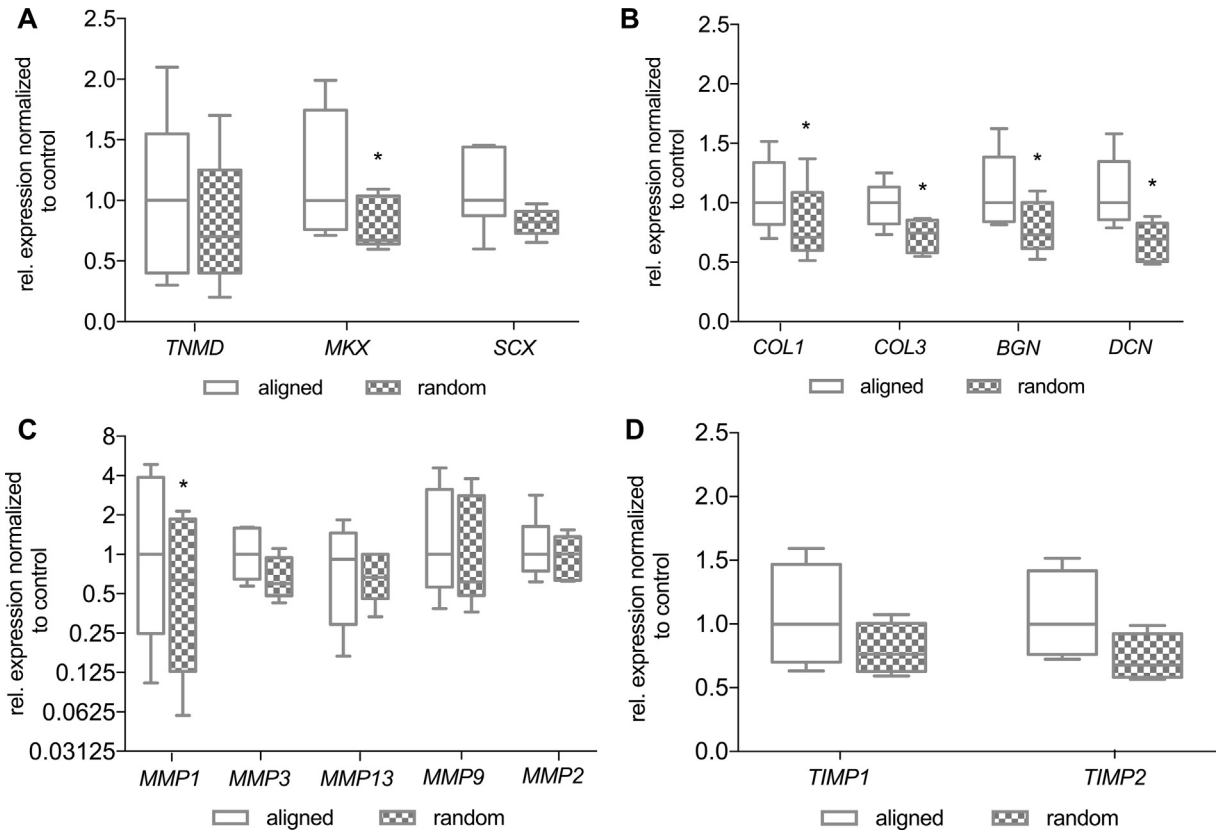

Fig. 2. Substrates with random fiber organization reduce tenogenic gene expression and markers of matrix remodeling. (A) TFs cultured on random substrates (checkerboard box), compared to aligned substrates (white box), displayed significantly decreased expression levels of MKX, and non-significant downregulation of TNMD and SCX. (B) A significant downregulation of the expression of ECM components (COL1 and -3, BGN, DCN) and of MMP1 (C), but not of their inhibitors (TIMP1 and -2) (D) was further detected in cells cultured on randomly distributed fiber substrates. Results were normalized to TFs seeded on aligned PCL nanofiber substrates (white bars) by dividing through its median. Mann-Whitney-U-tests were performed to analyze the differences between the conditions. (n = 6 independent tendon donors, $^{*}P \leq 0.05$, $^{**}P \leq 0.01$,$^{***}P \leq 0.001$.)

downregulation of scleraxis on the random matrix models (Fig. 3C). Expression levels of tenomodulin (*TNMD*) remained unchanged, independent of topography and macrophage phenotype. TFs co-cultured with M1 or M2 macrophages also featured downregulated gene expression levels related to collagen 1, 3, biglycan and decorin, which was more pronounced in TFs on aligned substrates (Fig. 3D). Interestingly, decorin was significantly downregulated when co-cultured with M2 macrophages on the aligned substrates, but significantly upregulated when cultured with M1 macrophages on the random substrates (Fig. 3D). Most strikingly, M1 macrophages induced a significant upregulation of matrix degrading enzymes in TFs, whereas M2 macrophages only had a modest effect (Fig. 3E). In addition, a trend was observed for increased MMP expression when TFs were cultured on the randomly oriented surfaces, compared to those on aligned substrates. Independent of macrophage phenotype in co-culture, both *TIMP1* and *-2* displayed lower expression levels on aligned substrates and higher levels on random compared with the control condition (Fig. 3F). Collectively, these data point to a higher potential for matrix turnover in TFs seeded on random nanofiber substrates.

Given the propensity of the random substrates to upregulate MMP gene expression, and its central involvement in inflammation and wound healing, we aimed to determine if the observed differences at the gene expression level translate accordingly at the protein level. Conditioned medium was collected after 24 h and analyzed by gelatin zymography (Fig. 4A). First, protein levels of proteases in cell culture medium of TFs seeded on aligned and random substrates were compared revealing no significant differences except for MMP2, partially confirming the results at the gene expression level (Fig. 4B). In addition, MMP1 activity appeared to increase in the M1 co-cultures, when compared to the control or M2-coculture (Fig. 4C). Substrate alignment did not affect MMP1 activity (Fig. 4C). On the contrary, MMP2 activity was significantly decreased in co-cultures of aligned TFs with M1 macrophages (Fig. 4C). A similar trend was observed for M2-cocultures, with lower levels of TF on aligned substrate compared to significantly increased levels on the random counterpart (Fig. 4C). Most strikingly, MMP9 activity was significantly and consistently higher in all macrophage-TF co-cultures (Fig. 4C), where M2-TF co-cultures displayed the highest increase. Substrate alignment did not appear to effect MMP9 activity.

3.3. IL1B released by M1 macrophages increases TF matrix remodeling potential, with enhanced matrix protease signaling on randomly oriented fibers

In search for soluble factors potentially responsible for the increased MMP expression in TFs, the cytokine expression levels in M1 and M2 macrophages in the presence or absence of TFs was assessed (Fig. 5A, B). Upon macrophage polarization, gene expression levels of *TGFB1*, *TNF* and *IL1B* were measured. As expected, M1 macrophages displayed higher levels of the studied pro-inflammatory signaling molecules than M0 and M2 macrophages (Fig. 5A). Next, we analyzed the impact of TFs on the gene expression levels of these cytokines in M1 macrophages. Interestingly, a significant downregulation of *TNF* due to co-culture with TFs was detected, whereas the expression of *IL1B* was dramatically upregulated (Fig. 5B). Because these results suggest a role of IL1B in

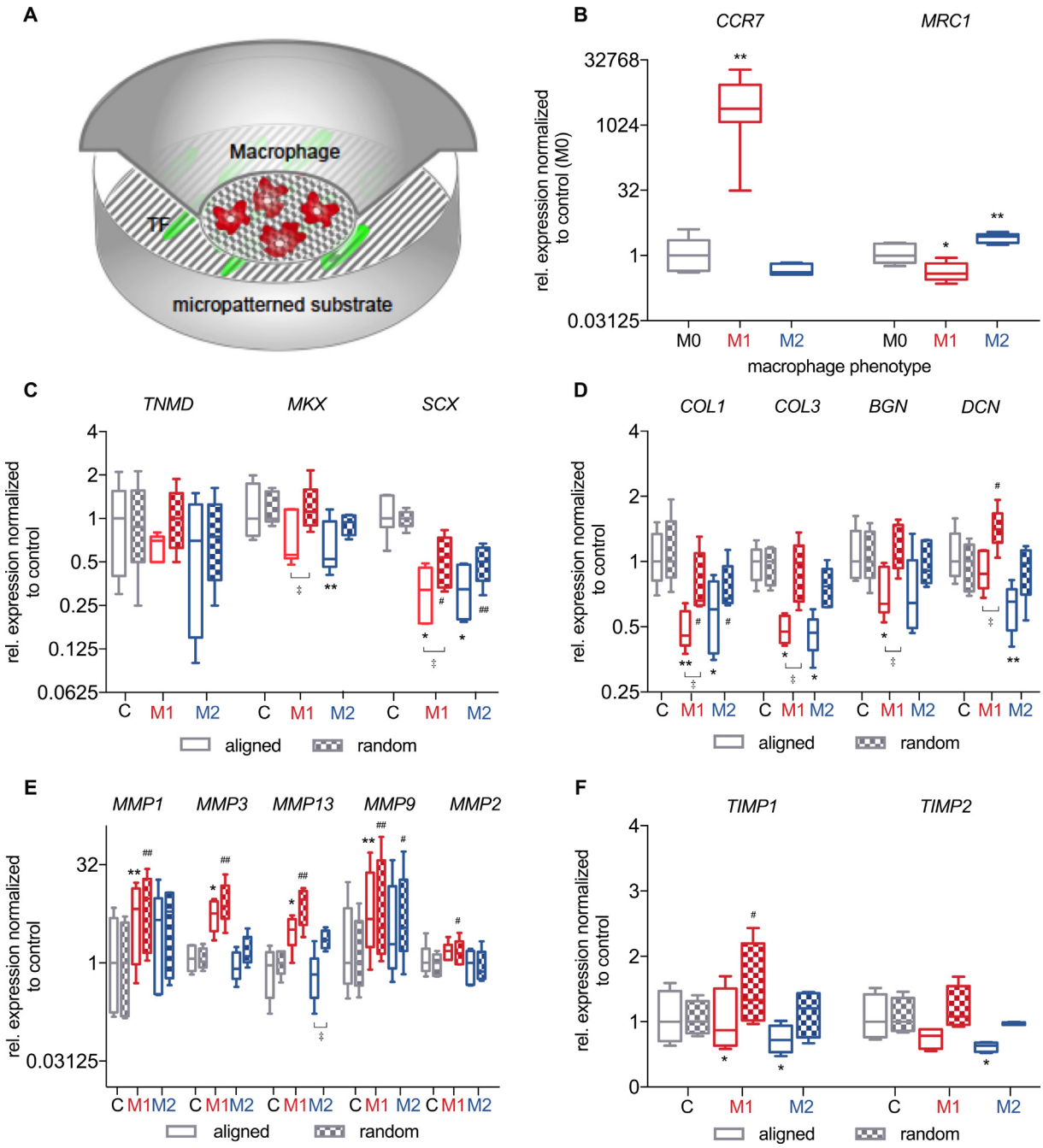

Fig. 3. M1 and M2 macrophages differently affect TF gene expression profiles, analyzed in a Transwell™ co-culture model system. (A) Schematic depiction of TFs (green) cultured on aligned or random PCL mats in indirect contact with M1 or M2 macrophages (red). (B) Boxplot displaying median, maximum and minimum gene expression values of chemically polarized M1 and M2 macrophages compared to naïve M0 macrophages (control) (n = 5). (C) TFs on aligned substrates co-cultured with macrophages displayed the largest downregulation of TF-marker expression. This was accompanied by a significant reduction in the expression levels of COL1, COL3, BGN and DCN (D). (E) MMP expression as a measure of ECM degradation potential, was significantly higher in M1 co-cultures, and most pronounced on random substrates. (F) Additionally, M1 and M2 macrophages caused a downregulation of TIMP expression in TFs cultured on aligned substrates compared to randomly oriented fibers. Results are presented as relative expression to TFs, monoculture on aligned or random substrates in RPMI medium only (control) in the absence of macrophages. Statistical analysis was performed using Friedman test and the Dunn's post hoc multiple comparison method comparing every treatment with its corresponding control, using Graphpad Prism 7.0a. (n = 6 independent tendon donors, $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

TF-induced MMP expression, we further analyzed the dose–response effect of IL1B on the MMP expression in TFs. Administration of IL1B indeed upregulated the expression of *MMP1, -3* and *-13* in human TFs, already at the lowest tested dose (10 ng/ml, Fig. 5C). Additionally, and in agreement with the Transwell™ co-culture results, a trend of increased MMP expression was observed for IL1B stimulated cells cultured on random substrates, when compared to aligned substrates (Fig. 5C).

3.4. TFs respond to IL1B signaling through phosphorylation and nuclear translocation of NFκB p65, with accelerated translocation on randomly oriented fibers

To more comprehensively investigate the observed cellular response upon stimulation by the pro-inflammatory cytokines IL1B, released by M1 macrophages, the activation of NFκB in the different conditions was assessed as a signaling pathway that could

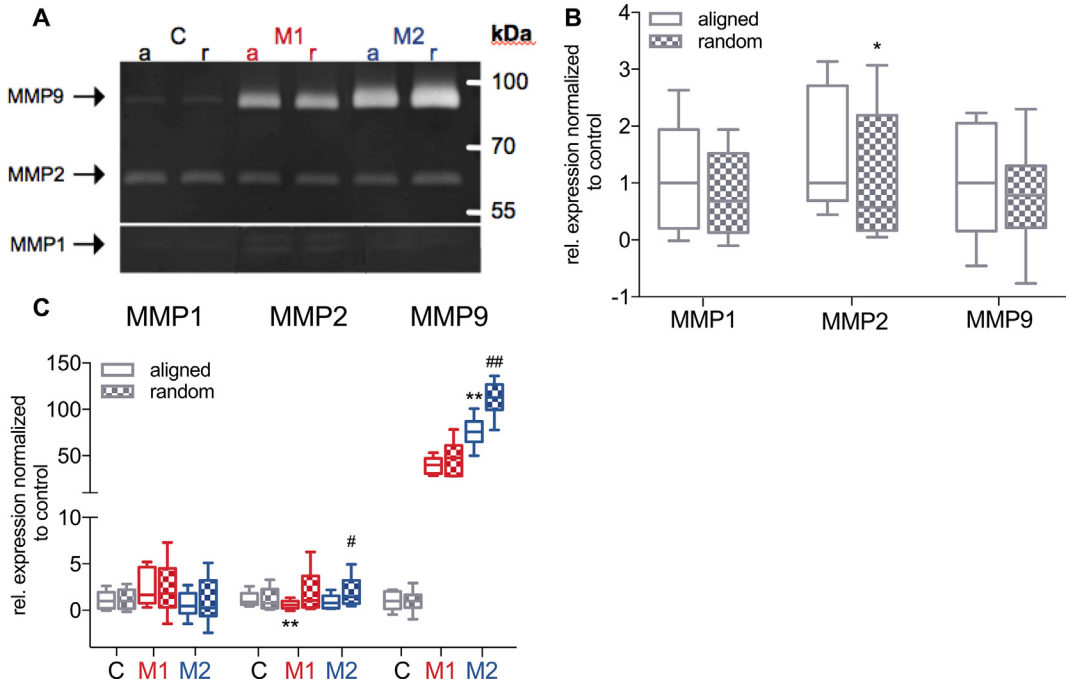

Fig. 4. Quantification of MMP activity in the supernatant of TFs-macrophages co-cultures. Gelatin zymography (A) reveals clear bands for MMP1, -2 and -9 activity in supernatant of TFs cultured with M1 or M2 macrophages. (B) Densitometry of the bands revealed small activity changes for MMP1 and -2, and a significant increase in MMP9, particularly in the TF-M2 macrophage co-cultures (C). Statistical analysis was performed using Mann-Whithney-U Test (B) and Friedman test with the Dunn's post hoc multiple comparison method comparing every treatment with its corresponding control. (n = 4, $^{*}P \leq 0.05$, $^{**}P \leq 0.01$).

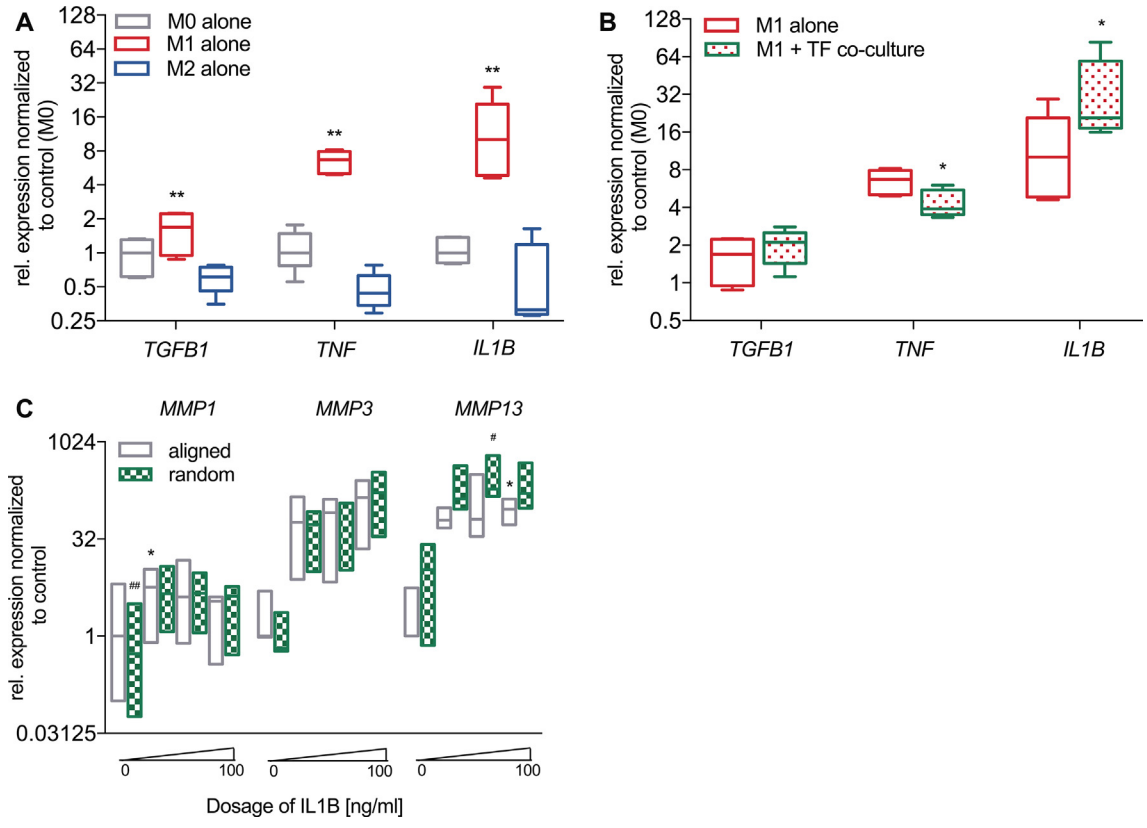

Fig. 5. Macrophage paracrine signaling may induce increased MMP expression by TF vie IL1B. (A) Gene expression analysis revealed that M1 macrophages express higher levels of TGFB1, TNF and IL1B (n = 5) than M0 and M2. (B) Co-culturing of M1 macrophages and TFs resulted in an additional significant increase in gene expression level of IL1B in M1-like macrophages, whereas TGFB1 levels remained unchanged and TNF levels significantly decreased (n = 5). (C) Exogenous addition of IL1B to TF cultures increased the expression of MMPs by TFs in a dose-dependent manner. Gene expression of each dosage is normalized to the control (0 ng/ml, aligned substrates; n = 3). Statistical analysis was performed using Friedman test with Dunn's multiple comparisons test comparing every treatment (co-culture/ IL1B stimulation) with the corresponding control, using Graphpad Prism 7.0a. (n = 3/5, $^{*}P$ 0.05, $^{**}P$ 0.01, $^{***}P$ 0.001.)

contribute to the observed increase in MMP-expression. Following stimulation, nuclear translocation of NFκB is known to be a rapid process ocurring within 60 min upon stimulation. In our experiments, 5 ng/ml of IL1B was added to the TFs seeded on both substrates for 3.5, 7.5, 15 and 30 min before fixation. The translocation of NFκB p65 into the nucleus was assesed by immunofluorescent staining of TFs exposed to IL1B for the different time periods (Fig. 6A and B). 30 min after the addition of 5 ng/ml IL1B, a clear translocation of p65 into the nucleus was observed in TFs cultured on both substrates (Fig. 6B). The quantitative anaylsis of the images revealed a faster translocation p65 into the nuclear compartment for the cells seeded on the pathological random substrates (Fig. 6C). M1 macrophages may therefore induce MMP-expression by TFs, possibly via IL1B paracrine signaling by activation and nuclear translocation of p65 of the NFκB pathway.

4. Discussion

Bioscaffold topographies can provide dominant cell-instructive cues, and electrospun nanofiber alignment offer large potential in this regard [29]. Previous studies demonstrated that highly aligned fibrous biomaterials tend to promote tenogenic cell differentiation while randomly oriented fibrous structures may promote chondrogenesis followed by tissue ossification [29,30,37]. Further, the onset of tendinopathy is characterized by a progressive disorganization of the extracellular matrix (ECM). This early disorganization is also accompanied by increased vascularization, inflammation and impaired mechanical function of the tissue as classified by the modified bonar grading of tendon disease [2,38]. Despite the high prevalence of this medical condition, the etiology and pathophysiological mechanisms that underlie aberrant tendon remodeling and poor healing remain largely unknown. The fact that matrix/scaffold topography is a dominant factor driving emergent tendon tissue behavior, particularly the highly disorganized matrix observed in tendinopathic tissue, inspired us to investigate a potential relationship between scaffold surface topography and sensitivity to inflammatory stimuli. We set out to test the hypothesis that cellular response to disorganized matrix architectures might play an important role in fibrotic signaling, and could potentially contribute to the progression of the disease.

To investigate the potential role of topography in tissue remodeling, we analyzed the impact of biomaterial scaffolds resembling healthy matrix (aligned PCL fibers) and degenerated matrix (random PCL fibers) environments on human primary tendon fibroblasts (TFs) isolated from healthy hamstring tendons. The PCL bioscaffolds used for this study were mechanically tested and proven to be stable against enzymatic degradation during the time course of our experiments (Supplementary Fig. S2). Morphological analysis of the cells revealed that TFs on random nanofiber scaffolds displayed polygonal shapes characteristic of tendinopathy, while on aligned substrates cells displayed highly aligned morphologies resembling those found in healthy tendon tissue. Additionally, at the gene expression level, we observed a tendency towards higher expression of tendon specific markers and genes related to ECM turnover in TFs seeded on aligned substrates, indicative of a more tenogenic phenotype. These findings confirm previous studies [31,39,40].

Moving beyond these confirmatory experiments, we then probed the potential of scaffold alignment to mediate the inflammatory response. It is known that the local microenvironment in healing tendon involves crosstalk between inflammatory immune cells and resident TFs that may eventually contribute to the onset of chronic tissue pathology [41–43]. At the onset of inflammation, M1 macrophages infiltrate the injured tendon to release pro-inflammatory cytokines [44,45]. As inflammation progresses, the M1-like macrophage population is thought to be gradually replaced by M2-like macrophages, which participate in the resolution phase of inflammation. In the present study, the hypothesis was tested that macrophage polarization, in particular towards the M1 inflammatory phenotype, can drive TFs toward enhanced matrix degrading potential. We further hypothesized that this would be most pronounced on random (compared to aligned) substrates. We tested these hypotheses by analyzing the cellular response of TFs cultured on substrates displaying different topographies when exposed to soluble factors secreted by M1 and M2 macrophages.

On both randomly oriented and on aligned nanofiber substrates, M1 macrophage co-culture provoked a pronounced shift toward increased potential for matrix turnover and degradation. More precisely, gene expression levels related to structural matrix proteins, such as *COL1*, *COL3* and *BGN* were downregulated and expression of genes encoding for matrix degrading proteins such as MMPs were upregulated in TFs exposed to M1 macrophages soluble factors. Importantly, there was a subtle, consistent, and significant shift toward ECM-turnover in cells cultured on 'pathology mimicking' random substrates compared to their aligned counterparts. These findings resonate with numerous studies reporting that tendon injury is immediately followed by targeted degeneration of specific components of the extracellular matrix [11,12,46]. Our experiments at the protein level revealed that these gene level changes resulted in secretion of proteases by TFs in a manner that was markedly enhanced by their interaction with inflammatory immune cells. This effect was especially pronounced on the randomly oriented electro-spun substrates that induced polygonal TF morphologies. Moreover, the highest levels of *TIMP1* and *-2* gene expression were observed in TFs seeded on the random substrates in indirect contact with M1 macrophages. Given the regulatory nature of TIMPs, these results may suggest a cellular attempt to adjust the catalytic activity of the secreted MMPs.

Co-culture with macrophages also led to alterations in the expression of stromelysin (*MMP3*), which displayed highest levels when in indirect contact with M1 macrophages. This enzyme is known to degrade matrix proteins characteristic of tendon matrix, such as collagen 3, proteoglycans and elastin, and has been shown to be elevated in degenerative tissue processes [47,48]. Also the gelatinases *MMP2* and *-9*, which are involved in the degradation of collagens [11], displayed highest activity upon co-culture with M1 macrophages. In addition to these results at the gene expression level, it is important to consider the MMP activity at the protein level since function of these enzymes is heavily regulated after secretion. Again, TFs on topographies mimicking tendinopathy in the presence of inflammatory macrophages exhibited the highest proteolytic activities.

Among the soluble factors that may be responsible for the observed increase in TF protease expression, we explored IL1B as a potential candidate. This pro-inflammatory cytokine is released by M1 macrophages and direct stimulation by recombinant IL1B led to a similar increase in protease expression by TFs. The effect of IL1B was further investigated by the nuclear translocation of its effector, the p65 subunit of the NFκB complex. Upon activation, the cytoplasmic p65 is phosphorylated and immediately translocated into the nucleus where it promotes transcription of specific genes [49]. It has been previously shown that cell features such as shape and the presence of neighboring cells have an impact on this translocation [50]. Although the described relationship between cell morphology and nuclear import dynamics is complex and involves many different factors, our data indicate that matrix topography may be an important factor in healing outcome, and potentially in the progression of tendinopathy. Our experiments showed that in fact, translocation of the p65 subunit into the

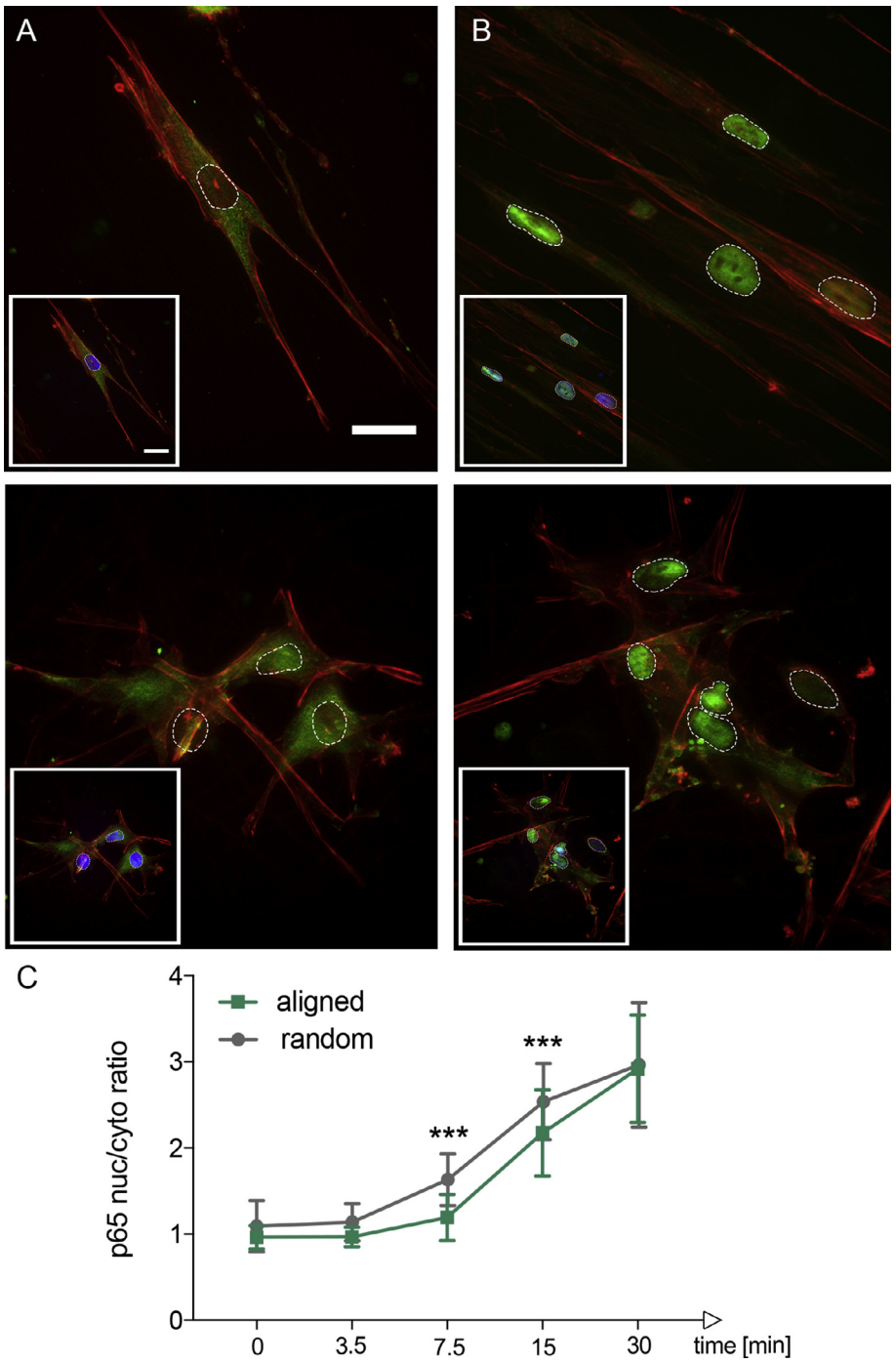

Fig. 6. IL1B induces nuclear translocation of NFκB p65 in TFs, with more rapid activity on random fibers. (A) Immunofluorescent staining of the p65 subunit of the NFκB complex (green) in TFs growing on substrates mimicking healthy and tendinopathic tendon. (B) Upon pro-inflammatory stimulation with 5 ng/ml of IL1B for 30 min p65 was translocated into the nucleus within the cells seeded on both substrates. (C) Pro-inflammatory stimulation with IL1B led to a significant increased translocation velocity of p65 into the nucleus in the cells seeded on the pathological/random substrates. The dotted lines indicate the location of the nuclei (DAPI, blue in the merged inserts). The ratios of p65 within the nucleus (p65 nuc) compared to p65 within the cytoplasm (p65 cyto) were calculated for 10 cells per patient and time point. Statistical analysis was performed using one-way ANOVA comparing the ratios of the cells on the random substrates with corresponding ratios of the cells on the aligned substrates at the same time point and corrected for multiple testing, using Graphpad Prism 7.0a. (n = 5, *P 0.05, **P 0.01, ***P 0.001). Scale bars represent 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

nucleus was significantly faster in cells seeded on the random substrates. These findings would point to an inflammation-facilitating role of randomly oriented biomaterial scaffolds that mimic the tendinopathic matrix, whereby TF/macrophages crosstalk can steer the system towards dysfunctional remodeling of the matrix.

Although inflammation is an essential part of the wound healing process, the development of chronic inflammation can drive aberrant remodeling of the ECM during tendon healing. This in turn can lead to scar formation and ultimate failure to recover tissue function after injury. Here, the dysregulated release of pro-inflammatory cytokines and growth factors by macrophages may result in the onset and exacerbation of tendinopathy in a healing tissue [51]. In our studies, M2-macrophages were observed to express significantly lower signaling molecule levels (e.g. *TGFB1*,

TNF and *IL1B*) than the inflammatory M1-macrophages. The growth factor TGFB1 was analyzed for its potential role in collagen synthesis, particularly in fibrotic processes [52]. Although TGFB1 activity has most widely been described as anti-inflammatory, recent studies have revealed its role to be more complex in nature, with functions that depend on physiological context [53,54]. For instance, recent studies have demonstrated that TGFB1 activity is altered in the presence of TNF [53], consistent with our observation that expression levels of TGFB1 were highest in conditions containing M1 polarized macrophages. While the markers used in the present study to verify macrophage polarization fell within reported ranges in the literature, it must be noted that the highly plastic nature of macrophages complicates efforts to rigidly classify phenotypic behaviors based on gene expression and cytokine expression [55]. Our results indicated that paracrine signaling by M2 macrophages results in a downregulation of matrix proteins in TFs and a significantly lower catabolic potential when compared to M1 paracrine signaling. It should be noted that in contrast to the trends observed in gene expression, protein level MMP9 was observed at higher levels in co-culture with M2 compared to M1 macrophages. This result highlights the complexity of MMP regulation, and the caution with which one should examine the data that emerge from assays on this family of proteases. Specifically, post-translational protease regulation, rather than the precise amount of MMPs in the extracellular milieu, is determinant in the net matrix synthesis or turnover of the tissue [56,57]. Because net matrix remodeling is thought to be determined by the balance between MMP and TIMP activity, we examined these ratios. We observed a trend of increased MMP2 to TIMP2 in cells seeded on the pathology-mimicking, randomly oriented substrates (Supplementary Fig. S3).

It is known that tendinopathic tissues display many changes in ECM composition and organization, including altered collagen and proteoglycan composition [58,59]. Biomechanical aspects of the ECM also vary depending on the pathological state [60], as does the relative involvement of the vascular and immune tissue compartments [2]. Among these many changes, we chose to focus on matrix alignment as a potentially important, but relatively unexplored characteristic of the diseased tendon matrix. While highly aligned surfaces have been reported to be beneficial for tenogenic expression and have been described as "pro-regenerative" [29–31], we are aware of no published studies that have examined biomaterial alignment as potentially predisposing a degenerative cell response. It is this aspect that we intended to isolate and investigate. Consequently, the experimental model system presented here includes topography cues that mimic the architecture of "healthy" versus "tendinopathic" collagen structures. However, it must be noted that this minimal model ignores numerous major features of tendon disease. Although highly simplified, the system nonetheless captures salient features of pathological *in vivo* cell behavior, including irregular cell morphology, increased catabolic matrix signaling, and a predisposition to inflammatory provocation. The present work demonstrates that MMP activation via inflammatory cytokines is more pronounced in TFs attached to biomaterials with a randomly structured fiber topography. Such scaffold topographies mimic the architecture of the tendinopathic extracellular matrix, and it is plausible that matrix organization plays a central role in perpetuation of tissue inflammation. We further suggest that randomly oriented electrospun scaffolds used in co-culture with macrophages is useful as an in vitro model of "minimal tendinopathy". As with all tendon research, efforts to assess the physiological or patho-physiological relevance of the in vitro model system were hindered by a lack of accepted tenogenic and pathogenic tendon markers. This lack acutely limits advances in tendon research, and represents a major bottleneck to be overcome. In future work, the model will be exploited to additionally probe the behavior of cells derived from pathological tissues. This would provide valuable perspective on the suitability of the markers used to assess phenotypic differences of non-pathological cells on these substrates. In addition, *in vivo* tendons are subjected to dynamic mechanical stimuli, which was not investigated in these studies, but which can be expected to interact with matrix structure in the regulation of highly mechanosensitive TFs. Using this model, we noted that M2-like macrophages induced a characteristic TF response in co-culture consistent with the paradigm that optimal tendon healing involves regulation by M2 macrophages that steer healing toward resolution of tissue remodeling [21,61]. While it is likely that some aspects of TF/macrophage crosstalk require direct contact between immune cells and stromal cells, recent studies focusing on the interplay between tenocytes and immune cells report similar system responses regardless of direct co-culture or indirect co-culture using Transwell™ systems, suggesting that soluble factors are key players in this crosstalk [62].

5. Conclusion

Understanding how matrix topography regulates the onset and eventual termination of cellular response in an inflammatory tissue is crucial to the understanding of tendon pathology, as well as for the development of therapeutic biomaterials. We have demonstrated that a paracrine switch from M1 to M2 macrophages differentially affects adherent tendon cell response in a substrate-dependent manner. Specifically, highly aligned electrospun fiber scaffolds tended to downregulate the expression of MMPs, potentially indicating a shift to less catabolic activity when compared to randomly oriented scaffolds. How these effects on gene expression may translate to altered matrix turnover remains ground for future work. In addition, the matrix topography affects TFs morphology as well as their sensitivity to and regulation by pro-inflammatory signaling. These novel biological insights are relevant to the design of nanofibrous scaffolds for use in tendon tissue engineering approaches, or the design of synthetic fibrous tissue grafts that lead to positive biological and clinical outcomes.

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Notes

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.actbio.2018.03.004.

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