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Short communication

Do mechanical strain and TNF-α interact to amplify pro-inflammatory cytokine production in human annulus fibrosus cells?

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ABSTRACT

During intervertebral disc (IVD) injury and degeneration, annulus fibrosus (AF) cells experience large mechanical strains in a pro-inflammatory milieu. We hypothesized that TNF-α, an initiator of IVD inflammation, modifies AF cell mechanobiology via cytoskeletal changes, and interacts with mechanical strain to enhance pro-inflammatory cytokine production. Human AF cells ($N=5$, Thompson grades 2–4) were stretched uniaxially on collagen-I coated chambers to 0%, 5% (physiological) or 15% (pathologic) strains at 0.5 Hz for 24 h under hypoxic conditions with or without TNF-α (10 ng/mL). AF cells were treated with anti-TNF-α and anti-IL-6. ELISA assessed IL-1β, IL-6, and IL-8 production and immunocytochemistry measured F-actin, vinculin and α-tubulin in AF cells. TNF-α significantly increased AF cell pro-inflammatory cytokine production compared to basal conditions (IL-1β:2.0 ± 1.4–84.0 ± 77.3, IL-6:10.6 ± 9.9–280.9 ± 214.1, IL-8:23.9 ± 26.0–5125.1 ± 4170.8 pg/ml for basal and TNF-α treatment, respectively) as expected, but mechanical strain did not. Pathologic strain in combination with TNF-α increased IL-1β, and IL-8 but not IL-6 production of AF cells. TNF-α treatment altered F-actin and α-tubulin in AF cells, suggestive of altered cytoskeletal stiffness. Anti-TNF-α (infliximab) significantly inhibited pro-inflammatory cytokine production while anti-IL-6 (atlizumab) did not. In conclusion, TNF-α altered AF cell mechanobiology with cytoskeletal remodeling that potentially sensitized AF cells to mechanical strain and increased TNF-α-induced pro-inflammatory cytokine production. Results suggest an interaction between TNF-α and mechanical strain and future mechanistic studies are required to validate these observations.

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1. Introduction

Intervertebral disc (IVD) disorders contribute to back pain which is a leading cause of global disability (Cheung et al., 2012; Jacobs, 2011; Vos et al., 2012). Painful IVD degeneration involves complex interactions between mechanical loading and inflammation yet few studies investigate these interactions. For example, excessive spinal loads that induce large dynamic bending moments and asymmetric loads are predictive of spinal functional impairment and pain (Marras et al., 2010). Annulus fibrosus (AF) defects and delamination induce strain 'concentrations', cell death, and pro-inflammatory cytokine production (Adams et al., 2015; Korecki et al., 2008; Michalek et al., 2010; Ulrich et al., 2007; Walter et al., 2011). In contrast, dynamic loading is considered healthy since it can enhance IVD matrix production; although this healthy response to load is altered with degeneration perhaps because dynamic compression facilitates transport of pro-inflammatory cytokines into healthy IVDs where they can persist and induce biomechanical changes (Sowa et al., 2012; Stokes and Iatridis, 2004; Wuertz et al., 2009; Walter et al., 2015a).

TNF-α and IL-1β are key pro-inflammatory cytokines that are increased in IVD degeneration and known to be produced by cells of the IVD and immune system (Allen et al., 2011; Le Maitre et al., 2007; Olmarker and Rydevik, 2001; Risbud and Shapiro, 2014; Seguin et al., 2005). TNF-α can stimulate the production of pro-inflammatory cytokines, matrix degrading enzymes, and pain mediators (Millward-Sadler et al., 2009; Purmessur et al., 2013; Seguin et al., 2005; Weiler et al., 2005). TNF-α is also implicated in

Abbreviations: AF, annulus fibrosus; IVD, intervertebral disc; IL, interleukin; TNF-α, tumor necrosis factor-α; DMEM, Dulbecco's Modified Eagles Media; ELISA, Enzyme linked immunosorbant assay

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painful spine conditions with infliximab and other TNF-α inhibitors showing improved pain and behavioral responses in clinical trials and animal studies (Allen et al., 2011; Likhitpanichkul et al., 2015; Risbud and Shapiro, 2014; Shamji et al., 2010; Nakamae et al., 2011). However, clinical trial results using infliximab are mixed possibly due to inadequate dosing or delivery (Risbud and Shapiro, 2014) highlighting a need for improved knowledge of how TNF-α affects IVD cells.

Physiological strains stimulated anti-catabolic effects on AF cells in vitro while pathological strains resulted in a detrimental responses (Gilbert et al., 2010; Sowa et al., 2011; Sowa et al., 2012; Gawri et al., 2014). Pathologically high mechanical strain on AF cells exposed to IL-1β induced greater catabolic gene expression compared to mechanical strain alone (Sowa et al., 2011). The roles that healthy and pathological strains play on AF cell pro-inflammatory response and cytoskeleton structure are unknown and improved knowledge of IVD cell mechanobiology under pro-inflammatory conditions may provide new insights on pathophysiology.

The objectives of this study were: 1) to investigate the interaction between TNF-α and tensile strain levels on human AF cells in vitro, with the hypothesis that TNF-α treatment interacts with pathological strains to amplify down-stream pro-inflammatory cytokines IL-1β, IL-6, and IL-8 via cytoskeletal changes; and 2) to test the effectiveness of infliximab, an anti-TNF-α drug, and atlizumab, an anti-IL-6 drug, on human AF cells with the hypothesis that infliximab is more effective than atlizumab in inhibiting the interacting effects of TNF-α and high strain. Cells were subjected to 5% and 15% strain to represent physiological and pathological strains, as previously defined for AF cells (Gilbert et al., 2011; Rannou et al., 2000; Sowa et al., 2011). Pro-inflammatory cytokines and cytoskeletal changes are our primary output measurements. In addition to TNF-α and IL-1β, we measured IL-8 levels and IL-6. IL-8 is associated with the recruitment of immune cells including macrophages, which can further intensify the matrix breakdown and result in granulation tissue formation (Burke et al., 2002; Kim et al., 2008). IL-6 is up regulated in IVD injury, degeneration and sciatica (Andrade et al., 2013; Ulrich et al., 2007; Wuertz and Haglund, 2013), and has been considered a clinical target since it can enhance the effects of TNF-α and IL-1β (Studer et al., 2011).

2. Methods

Primary human AF cells were isolated from 5 human IVDs from surgery and autopsy (mean ± SD age: 50 ± 16 years) with degeneration grades 2–4 (Thompson et al., 1990). For cell isolation outer AF tissue was macroscopically dissected, minced and digested for 1 h in 2 g/L pronase (Fisher-Scientific, Waltham, MA) and 4 h in collagenase type I (Fisher-Scientific, Waltham, MA) in high glucose Dulbecco's Modified Eagles Media (DMEM). The cell suspension was filtered (100 μm) and cell pellets were washed with PBS. To accelerate proliferation, cells were expanded under normoxic conditions (37 °C, 5% CO_2, 20% O_2) in high glucose DMEM (Fisher-Scientific, Waltham, MA), 10% FBS (Fisher-Scientific, Waltham, MA), 50 ug/mL ascorbic acid (Fisher-Scientific, Waltham, MA), 1% penicillin/streptomycin (Fisher-Scientific, Waltham, MA), 2 μL/mL primocin (Invivogen, San Diego, CA), at 37 °C, 5% CO_2 with media changes each 3–4 days. Experimental media conditions better simulated IVD physiologic conditions by using hypoxia (37 °C, 5% CO_2, 5% O_2) as well as serum-free media, high glucose DMEM, 1% ITS (Fisher-Scientific, Waltham, MA), 50 μg/mL Ascorbic acid, 1% Pen/Strep, and 2 μL/mL primocin. For experiments, AF cells (passage 1–2; 100,000 cells/mL) were seeded onto silicone-membrane chambers (STREX, B-Bridge, Cupertino, CA) coated with rat-tail collagen I (BD Biosciences, San Jose, CA) for a one day incubation prior to stretch to enable cell confluency and equilibration under experimental conditions.

For TNF-α treatment, 10 ng/mL TNF-α (Fisher-Scientific, Waltham, MA) was added to serum-free media immediately prior to incubation. This TNF-α concentration was shown to increase proinflammatory cytokine and matrix degrading enzyme production in IVD cells (Millward-Sadler et al., 2009; Walter et al., 2015b). For mechanical treatment, Strex chambers were exposed to physiological (5%) or pathological (15%) cyclic tensile uniaxial strain at a 0.5 Hz frequency for 24 h, and controls were unstrained.

Enzyme linked immunosorbant assay (ELISA) specific for human TNF-α, IL-1β, IL-6, and IL-8 (MSD N45025B-1, Rockville, MD) assessed concentrations of pro-inflammatory cytokines within culture medium 24 h after treatment.

Immunocytochemistry was performed on cells 24 h after initiation of treatments to determine acute effects on F-actin, vinculin, and α-tubulin ($N=3$). AF cells were fixed using 16% paraformaldehyde, and stained for F-actin, α-tubulin, and vinculin (F-actin: Phalloidin-TRITC: Sigma Cat#P1951, 1:100; α-tubulin:α-tubulin-Alexa Flour 488: eBioScience Cat#53-4502-80, 1:50; vinculin:mouse anti-vinculin: Sigma Cat#V4505, 1:100; Alexa Flour 488 goat anti-mouse: Life Technologies Cat#A1001, 1:200). Viability was determined with live/dead staining (2 μL/mL calcein, 1 μL/mL ethidium homodimer, 1 μL/5 mL Hoechst). Apoptosis was measured by caspase-3 activity (rabbit cleaved caspase-3:Cell signaling Cat# mAB9664, 1:100; Alexa Flour 594 goat anti-rabbit:Invitrogen Cat#A-11037, 1:1000). Imaging was performed using confocal (Leica SP5 DM, Wetzlar, Germany) and epi-fluorescence imaging (AxioImager Z1, Zeiss, Jena, Germany).

Anti-inflammatory treated cells were exposed to 10 ng/mL TNF-α and 1000 ng/mL infliximab (anti-TNF-α) or 1000 ng/mL anti-IL-6 added to fresh serum-free media immediately prior to incubation or application of tensile strain. Infliximab and atlizumab doses were selected based on pilot 3 day dose response studies showing largest effect with no observed cell death.

Cytokine concentrations were normalized to paired controls and compared between treatment groups. Student's paired t-tests were performed to compare cytokine concentrations in response to unstrained basal and TNF-α treatment ($N=5$). One-way ANOVA with Tukey's post-hoc tests for multiple comparisons were performed to compare cytokine concentrations between 0%, 5%, and 15% strains in the presence of TNF-α ($N=5$). $p<0.05$ was considered significant.

3. Results

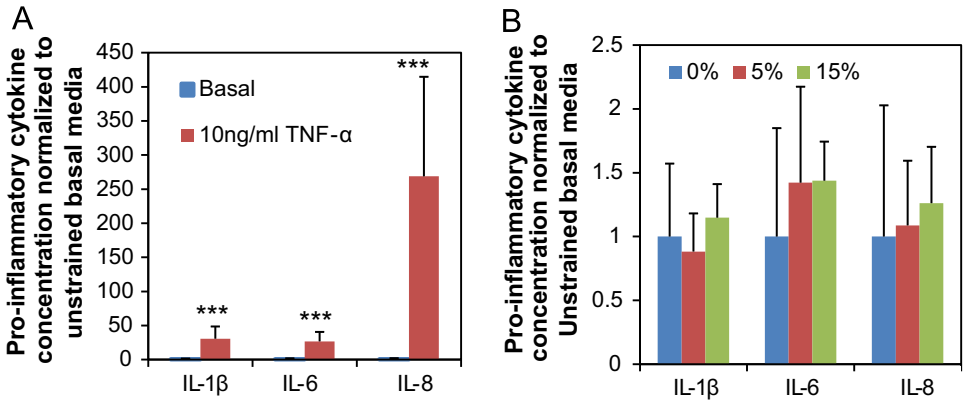
TNF-α treatment significantly increased IL-1β, IL-6 and IL-8 concentrations (IL-1β:84.15 ± 77.3, IL-6:280.9 ± 214.1, IL-8:5125.1 ± 4170.8 pg/ml) compared to basal media, which had low concentrations (IL-1β:1.9 ± 1.4, IL-6:10.6 ± 9.9, IL-8: 23.9 ± 26.0 pg/ml) (Fig. 1). Mechanical strain at 5% and 15% showed no significant effect on pro-inflammatory cytokine production compared to unstrained groups. Combined TNF-α treatment with 15% strain showed a significant increase in IL-1β and IL-8 concentrations at 15% compared to 5% strain (Fig. 2).

TNF-α treatment on AF cells increased F-actin intensity compared to basal, however no changes in vinculin staining were apparent (Fig. 3). The α-tubulin staining demonstrated a more diffusely connected microtubule network for the TNF-α treated cells compared to basal. In basal medium, no apparent differences in F-actin or vinculin staining of AF cells at 5% and 15% strain were observed compared to unstrained (Fig. 4). Cells subjected to TNF-α and 15% strain exhibited cytoskeletal changes similar to TNF-α alone (Fig. 5). Cells subjected TNF-α and 15% strain had increased cell roundedness, however, no staining for dead cells or cleaved caspase-3 was observed for any groups.

Infliximab non-significantly decreased IL-1β and IL-8 levels compared to TNF-α-stimulated AF cells undergoing 15% strain, and significantly decreased IL-1β and IL-8 levels compared to anti-IL-6 treatment (Fig. 6).

4. Discussion

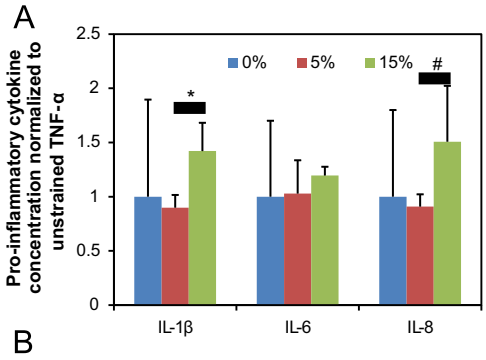
The AF cell niche changes following injury and degeneration to create large deformations and defects that expose AF cells to high levels of pro-inflammatory cytokines and pathological strains. The most important findings were that human AF cells exposed to TNF-α responded to pathological strains with increased IL-1β and IL-8 production and a modified AF cytoskeletal network with enhanced stress fiber formation from actin polymerization and disrupted microtubule network. Results suggested that TNF-α induced cytoskeletal changes in human AF cells suggestive of enhanced mechanosensitivity, although this hypothesis requires further testing. Anti-TNF-α treatment inhibited pro-inflammatory cytokine production compared to anti-IL-6, suggesting anti-



C

	Pro-inflammatory cytokine concentration (pg/mL)		
	IL-1β	IL-6	IL-8
Unstrained basal media	2.0± 1.4	10.6± 9.9	23.9± 26.0

Fig. 1. TNF-α increased pro-inflammatory cytokine production while stretch did not. (A) TNF-α (10 ng/mL) treatment alone significantly increased pro-inflammatory cytokine concentrations produced by human AF cells ($N=5$) in the absence of stretch. (B) Mechanical strain at physiological (5%) or pathological (15%) levels in the absence of TNF-α treatment exhibited no significant effect on pro-inflammatory cytokine concentration as compared to basal media. (C) Absolute values of pro-inflammatory cytokine concentrations measured in basal media. (mean ± SD; $^{***}p < 0.005$).



B

	Pro-inflammatory cytokine concentration (pg/mL)		
	IL-1β	IL-6	IL-8
Unstrained TNF-α media	84.0± 77.3	280.9± 214.1	5125.1± 4170.8

Fig. 2. TNF-α and pathological mechanical strain interacted to substantially increase pro-inflammatory cytokine production. (A) Production of pro-inflammatory cytokines by TNF-α treated human AF cells ($N=5$) in the presence of 15% strain significantly increased for IL-1β and IL-8 compared to 5% strain but not 0% strain. (B) This was particularly notable given the high cytokine concentrations present for the unstrained TNF-α group. (mean ± SD; $p < 0.05$).

inflammatory treatments with appropriate targets and dose can inhibit these effects.

Little data exists on AF cytoskeletal changes to pro-inflammatory cytokines. TNF-α increased chondrocyte cell stiffness due to increases in F-actin staining (Chen et al., 2015) that was similar to our observations and together suggest TNF-α induced AF cell stiffening could be responsible for heightened AF cell mechanosensitivity. TNF-α and lipopolysaccharide induced permanent alterations in nucleus pulposus cytoskeletal mechanics (Maidhof et al., 2014), suggesting TNF-α-induced cytoskeletal changes may be generalizable to other pro-inflammatory conditions and long-lasting; however it is impossible to compare these NP cell-volume and hydraulic permeability values to cytoskeletal measurements in this study. Similar TNF-α induced cytoskeletal changes including re-organization of F-actin (Koukouritaki et al., 1999; Petrache et al., 2001) and microtubules (Petrache et al., 2003) was observed in other cell types. Disruption to microtubule networks, as observed in this study, could reduce uniformity of the load distribution within the cytoskeleton leading to higher stress concentration at the connection with the nucleus (Maniotis et al., 1997), providing a counter-hypothesis to cell-stiffening as a cause for heightened mechanosensitivity. Vinculin, an intracellular attachment protein linking integrins on the cell membrane to the F-actin cytoskeleton, showed no notable changes in response to TNF-α, suggesting limited changes in vinculin-dependent cell matrix connectivity although it is possible that other cell adhesion proteins may be affected by TNF-α. In the context of the literature, the alterations in cytoskeletal structures observed here support the concept that heightened mechanosensitivity of AF cells following TNF-α treatment resulted from AF cell stiffening. Future studies are required to validate these observations with quantitative data and to mechanistically test which cytoskeletal structures are responsible for these observed effects.

AF cell strain is known to interact with pro-inflammatory cytokines, and the current results help explain the finding that AF cells obtained from a rabbit IVD degeneration model demonstrated enhanced pro-inflammatory responses to mechanical and inflammatory stimuli (Sowa et al., 2012). Specifically, AF cells in degenerated rabbit IVDs may have been chronically exposed to TNF-α resulting in heightened mechanosensitivity. Low magnitudes but not high magnitudes of strain had a somewhat protective effect of a pro-inflammatory challenge with IL-1β (Sowa et al., 2011). Excessive pro-inflammatory cytokine exposure is particularly harmful to the IVD due to its low cellularity and limited repair potential (Kandel et al., 2008; Zhu et al., 2012). For example, IVDs in organ culture subjected to TNF-α resulted in cell senescence, irrecoverable matrix changes with proteoglycan degradation, a catabolic shift in gene expression, and biomechanical alterations (Purmessur et al., 2013; Walter et al., 2015a; Walter et al., 2014). The current results are consistent with the literature that pro-inflammatory cytokine production by IVD cells are likely to be an important contributor to the inflamed environment associated

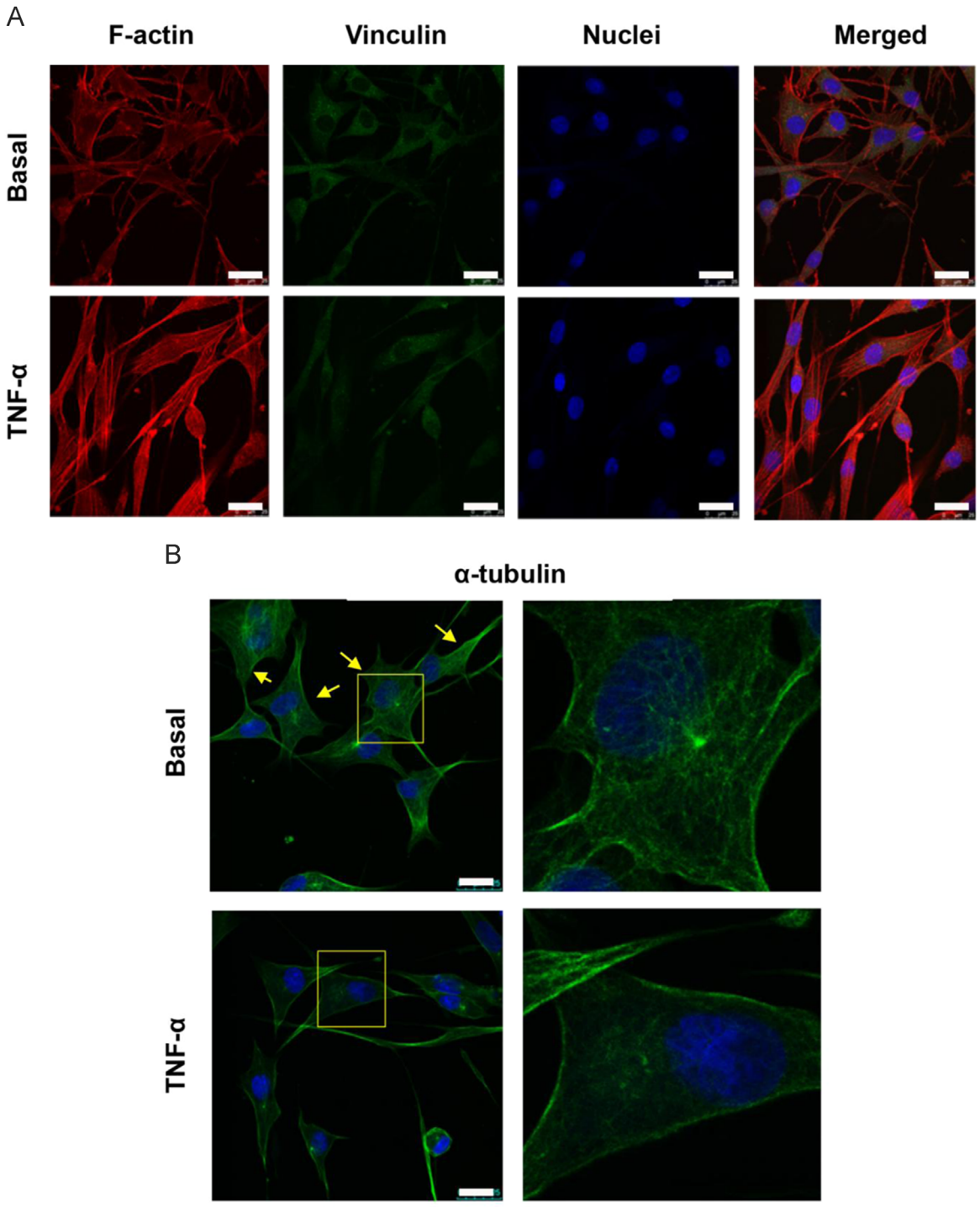


Fig. 3. TNF-α increased F-actin staining and microtubule disruption. Immunocytochemistry of F-actin and vinculin on human AF cells treated with basal media and TNF-α. F-actin staining intensity increased with TNF-α and there was no difference in vinculin staining. (B) An extensive microtubule network was evident using α-tubulin staining on AF cells for the basal media group (yellow arrows). The microtubule network was disrupted with TNF-α treatment, as evidenced by more diffuse α-tubulin staining which was particularly evident at higher optical zoom. (Magnification = 60X, Scale = 25 μm). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

with IVD degeneration with the potential to permanently alter IVD cell mechanobiology.

Anti-TNF-α was more effective than anti-IL-6 as it inhibited direct and indirect effects of TNF-α on the AF cell pro-inflammatory response. Infliximab inhibits pro-inflammatory cytokine production by binding to and sequestering extracellular TNF-α. Atlizumab targets the IL-6 receptor and was selected to see if autocrine IL-6 signaling contributed to pro-inflammatory cytokine production since TNF-α and IL-6 were previously shown to act synergistically (Studer et al., 2011). Atlizumab did not have an effect in our cell culture studies suggesting that few interactions between TNF-α and IL-6 were present in our system. An alternate anti-IL-6 drug capable of binding and sequestering IL-6 or greater doses may have larger effects.

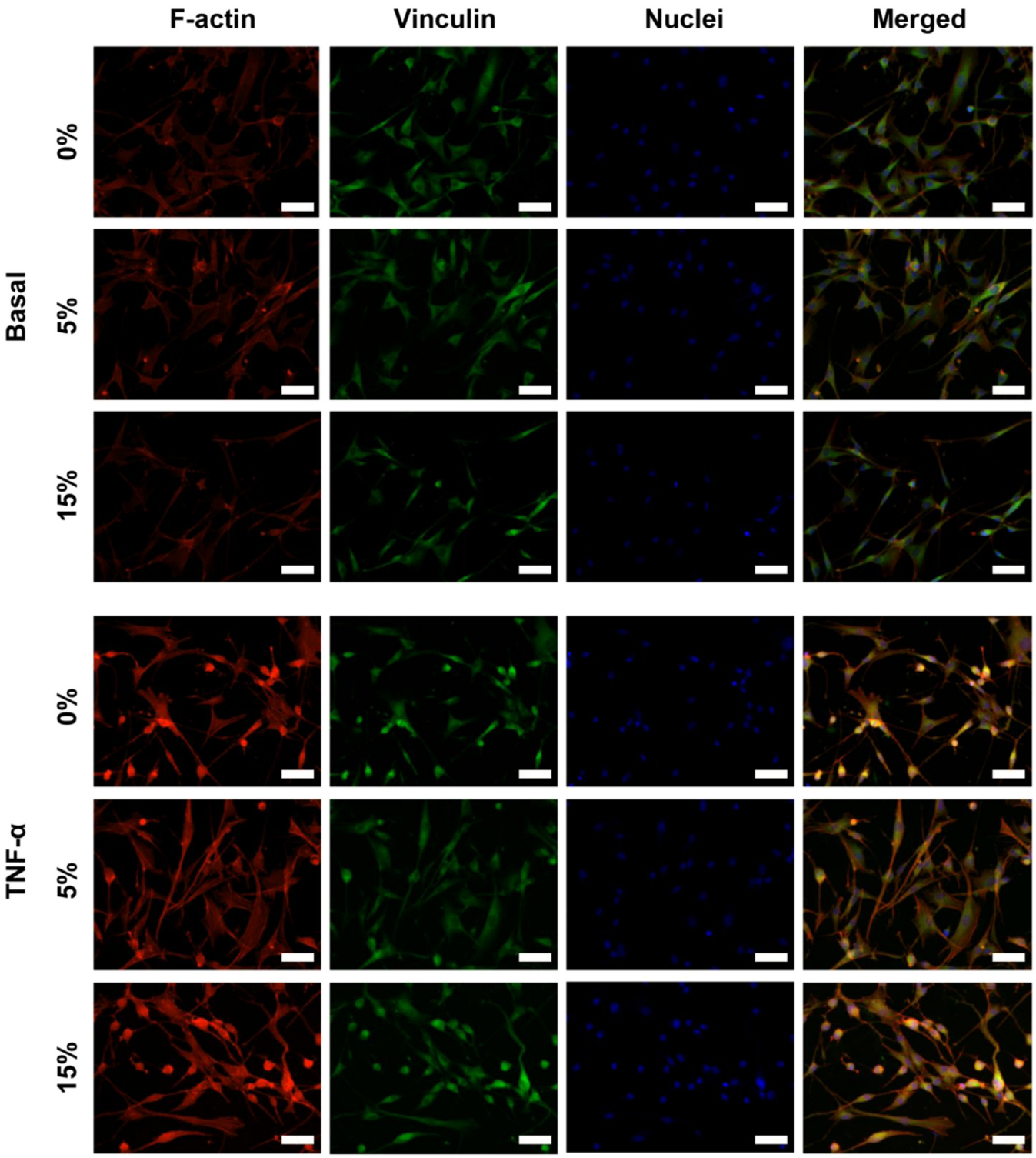


Fig. 4. TNF-α but not mechanical stretch induced F-actin changes in human AF cells. Immunocytochemistry for F-actin and vinculin in human AF cells seeded at 100,000 cells/mL immediately after 24 h of stretch with and without TNF-α. F-actin and vinculin staining exhibited no apparent differences in cytoskeletal organization across all strain magnitudes. TNF-α treatment increased F-actin staining intensity but there were no discernable differences between strains in cytoskeletal structure with TNF-α treatment. (Magnification = 20X, Scale = 100 μm).

Future mechanistic studies with additional pharmacologic blocking and a larger set of inflammatory outcome measures would enhance understanding of the complex interactions between inflammation and mechanobiology. Results show changes in actin fibers and microtubule network rearrangement after 24 h justifying this relevant time point for measurements, although additional timepoints of cytoskeletal analyses with more anti-inflammatory treatments would allow broader interpretations. A greater sample size is also warranted to address the large human variance and validate the observed findings.

Conflict of interest statement

The authors declare that they have no competing interests.

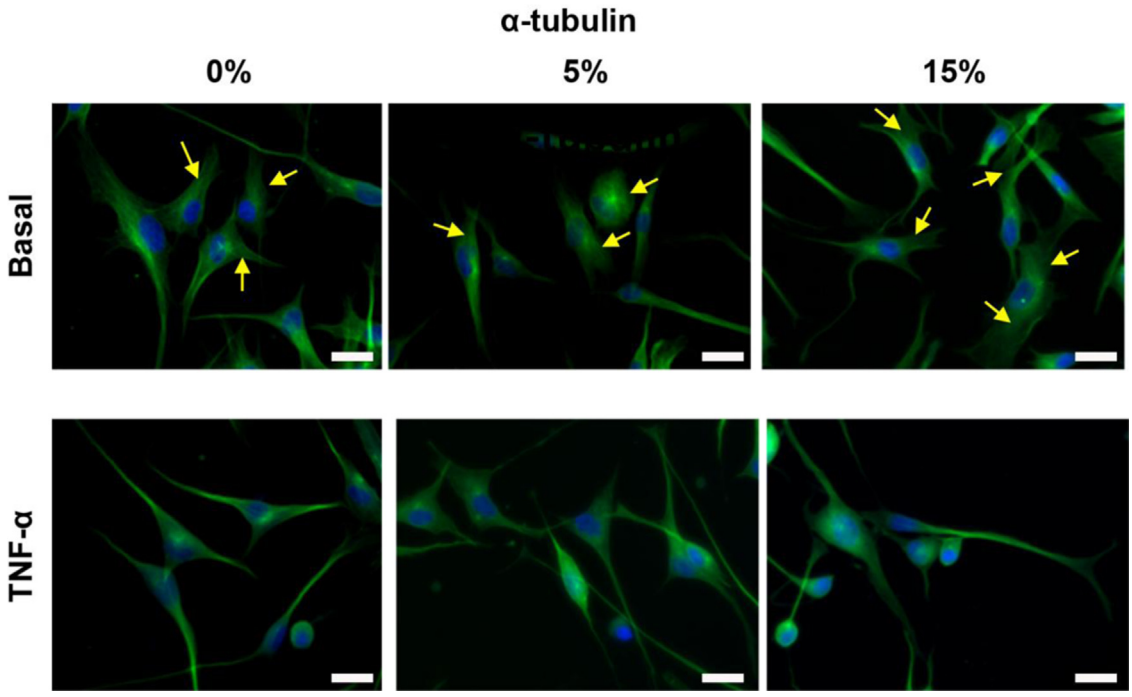
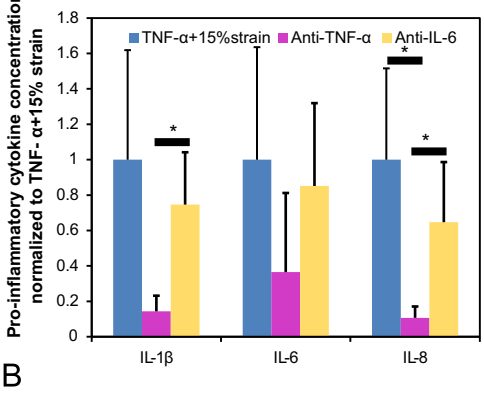


Fig. 5. Microtubules were disrupted in AF cells following TNF-α treatments and stretch. An extensive microtubule network was clearly demonstrated with α-tubulin staining under all mechanical strain conditions in the basal media group (yellow arrows). Microtubules were disrupted in AF cells subjected to TNF-α treatment with more diffuse α-tubulin staining. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

A



B

	Pro-inflammatory cytokine concentration (pg/mL)		
TNF-α+15% strain	IL-1β	IL-6	IL-8
	103.6± 64.1	273.6± 173.9	5907.9± 3046.7

Fig. 6. Pro-inflammatory cytokine production was reduced by anti-TNF- α but not anti-IL-6. Infliximab decreased the concentrations of pro-inflammatory cytokines produced by AF cells ($N=5$) exposed to TNF-α (10 ng/mL) and 15% stretch. There was a significant effect on IL-8 concentration for cells treated with anti-TNF-α, but not for anti-IL-6. Anti-IL-6 had a significantly smaller reduction of pro-inflammatory cytokine levels. There was a significant difference between the effects of Anti-TNF-α and Anti-IL-6 on the concentration of pro-inflammatory cytokines. (mean ± SD; $^{*}p=0.01$,$^{\#}p=0.08$).

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