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SUMOylated T β L1/T β LR1 interacts with β -catenin in palmar fascia fibrosis: a novel therapeutic target

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Abstract

Dupuytren's Disease (DD) is a benign fibrosis of the palmar fascia, the connective tissue beneath the skin of palm and digits. DD leads to loss of hand function and affects 4 - 6% of the US population alone. Current treatments focus on removing diseased tissue through surgery — however, post-surgery disease recurrence rates exceed 30% with no known cure. Previous studies found that fibroproliferative diseases such as DD contain fibroblasts with abnormally high levels of β -catenin, similar to certain malignancies such as colon cancer. In colon cancer cells, nuclear translocation of β -catenin trans-activates genes responsible for cellular proliferation, and this process is facilitated by the transducin β -like proteins T β L1 and T β LR1 that are post-translationally modified with small ubiquitin-like modifiers (SUMOylation). The anti-cancer drug, BC2059 (Tegavivint), has been developed to competitively inhibit interactions between β -catenin and SUMOylated T β L1/T β LR1 and is currently undergoing Phase-I clinical trials. It is currently unknown whether the SUMOylated T β L1/T β LR1 complex mediates β -catenin nuclear translocation in DD. In this study, we investigated whether β -catenin interactions with SUMOylated T β L1/T β LR1 were present in primary fibroblasts derived from patients with DD, and if cytokine treatments mimicking inflammation during fibrosis modified these interactions. Through western blotting, proximity ligation assays, and confocal microscopy, we confirmed that in DD fibroblasts— the levels of SUMOylated T β L1/T β LR1 increased in response to cytokines. Moreover, we observed that the interactions between β -catenin and T β L1/T β LR1 were significantly increased. The novel finding of cytokine-induced SUMOylation of T β L1/T β LR1 presents a mechanistic link between inflammation and fibroproliferation. Given the prevalence and debilitating nature of DD, there exists a need to identify new therapeutic

targets to prevent disease progression and recurrence — the results of this study identify SUMOylated T β L1/T β LR1 interactions with β -catenin as a feasible target and provide a strong rationale to cross-purpose anti-cancer drugs such as BC2059 to treat DD.

Introduction

Dupuytren's Disease

Dupuytren's Disease (DD) is a chronic, heritable fibrosis of the palmar fascia —the fibrous layer of connective tissue found underneath the skin of the palm and digits. During the progression of DD, the palmar fascia undergoes excessive deposition of collagen, eventually thickening to the point where some patients experience permanent hand and/or finger contractures¹. The prevalence of DD is estimated to be up to 7% in the United States of America, and as high as 32% in some regions of Europe^{2, 3}. Current treatments for DD primarily focus on directly removing diseased palmar fascia, either through invasive surgical interventions (fasciectomy), or enzymatic breakdown using minimally invasive collagenase injections^{1, 4}. Unfortunately, post-surgery disease recurrence rates are high, typically exceeding 30% and as a result, DD is presently incurable⁵. Given the prevalence and debilitating nature of DD, there exists a crucial need to identify new therapeutic targets in order to prevent disease progression and recurrence.

Canonical β -catenin signalling

Although DD is ultimately benign, it shares certain molecular characteristics with malignant tumours, particularly increased β -catenin levels⁶. β -catenin is a dual function trans-activating factor that regulates gene transcription and coordinates cell-to-cell adhesion⁷. It is normally under regulation and tagged for degradation through phosphorylation through a destruction complex consisting of the scaffolding proteins Axin, Adenoma Polyposis Coli (APC), glycogen synthase kinase 3 β (GSK3 β), and casein kinase 1 (CK1). Ultimately,

phosphorylated β -catenin undergoes degradation through the 26S proteasome ubiquitination pathway⁷. Inactivation of the destruction complex occurs canonically through Wnt-signalling, where β -catenin accumulates in the cytoplasm and translocates into the nucleus to trans-activate genes involved in cell proliferation, differentiation, and adhesion— normally, this process is crucial in normal embryonic development⁷. However, abnormal Wnt-signalling has been implicated in various malignancies such as colon cancer as a result of mutations in the APC gene—leading to a dysfunctional destruction complex and over-accumulation of β -catenin in the cytoplasm^{6,7}.

The role of β -catenin in normal and abnormal wound healing

Fibroproliferative diseases such as DD are an exaggerated response to wound healing⁷. In addition to its role in the development of certain cancers, β -catenin is involved in the normal wound healing process, which can be characterized by three major phases: the inflammatory response, the proliferative phase, and the remodelling phase⁷ (Figure 1). The inflammatory response is necessary to mediate the break down of debris and clearance of bacteria by neutrophils and macrophages. To begin the transition from the inflammatory phase to the proliferative phase, a number of growth factors and anti-inflammatory cytokines such as transforming growth factors α and β (TGF α and TGF β), epidermal growth factor (EGF), and insulin-like growth factor (IGF) are released by macrophages at the wound to stimulate fibroblast migration and proliferation. During the proliferation phase, recruited fibroblasts differentiate into myofibroblasts, which then function during the tissue-remodelling phase to deposit collagen bundles that compact or contract the wound. Notably, within the proliferative phase of normal wound healing — it is believed that TGF β mediates β -catenin signalling in fibroblasts through

inactivation of GSK3 β , and that fibroblast proliferation and subsequent tissue remodelling is dependent on β -catenin signalling. Ultimately, dysregulation of β -catenin-signalling during fibroblast proliferation may be a factor in the abnormal wound healing response leading to the development of fibroproliferative disorders such as DD^{16, 17, 18}.

Previous studies have shown that the fibroblasts inducing palmar fascia fibrosis (DD fibroblasts) exhibit markedly increased cytoplasmic and nuclear β -catenin levels²⁴. Unpublished findings in our lab have demonstrated that β -catenin nuclear translocation in DD may lead to its association with certain pro-fibrotic and pro-malignant genes, such as *WT1* (encoding Wilm's Tumour One). A high level of the WT1 protein is known to be a common characteristic among various cancers, and more recently, in DD^{8, 9}. The potential consequences of aberrant β -catenin nuclear translocation in DD, therefore, make it a valuable therapeutic target to prevent disease progression and/or recurrence. However, the specific molecular mechanisms that are responsible for its nuclear translocation are unclear.

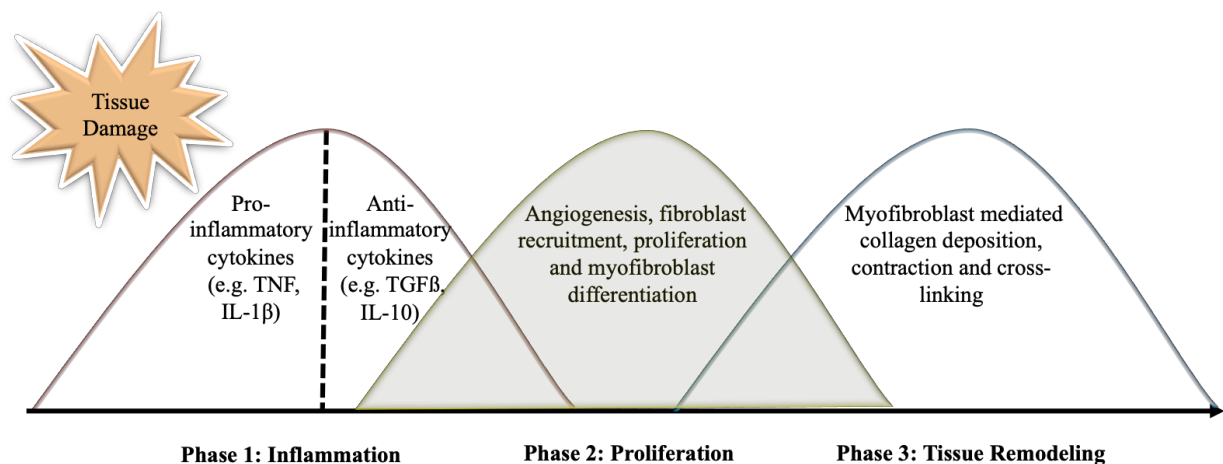


Fig 1. The normal wound healing process.

Mechanisms behind the nuclear translocation of β -catenin

Recently, the conjugation of small ubiquitin-like modifiers (SUMO1) to Transducin β -like 1 (T β L1) and Transducin β -like receptor 1 (T β LR1) have been implicated in the nuclear translocation of β -catenin in colon cancer cells exhibiting high β -catenin levels and aberrant β -catenin signaling. More specifically, it is believed that only after SUMO1 conjugation (SUMOylation) of the T β L1/T β LR1 complex will β -catenin associate with the complex and localize into the nucleus¹⁰ (Figure 2). Small molecule inhibitors, such as BC2059 (Tegavivint), have been developed to competitively inhibit SUMOylated T β L1/T β LR1 interactions with β -catenin as an anti-cancer therapeutic¹¹, and are already in phase I clinical trials²⁸. The aim of this study was to identify parallels in the molecular characteristics associated between DD and cancer to determine the feasibility of cross-purposing anti-cancer drugs such as BC2059 for the treatment of DD and other fibroproliferative disorders.

Based on the findings that there are increased levels of cytoplasmic and nuclear β -catenin in DD, we hypothesized that in DD, β -catenin nuclear translocation is facilitated by the SUMOylated T β L1/T β LR1 complex, and that inhibition of this complex will prevent DD progression and recurrence.

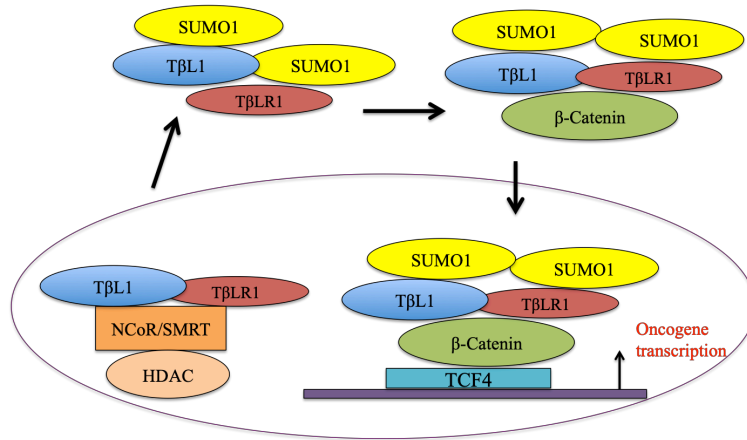


Fig 2. Nuclear translocation of β-catenin in cancer.

TβL1/TβLR1 SUMOylation induces their dissociation from the Nuclear hormone receptor Co-Repressor (NCoR) / Silencing Mediator of Retinoic acid and Thyroid hormone receptor (SMRT) complex and translocation to the cytoplasm. Cytoplasmic stabilization of β-catenin by Wnt promotes the formation of β-catenin/SUMO-TβL1/SUMO-TβLR1 complexes, translocation to the nucleus and transactivation of gene transcription. Adapted from Choi *et al.*, (2011).

Materials and Methods

Clinical Specimen Collection

Surgically resected palmar fascia tissue samples were obtained from patients with Dupuytren's Disease (DD) and patients undergoing surgery for carpal-tunnel release (CT) at the XXX hospital. All patients signed consent forms allowing their tissues to be used for research, and the XXX Research Ethics Board approved sample collection for Health Sciences Research involving Human Subjects (XXX). Lab numbers were assigned to samples prior to processing in order to meet patient de-identification and confidentiality agreements.

Primary Cell Culture

DD fibroblasts were extracted from the palmar fascia of DD patients, while the palmar fascia from patients without any history of DD undergoing carpal tunnel release was used to extract phenotypically normal fibroblasts (CT fibroblasts) for use as allogeneic controls. Tissue samples were dissected and placed onto 100 mm culture dishes containing α -MEM medium (Life Technologies) supplemented with 1% antibiotic-antimycotic solution (Life Technologies), 1% L-Glutamine (Life Technologies), and 10% fetal bovine serum (FBS; Life Technologies) at 37°C in 5% CO₂. The cellular outgrowths from tissue fragments were then passaged routinely by trypsinization using 0.25% trypsin (Gibco). All primary cell cultures were used up to a maximum of seven passages, where afterward they were discarded.

Cytokine Treatments

DD and CT fibroblasts were seeded at a density of 2.2×10^6 cells in 100 mm dishes and serum-starved in serum-free α -MEM media for at least 24 hours prior to treatment with human pro-inflammatory cytokine mixtures (Cytomix 1), or human anti-inflammatory cytokine mixtures (Cytomix 2). The human cytokines comprising Cytomix 1 and 2 were purchased from PeproTech. Cytomix 1 was comprised of tumour necrosis factor (TNF), interleukin 1 beta (IL-1 β), and interferon gamma (IFN- γ) at a stock concentration of 1 ug/mL. Cytomix 2 was comprised of interleukin-4 (IL-4), interleukin 10 (IL-10), and transforming growth factor beta (TGF β 1), at a stock concentration of 1 ug/mL. DD and CT fibroblasts were treated separately at 24 and 48h with Cytomix 1 or Cytomix 2, at a dose of 0.5 ng/ml.

Antibodies

The following primary antibodies were used: rabbit polyclonal SUMO1 antibody (ab11672, Abcam, 1:100 for proximity ligation assays), mouse monoclonal T β L1 antibody (sc-137083, SantaCruz 1:150 for immunocytofluorescence and proximity ligation assays), mouse monoclonal T β LR1 antibody (ab117761, Abcam, 1:150 for immunocytofluorescence and proximity ligation assays), rabbit monoclonal non-phosphorylated active β -catenin antibody (D13A1, Cell Signalling Technology, 1:1000 for immunoblotting; 1:200 for proximity ligation assays). The following secondary antibodies for western immunoblotting were used: horseradish peroxidase (HRP)-linked horse anti-mouse IgG (#7076, Cell Signaling Technology, 1:1000), and HRP-linked goat anti-rabbit IgG (#7074, Cell Signaling Technology, 1:1000). For

immunocytofluorescence, the following secondary antibodies were used: anti-rabbit Alexa Fluor 568 (Molecular Probes, 1:400) and anti-mouse Alexa Fluor 488 (Molecular Probes, 1:400).

Cell Lysate Preparation

Cell pellets stored at -80°C were resuspended in RIPA cell lysis buffer (Teknova) supplemented with protease inhibitor cocktail (Sigma-Aldrich), 0.1M sodium fluoride, 10 mM sodium orthovanadate, and 10 mM phenylmethane sulfonyl fluoride (PMSF). Resuspended cells were then needle aspirated five times and placed on ice for 30 minutes. Cell lysates were centrifuged at 5000 RPM to remove insoluble material, and total protein concentrations were determined by Bicinchoninic Assay (BCA, ThermoScientific). Protein lysates were used directly for western immunoblotting.

Western Immunoblotting and Densitometry Analysis

Protein samples were separated using stain-free Mini Protean TGX precast gels (BioRad), and transferred onto a nitrocellulose membrane using the Invitrogen iBlot. Membranes were visualized by chemiluminescence on a Chemidoc XRS+ (BioRad). Band intensity was normalized using total protein lane quantification, using the total protein imaged on the stain-free membrane²⁵. Relative densitometry analysis was performed using ImageLab 6.0 (BioRad).

Immunocytofluorescence

Cells were fixed with 4% paraformaldehyde using Nunc Lab-Tek 8-well chamber slides (ThermoFisher) at 60-70% confluency. Cells were washed with phosphate-buffered saline (PBS) three times for five minutes and permeabilised by incubating in 0.1% Triton X-100 in PBS for 30 minutes. Samples were blocked for ten minutes (Background Sniper, Biocare Medical) and then

rinsed three times for five minutes in PBS. All incubations were completed at room temperature. Primary antibodies were then added for overnight incubation at 4°C. Alexa Fluor 488 or 568 secondary antibodies were added to the cells for one hour in PBS containing 1% bovine serum albumin (BSA) and 0.1% Tween-20 detergent, and subsequently washed in PBS for three times for five minutes in PBS. Glass slides were then mounted onto the chamber slides with Duolink mounting media containing DAPI counterstain (Sigma-Aldrich).

Images were captured on a Nikon A1R+ hybrid resonant/galvano point scanning confocal microscope 20X objective lens, using the NIS Elements AR software for data acquisition at room temperature. The Alexa Fluor 568 was excited with the 15 MW 561 nm diode-pumped solid-state laser, while the Alexa Fluor 488 was excited with the 15 MW 488 nm diode laser. The DAPI signal was excited using the 15 MW 405 nm diode laser. The system is equipped with four Photomultiplier tubes for standardized fluorescence detection.

Proximity Ligation Assays (PLA)

Cells were fixed with 4% paraformaldehyde using Nunc Lab-Tek 8-well chamber slides (ThermoFisher) at 60-70% confluency. Cells were washed with phosphate-buffered saline (PBS) three times for five minutes and permeabilised by incubating in 0.1% Triton X-100 in PBS for 30 minutes. Cells were then rinsed with three times for five minutes in PBS and then treated with blocking solution (Duolink Sigma-Aldrich) for 60 minutes at 37°C. All incubations were completed at room temperature. Primary antibodies were then added for overnight incubation at 4°C. Combinations of each primary antibody are as follows: i) β -catenin rabbit polyclonal Cell-Signalling technology 9562L (1:200) and T β L1 mouse monoclonal Santa Cruz H-11 (1:100), ii) β -catenin rabbit polyclonal Cell-Signalling technology 9562L (1:200) and T β LR1 mouse

monoclonal Abcam 117761 (1:200), iii) T β L1 mouse monoclonal Santa Cruz H-11 (1:100) and SUMO1 rabbit polyclonal Abcam 11672 (1:400), iv) T β LR1 mouse monoclonal Abcam 117761 (1:200) and SUMO1 rabbit polyclonal Abcam 11672 (1:400). The secondary probes were diluted 1:5 in the antibody diluent (Duolink Sigma-Aldrich) and incubated for one hour at 37°C. The species of the secondary probes used were Anti-Rabbit PLUS and Anti-mouse MINUS (Duolink Sigma-Aldrich). The subsequent ligation and amplification reactions were performed according to the manufacturer's instructions. Cells were then mounted on slides with mounting media containing DAPI counterstain (Sigma-Aldrich).

Images were captured on a Nikon A1R+ hybrid resonant/galvano point scanning confocal microscope 60X objective lens, using the NIS Elements AR software for data acquisition. Five regions were randomly selected per slide and Z-stack images were captured for quantitative analysis

Three-Dimensional Analysis of PLA Signals

The 3D analysis was performed using NIS Elements AR software. The default 3D-thresholding function was used to create an iso-surface of the nuclear (DAPI) signals. The PLA signals were then iso-surfaced using the default 3D spot-detection function. PLA interactions per cell were calculated by dividing the total number detected PLA signals by the number of cells in each image (based on DAPI signal).

Statistical Analyses

Statistical analyses were conducted using GraphPad Prism 7 statistical software. For the immunoblotting experiments, ordinary one-way Analysis of Variance (ANOVA) was used in

order to determine any significant treatment effects at similar time points for the immunoblotting experiments, where significance was designated at $p < 0.05$. When significance was detected, post-hoc analyses were performed using the Dunnett's test to compare the effect of each treatment at a specific time point with a control group at the same time point. For the PLA experiments, the unpaired T-test was used to determine any significant treatment effects between treatment and control, where significance was designated at $p < 0.05$.

Results

Fibrosis-associated cytokines enhance β -catenin protein levels in DD and CT fibroblasts

As with other fibroproliferative disorders, DD is the result of an exaggerated, abnormal wound healing response to chronic inflammation⁷. Therefore, we created cell culture conditions that would best mimic the chronic inflammatory environment in order to maximize and stabilize β -catenin levels.

To mimic the pro-inflammatory phase of tissue repair, in which chronic activation is associated with cytotoxicity and damage¹⁴, we treated DD and CT fibroblasts with a pro-inflammatory cytokine mixture consisting of TNF (tumour necrosis factor), IL1 β (interleukin-1 beta), IFN γ (interferon gamma), termed “Cytomix 1”. To mimic the anti-inflammatory phase of tissue repair, where inflammation is dampened and tissue remodeling is initiated¹⁴, we treated DD and CT fibroblasts with an anti-inflammatory cytokine mixture consisting of IL4 (interleukin 4), IL10 (interleukin 10), and TGF β (transforming growth factor beta), termed “Cytomix 2”. The concentrations of our cytokine mixtures were dosed at 0.5 ng/ml, approximating the physiological levels detected in fresh DD tissue¹⁵. There were no noticeable differences in the levels of active β -catenin when treated with Cytomix 1 and 2 at 24 hours, in both DD and CT fibroblasts (Figure 3B, 4B, respectively). A modest 2-fold increase in β -catenin levels was observed after Cytomix 1 treatment at 48 hours in DD fibroblasts (Figure 3D), however CT fibroblasts remained relatively unaffected (Figure 4D). After 48-hour Cytomix 2 treatment, we observed in DD fibroblasts significant 3-fold increase in β -catenin levels (Figure 3D), and a significant 2-fold increase in β -catenin levels in CT fibroblasts (Figure 4D). Taken together,

exposing DD and CT fibroblasts to Cytomix 2 successfully increased β -catenin levels, relative to Cytomix 1 treatments.

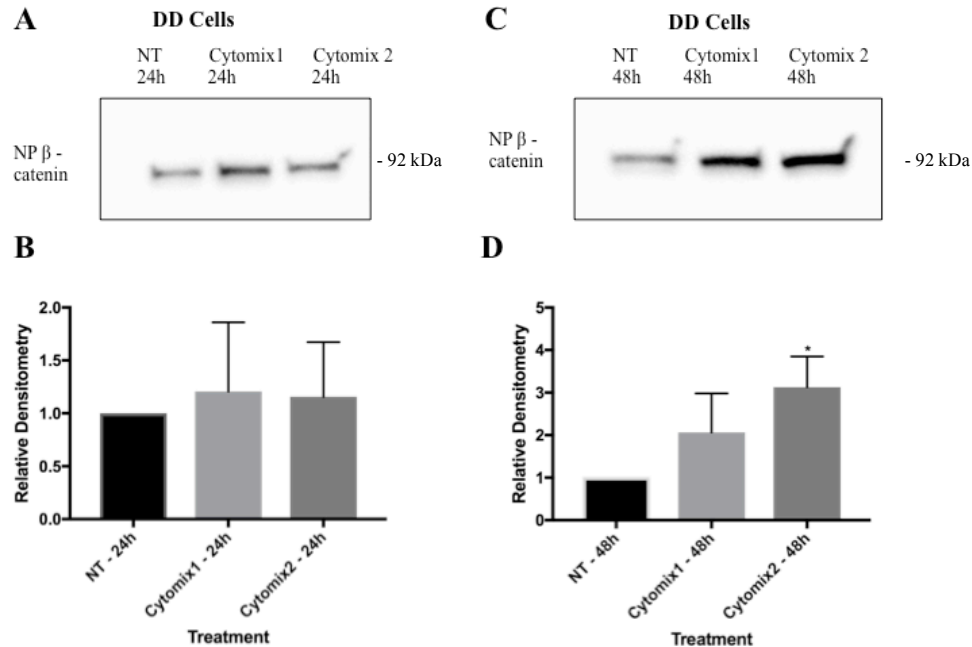


Fig 3. Anti-inflammatory cytokines (Cytomix 2) increase levels of β -catenin in DD fibroblasts.

(A, C) Immunoblots for active non-phosphorylated (Ser33/37/Thr41) β -catenin in DD fibroblasts. All groups were treated cultured in serum-free media for 24 hours prior to treatment. Treatments: no treatment for 24 hours or 48 hours (NT); TNF, IL1 β , IFN γ (Cytomix1) (0.5 ng/ml) added for 24 or 48 hours; IL4, IL10, TGF β 1 (Cytomix2) (0.5 ng/ml) added for 24 or 48 hours. Total protein imaged on the stain-free membrane was used as a loading control to normalize band intensity. Results are shown from the same probed blot.

(B, D) Relative densitometry analysis for active β -catenin in DD fibroblasts treated with Cytomix 1 or 2 at 24 and 48 hours, respectively. Data derived from one patient (N=1) assessed in triplicate (n=3). Total lane protein quantification was used to normalize band intensity. Densitometry analysis was tested by one-way ANOVA followed by Dunnett's multiple comparison test. Asterisks indicate significant differences to control (*P<0.05, *** P<0.0002). Values shown are mean +/- SD.

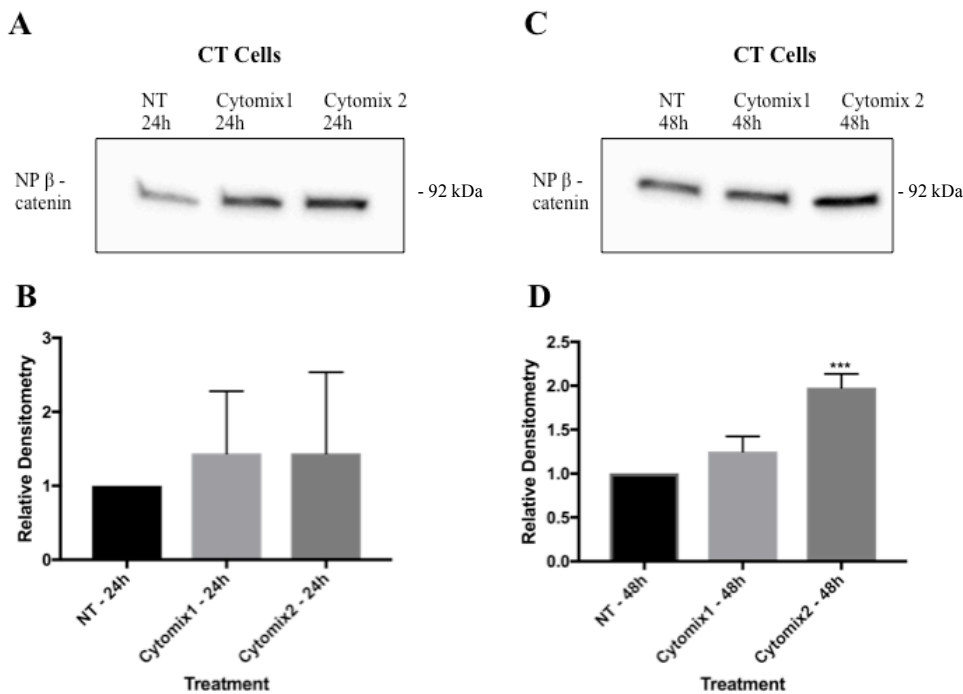


Fig 4. Anti-inflammatory cytokines increase levels of β -catenin in CT fibroblasts.

(A, C) Immunoblots for active non-phosphorylated (Ser33/37/Thr41) β -catenin in CT fibroblasts. All groups were cultured in serum-free media for 24 hours prior to treatment. Treatments: no treatment for 24 hours or 48 hours (NT); TNF, IL1 β , IFN γ (Cytomix1) (0.5 ng/ml) added for 24 or 48 hours; IL4, IL10, TGF β 1 (Cytomix2) (0.5 ng/ml) added for 24 or 48 hours. Total protein imaged on the stain-free membrane was used as a loading control to normalize band intensity. Results are shown from the same probed blot.

(B, D) Relative densitometry analysis for active β -catenin in CT fibroblasts treated with Cytomix 1 or 2 at 24 and 48 hours, respectively. Data derived from one patient (N=1) assessed in triplicate (n=3). Total lane protein quantification was used to normalize band intensity. Densitometry analysis was tested by one-way ANOVA followed by Dunnett's multiple comparison test. Asterisks indicate significant differences to control (*P<0.05, *** P<0.0002). Values shown are mean +/- SD.

Cytoplasmic localizations of T β L1 are increased in DD fibroblasts relative to CT fibroblasts

To assess whether the SUMOylated T β L1/T β LR1 complex facilitates β -catenin nuclear translocation in DD fibroblasts, the cytoplasmic localization of T β L1 was visualized using immunofluorescence confocal microscopy. Primary mouse monoclonal antibodies to T β L1 and Alexa Fluor 568 secondary antibodies were used to visualize the cytoplasmic and nuclear localization of T β L1 by immunofluorescence confocal microscopy. As demonstrated in Figure 5A, both cytoplasmic and nuclear localization of T β L1 was evident in DD fibroblasts, while T β L1 localization was found to be predominantly nuclear in CT fibroblasts, as seen in Figure 5B.

T β LR1 localization is cytoplasmic and nuclear in both DD and CT fibroblasts

To assess whether the SUMOylated T β L1/T β LR1 complex facilitates β -catenin nuclear translocation in DD fibroblasts, the cytoplasmic localization of T β LR1 was visualized using immunofluorescence confocal microscopy. Primary mouse monoclonal antibodies to T β LR1 and Alexa Fluor 568 secondary antibodies were used to visualize T β LR1 localization by confocal microscopy. As demonstrated by Figure 6A and 6B, T β LR1 localization was both cytoplasmic and nuclear in DD and CT fibroblasts, respectively.

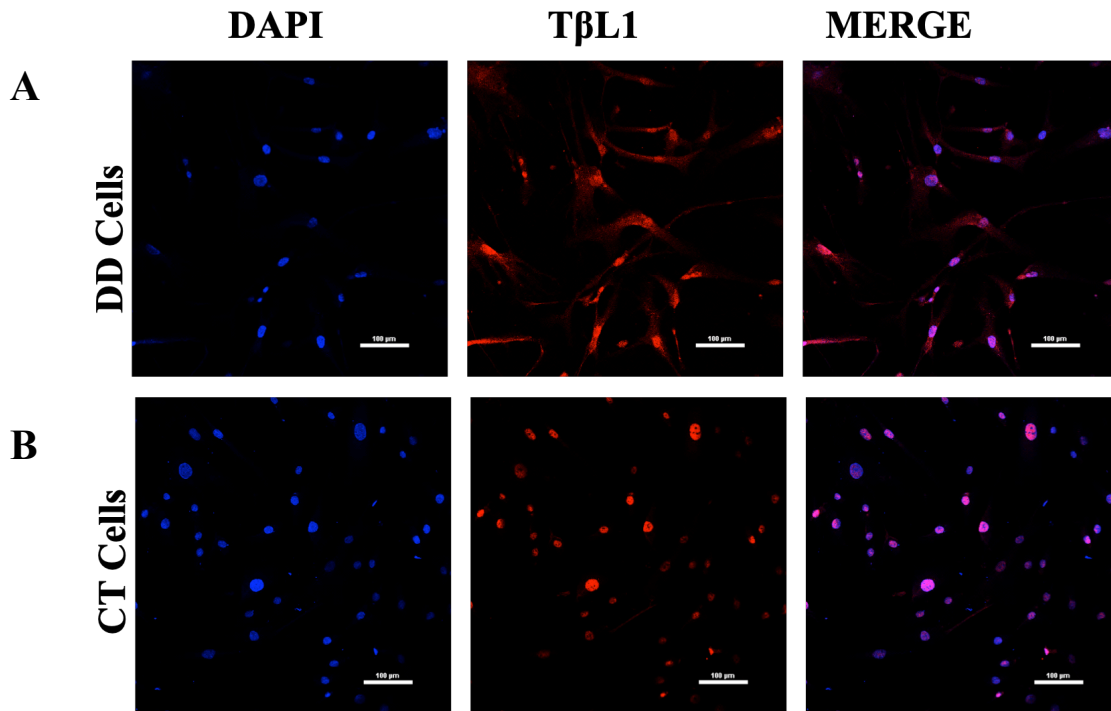


Fig 5. TβL1 cellular localization is more cytoplasmic in DD fibroblasts relative to CT fibroblasts.

(A, B) DD and CT fibroblasts, respectively, were stained with a mouse monoclonal anti-TβL1 antibody and the secondary antibody Alexa Fluor anti-mouse 568. Images were captured by confocal microscopy using a 20X objective lens. Scale bars: 100 microns.

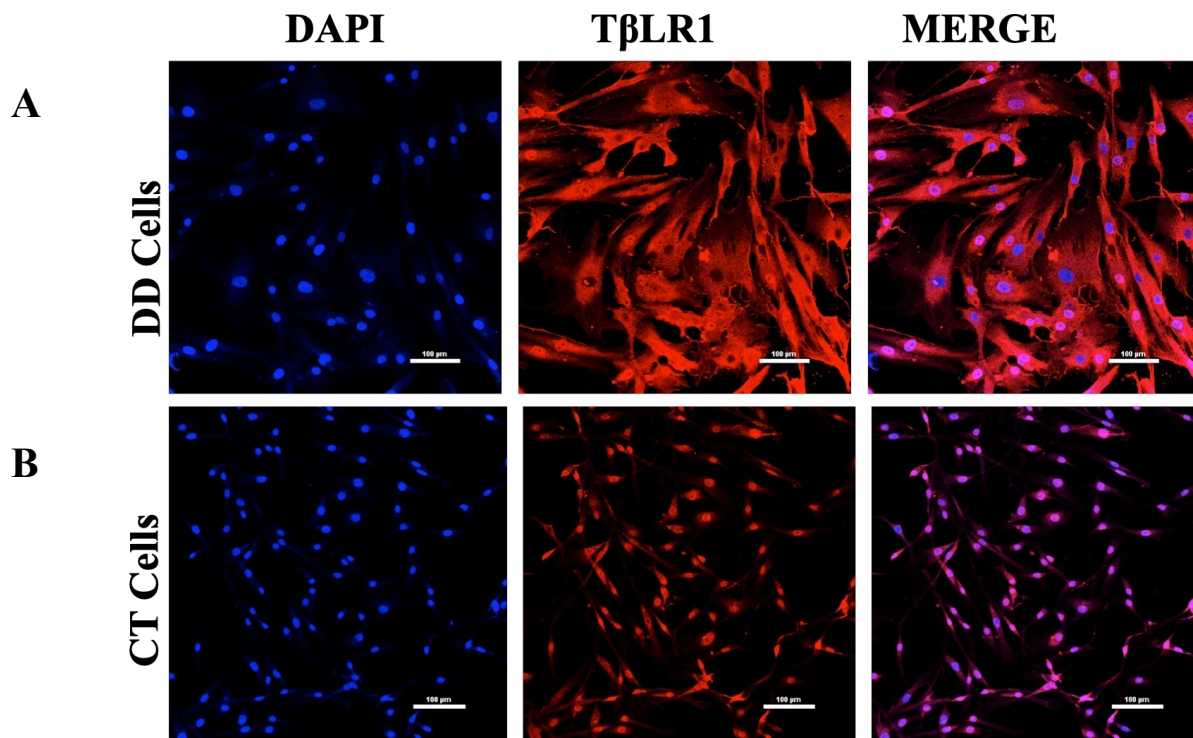


Fig 6. T β LR1 cellular localization is cytoplasmic and nuclear in both DD and CT fibroblasts.

(A, B) DD and CT fibroblasts, respectively, were stained with a mouse monoclonal anti-T β LR1 antibody, and secondary antibody Alexa Fluor anti-mouse 568. Images were captured by confocal microscopy using a 20X objective lens. Scale bars: 100 microns.

Cytokine treatments enhance PLA signals for SUMO1 and TβL1 in both DD and CT fibroblasts, but not for SUMO1 and TβLR1

To assess whether the SUMOylated TβL1/TβLR1 complex is active in DD and CT fibroblast, and whether the formation of these complexes changes in response to anti-inflammatory cytokines, we conducted PLAs to determine proximity (<40 nm) between TβL1 and SUMO1, as well as TβLR1 and SUMO1 by confocal microscopy. To maximize and stabilize β-catenin levels and effectively simulate the tissue repair environment *in vitro*, DD and CT fibroblasts were treated with Cytomix 2 for 48 hours. PLA signals for TβL1 and SUMO1 were significantly increased in both DD and CT fibroblasts (P = 0.0002) upon treatment with Cytomix 2 for 48 hours (Figure 7). However, PLA signals for TβLR1 and SUMO1 remained unchanged in both DD and CT fibroblasts upon treatment with Cytomix 2 for 48 hours (Figure 8).

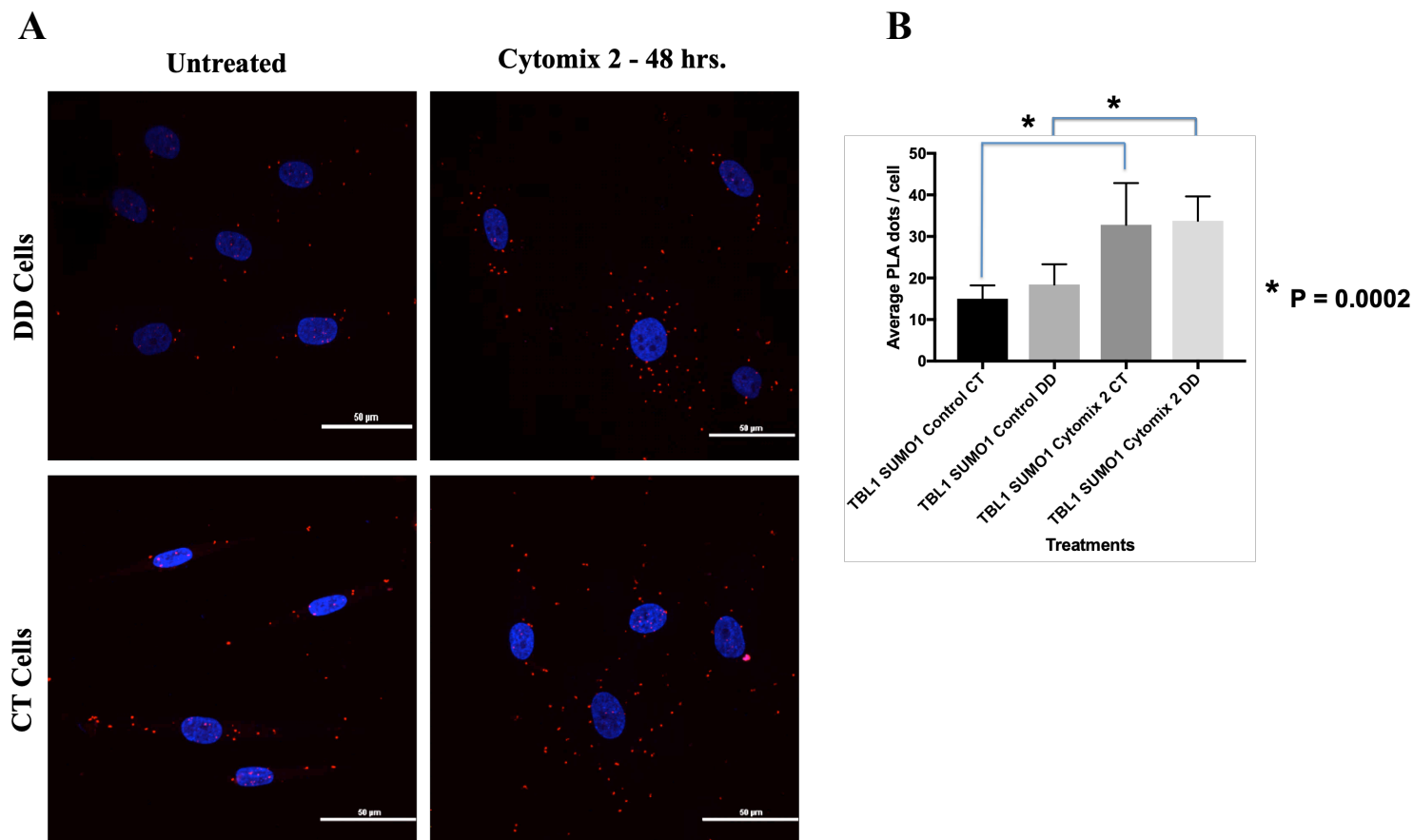


Fig 7. Interactions between TβL1 and SUMO1 are increased in DD and CT fibroblasts in response to Cytomix 2.

(A): Transducin β-like 1 (TβL1) and small ubiquitin-like modifier 1 (SUMO1) are visualized by proximity ligation assay (PLA). Representative images are shown where PLA signal (red) indicates close proximity (<40 nm) between two proteins. DD and CT fibroblasts were treated with Cytomix 2, consisting of interleukin-4, interleukin-10, and transforming growth factor-1 beta. Images were captured by confocal microscopy using a 60X objective lens. Scale bars: 50 microns.

(B): Quantification of PLA signal. Data derived from one patient (N=1; n=5). An unpaired t-test was performed to test for significance between control and Cytomix 2 treatment. Values shown are mean +/- SD.

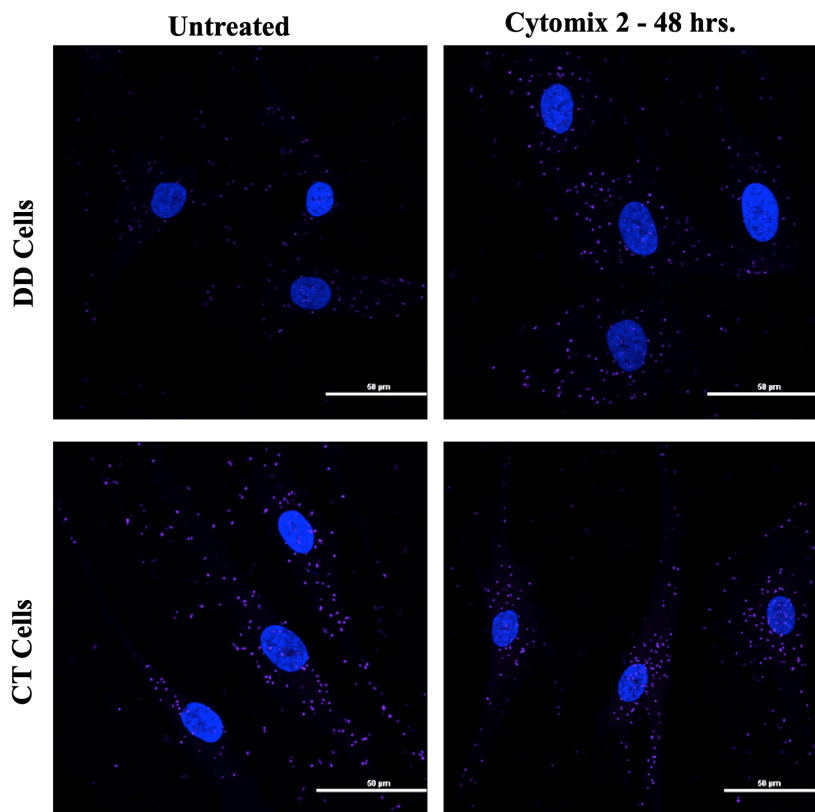
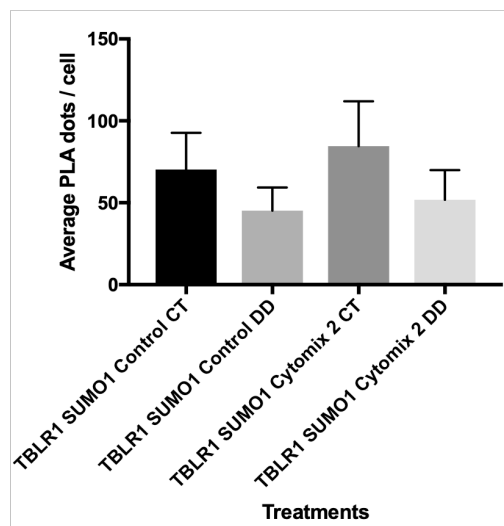
A**B**

Fig 8. Interactions between T β LR1 and SUMO1 are unchanged in DD and CT fibroblasts in response to Cytomix 2.

(A): Transducin β -like receptor-1 (T β LR1) and small ubiquitin-like modifier 1 (SUMO1) are visualized by proximity ligation assay (PLA). Representative images are shown where PLA signal (purple) indicates close proximity (<40 nm) between two proteins. DD and CT fibroblasts were treated with Cytomix 2, consisting of interleukin-4, interleukin-10, and transforming growth factor-1 beta. Images were captured by confocal microscopy using a 60X objective lens. Scale bars: 50 microns.

(B): Quantification of PLA signal. Data derived from one patient (N=1; n=5). An unpaired t-test was performed to test for significance between control and Cytomix 2 treatment. Values shown are mean \pm SD.

Cytokine treatments enhance PLA signals for β -catenin and T β L1 as well as β -catenin and T β LR1 in both DD and CT fibroblasts

To assess whether the SUMOylated T β L1/T β LR1 complex is active in DD and CT fibroblasts, and whether these complexes interact with β -catenin, PLAs were conducted to determine proximity (<40 nm) between β -catenin and T β L1 as well as β -catenin and T β LR1 by confocal microscopy. To maximize and stabilize β -catenin levels and effectively simulate the tissue repair environment *in vitro*, DD and CT fibroblasts were treated with Cytomix 2 for 48 hours. PLA signals for T β L1 and β -catenin were significantly increased in both DD and CT fibroblasts in response to Cytomix 2 treatment for 48 hours ($P = 0.0003$) (Figure 9). PLA signals for T β LR1 and β -catenin were also significantly increased in both DD and CT fibroblasts in response to Cytomix 2 treatment for 48 hours ($P < 0.02$) (Figure 10).

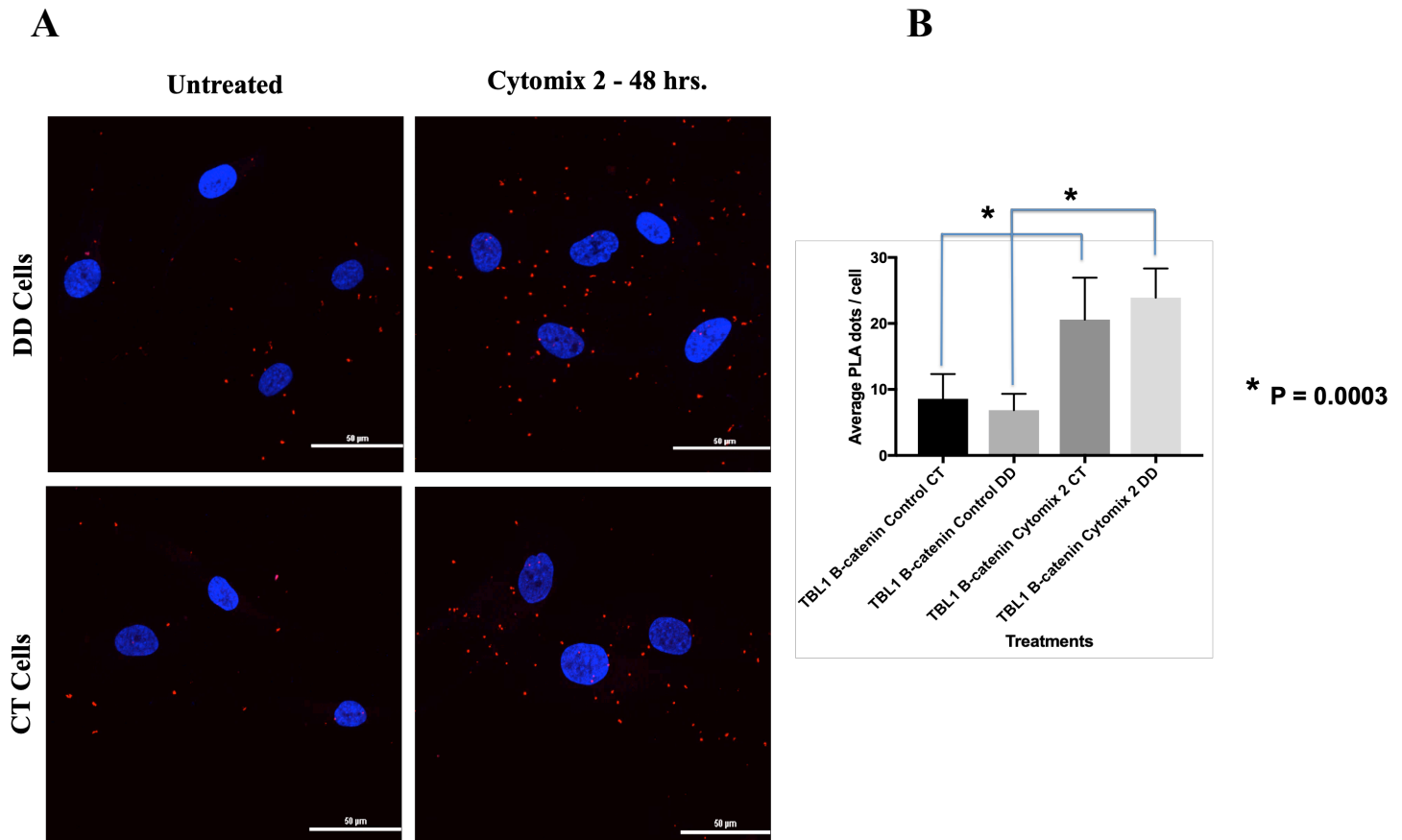


Fig 9. Interactions between TβL1 and β-catenin are increased in DD and CT fibroblasts in response to Cytomix 2.

(A): Transducin β-like 1 (TβL1) and β-catenin are visualized by proximity ligation assay (PLA). Representative images are shown where PLA signal (red) indicates close proximity (<40 nm) between two proteins. DD and CT fibroblasts were treated with Cytomix 2, consisting of interleukin-4, interleukin-10, and transforming growth factor-1 beta. Images were captured by confocal microscopy using a 60X objective lens. Scale bars: 50 microns.

(B): Quantification of PLA signal. Data derived from one patient (N=1; n=5). An unpaired t-test was performed to test for significance between control and Cytomix 2 treatment. Values shown are mean +/- SD.

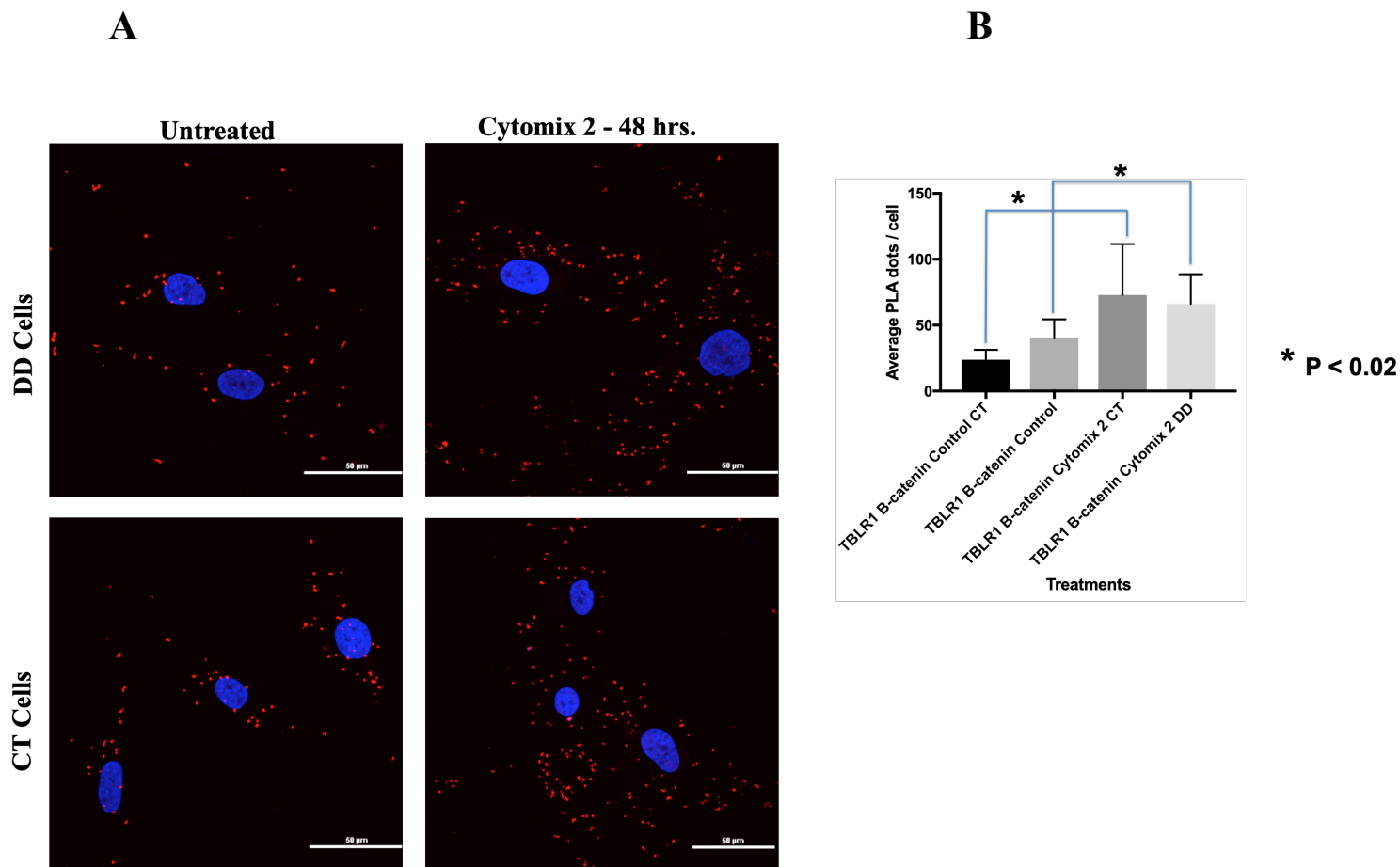


Fig 10. Interactions between TβLR1 and β-catenin are increased in DD and CT fibroblasts in response to Cytomix 2.

(A): Transducin β-like receptor-1 (TβLR1) and β-catenin are visualized by proximity ligation assay (PLA). Representative images are shown where PLA signal (red) indicates close proximity (<40 nm) between two proteins. DD and CT fibroblasts were treated with Cytomix 2, consisting of interleukin-4, interleukin-10, and transforming growth factor-1 beta. Images were captured by confocal microscopy using a 60X objective lens. Scale bars: 50 microns.

(B): Quantification of PLA signal. Data derived from one patient (N=1; n=5). An unpaired t-test was performed to test for significance between control and Cytomix 2 treatment. Values shown are mean +/- SD.

Discussion

In this study, we investigated whether any parallels existed with respect to abnormal β -catenin signalling in DD relative to cancer, in order to assess the feasibility and potential of cross-purposing anti-cancer drugs to target β -catenin nuclear translocation in fibroproliferative diseases. We determined that the cellular localization of T β L1 and T β LR1 are primarily cytoplasmic in DD fibroblasts— translating into an increased availability for the formation of T β L1/T β LR1/ β -catenin complexes that can then translocate into the nucleus relative to CT fibroblasts. Furthermore, in response to treatment with anti-inflammatory cytokines, DD and CT fibroblasts both demonstrated increased levels of SUMOylated T β L1 and T β LR1, as well as higher levels of T β L1 and T β LR1 interactions with β -catenin.

To maximize and stabilize β -catenin levels, DD and CT fibroblasts were treated with fibrosis-associated cytokines to mimic chronic inflammation. DD fibroblasts demonstrated higher sensitivity to 48-hour Cytomix 1 treatment, as a modest two-fold increase in β -catenin levels was observed (Figure 3D) relative to CT fibroblasts (Figure 4D). As mentioned previously, DD is a hyperproliferative disorder resulting from an exaggerated, abnormal wound healing response to chronic inflammation⁷. The heightened sensitivity of DD fibroblasts to pro-inflammatory cytokines in Figure 3D supports this hypothesis. In response to 48-hour Cytomix 2 treatment, DD fibroblasts experienced a significant 3-fold increase in β -catenin levels (Figure 3D), and CT fibroblasts experienced a significant 2-fold increase (Figure 4D). The increase in β -catenin levels observed after Cytomix 2 treatment may be attributed to TGF β , as previous studies determined that fibroblasts treated with TGF β experienced an increase in β -catenin levels^{13, 17}.

TGF β is an important anti-inflammatory cytokine during the wound healing process and is linked to β -catenin signalling to modulate wound repair and tissue remodeling¹⁸.

We determined that upon treatment with Cytomix 2 —consisting of the anti-inflammatory fibrosis-associated cytokines IL4, IL10, and TGF β 1— resulted in increased levels of SUMOylated T β L1 in DD and CT fibroblasts (Figure 7). Furthermore, interactions between T β L1 and T β LR1 with β -catenin were increased in DD and CT fibroblasts upon treatment with Cytomix 2 (Figures 9 & 10). To our knowledge, the facilitation of β -catenin nuclear translocation by the SUMOylated T β L1/T β LR1 has only been studied in the context of cancer cells. Our novel findings implicating β -catenin nuclear translocation by SUMOylated T β L1/T β LR1 have not yet been studied in the context of fibrosis and chronic inflammation. Based on these new findings, along with the role that TGF β plays in modulating wound repair and tissue remodelling^{16, 17, 18} — we hypothesize that the anti-inflammatory cytokines IL4, IL10, and TGF β 1 may contribute to the transition between the inflammation and proliferation phases in both normal and fibrotic tissue repair. More specifically, the anti-inflammatory cytokines may lead to increased interactions between SUMOylated T β L1/T β LR1 and β -catenin, thereby resulting in the β -catenin-mediated transactivation of genes involved in cellular proliferation and wound healing during normal and abnormal tissue repair (Figure 11).

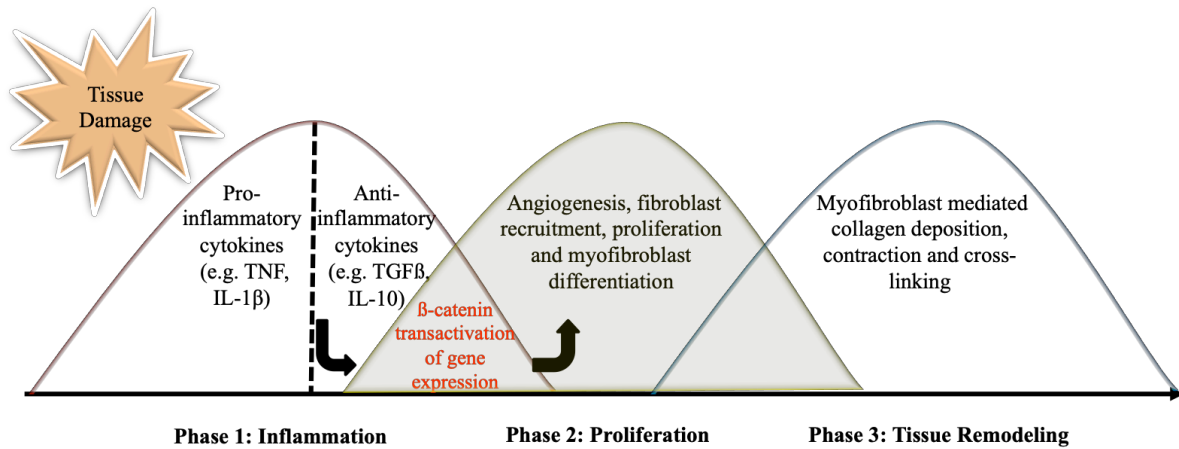


Fig 11. The presence of anti-inflammatory cytokines during the transition from the inflammation to proliferation phase of wound healing mediates β -catenin nuclear translocation as a result of the SUMOylated T β L1/T β LR1 complex.

Limitations and Future Directions

We have presented here evidence that SUMOylated T β L1 and SUMOylated T β LR1 exist at higher levels in both DD and CT fibroblasts upon treatment with anti-inflammatory cytokines, which correlates with increased protein levels of β -catenin. Additionally, T β L1 and T β LR1 interactions with β -catenin are also more abundant as a result. PLAs were used to detect these protein-protein interactions, as this technique detects proximity between two proteins that is < 40 nm. However, it is important to detect these interactions with other techniques such as immunoprecipitation, in order to validate our PLA findings. Additionally, in order to directly confirm the mechanisms behind BC-2059 and its specificity towards targeting specific β -catenin interactions with SUMOylated T β L1 and T β LR1 — it is important to test whether interactions between T β L1 and T β LR1 with β -catenin are effectively disrupted in drug-treated DD fibroblasts.

The protein-protein interactions were primarily detected in fibroblasts cultured *in vitro*, and this may present a limitation of this study. Although the levels of β -catenin were stabilized through treatments with fibrosis-associated cytokines in order to mimic the tissue repair environment and maintain physiological relevance — these findings can be strengthened by moving towards an *ex vivo* experimental approach. Performing immunostaining and PLAs in fresh palmar fascia tissue *ex vivo* may strengthen our findings by providing increased physiological relevance²⁷.

Conclusion

In summary, we have presented evidence of the specific mechanisms that may be involved in β -catenin nuclear translocation during the anti-inflammatory phase of tissue repair. Our novel findings present the SUMOylated T β L1/T β LR1 complex as a new therapeutic target for DD and other fibroproliferative diseases. Small molecule inhibitors such as BC2059 that disrupt interactions between SUMOylated T β L1/T β LR1 and β -catenin are in development for the treatment of cancer and are already undergoing phase-I clinical trials²⁸. Our observations here show that similar mechanisms may be involved during the abnormal tissue repair process associated with the development of DD. At present, there are no therapies that focus on targeting early disease development and preventing post-surgical disease recurrence in DD. The results of this study identify SUMOylated T β L1/T β LR1 interactions with β -catenin as a novel therapeutic target for DD and other fibroproliferative diseases.

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