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TRPV2 suppresses Rac1 and RhoA activation and invasion in rheumatoid arthritis fibroblast-like synoviocytes

Teresina Laragione, Carolyn Harris, Percio S. Gulko*

Division of Rheumatology, Department of Medicine, Icahn School of Medicine at Mount Sinai, New York, NY, 10029, USA

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ABSTRACT

The TRPV2 cation channel has been recently implicated in the regulation of arthritis severity, joint damage, and in the invasive behavior of the fibroblast-like synoviocyte (FLS). However, its mechanism of action was unknown. In this study we characterize the cell signaling events mediating the TRPV2 suppressive activity in FLS invasiveness. Studies with FLS cell lines derived from patients with RA revealed that TRPV2-specific stimulation significantly reduced FLS adhesion to different extracellular matrices that shared binding to αv, β1 and β3 integrins. Localization of these integrins to the plasma membrane and numbers of thick and organized actin filaments were diminished by TRPV2 specific stimulation, and cells developed a round and non-polarized morphology. TRPV2 stimulation significantly reduced levels of activated RhoA, Rac1 and cofilin. RhoA activators were able to overcome the TRPV2-induced suppression on both RhoA activation and invasion. These new discoveries suggest that TRPV2 regulates key intracellular processes implicated in cell invasion in arthritis and other processes such as cancer, and has the potential to become a useful target for drug development.

1. Introduction

Rheumatoid arthritis (RA) affects nearly 1% of the population [29] and is associated with increased risk for disability, for reduced quality of living, as well as reduced survival [34]. New and more effective therapies have significantly improved disease control, yet remission is rarely achieved underscoring the need for better treatments. Current treatments for RA target the immune response, rendering patients increasingly susceptible to infections. Better understanding the disease pathogenesis, particularly the regulation of arthritis severity and articular damage has the potential to generate new and better targets for therapies aimed at improving disease control and outcome.

Fibroblast-like synoviocytes (FLS) have a central role in the RA synovial hyperplasia and disease pathogenesis [1]. The numbers of FLS are significantly increased in synovial tissues of RA patients and have an aggressive and invasive behavior resembling that of cancer cells, invading and destroying cartilage and bone and releasing pro-inflammatory cytokines, chemokines and Matrix Metallaproteinases (MMPs) [1,19]. While the RA FLS behavior is not completely understood, its *in vitro* invasive properties correlate with radiographic damage in RA [33] and with histologic changes in rodent models of arthritis [12]. Therefore, understanding the regulation of FLS invasiveness has the potential to generate new therapies aimed at reducing articular damage and improving disease control [8,14].

We have previously determined that the non-specific cation channel TRPV2 (transient receptor potential vanilloid subfamily, type 2 channel) is expressed in RA FLS and acts as a suppressor of cell invasion [13]. TRPV2 is a non-selective cation channel belonging to the TRPV family, a multifunctional set of membrane proteins implicated in sensory functions between cells and their environment [22]. TRPV channels are activated by different physical or chemical stimuli. However, human and mouse TRPV2 do not have any specific roles in sensing thermo or painful stimuli [20,21]. *In vitro* TRPV2 activation by synthetic agonists suppressed the invasiveness of RA FLS and decreased expression levels of MMP2 and MMP3 [13]. *In vivo* TRPV2 activation decreased disease severity in both collagen-induced arthritis (CIA) and KRN serum-induced arthritis in mice, and reduced numbers of synovial-infiltrating inflammatory cells and numbers of synovial vessels (Angiogenesis) [13].

In this study we characterized the cell signaling events mediating the TRPV2 suppressive activity in RA FLS and discovered a new role for this channel in the regulation of integrins and small-GTPases.

* Corresponding author at: Division of Rheumatology, Icahn School of Medicine at Mount Sinai, One Gustave L. Levy Place, Box 1244, New York, NY 10029, USA.
E-mail address: percio.gulko@mssm.edu (P.S. Gulko).

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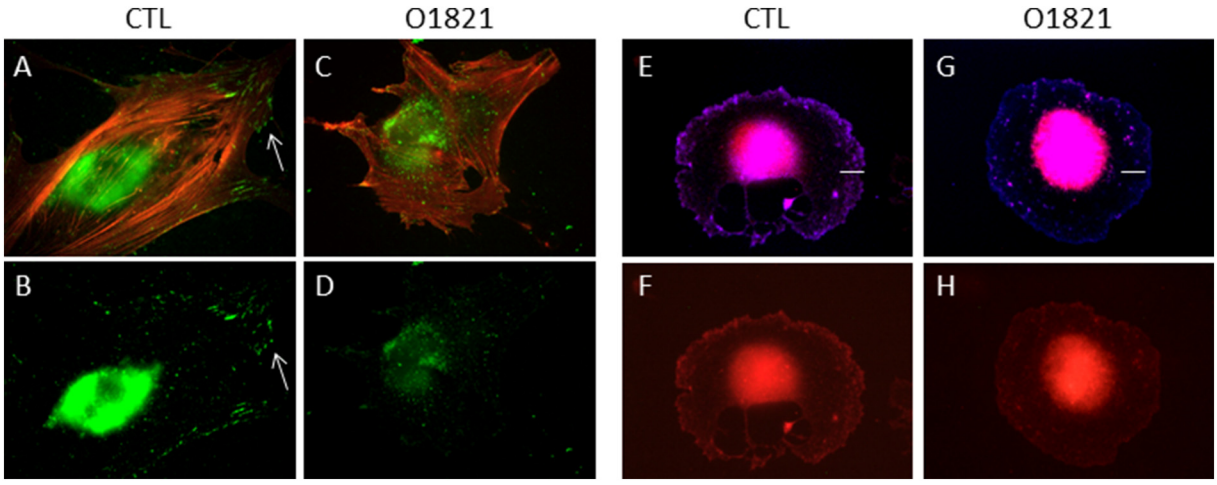

Fig. 1. O1821 inhibits cell elongation, lamellipodia formation and decreases TRPV2 localization at the plasma membrane. (A–B) RA FLS plated on plastic coverslips and pre-treated 10% FBS with vehicle had elongated morphology, thick actin fibers and polarized formation of lamellipodia (arrows), while (C–D) O1821-treated cells had a round morphology with disorganized actin fibers and no lamellipodia. Red: phalloidin staining, green: p-FAK staining. (RA FLS, n = 4). (E–F) RA-FLS were pre-treated with FBS 10% with vehicle had increased levels of TRPV2 at the cell periphery/plasma membrane. (G–H) O1821-treated cells had significantly reduced localization of TRPV2 at the cell periphery. Red: TRPV2 staining, blue: phalloidin staining, white bar points to the difference in the amount of TRPV2 localization to the cell periphery/plasma membrane (RA FLS n = 3; representative cells are shown at 600× magnification). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2. Results

2.1. Activation of TRPV2 interfered with cytoskeleton and lamellipodia formation in RA FLS

RA FLS cultured in 10% FBS had the typical morphology of invading cells characterized by an elongated shape, thick actin stress fibers, and lamellipodia present at the edge of the cells and co-localizing with pFAK (n = 4 different RA FLS cell lines; Fig. 1A–B). RA FLS treated with O1821 became round-shaped, with decreased numbers of thick actin filaments and increased presence of thinner and disorderly distributed filaments. pFAK was predominantly present in the center of the cell and

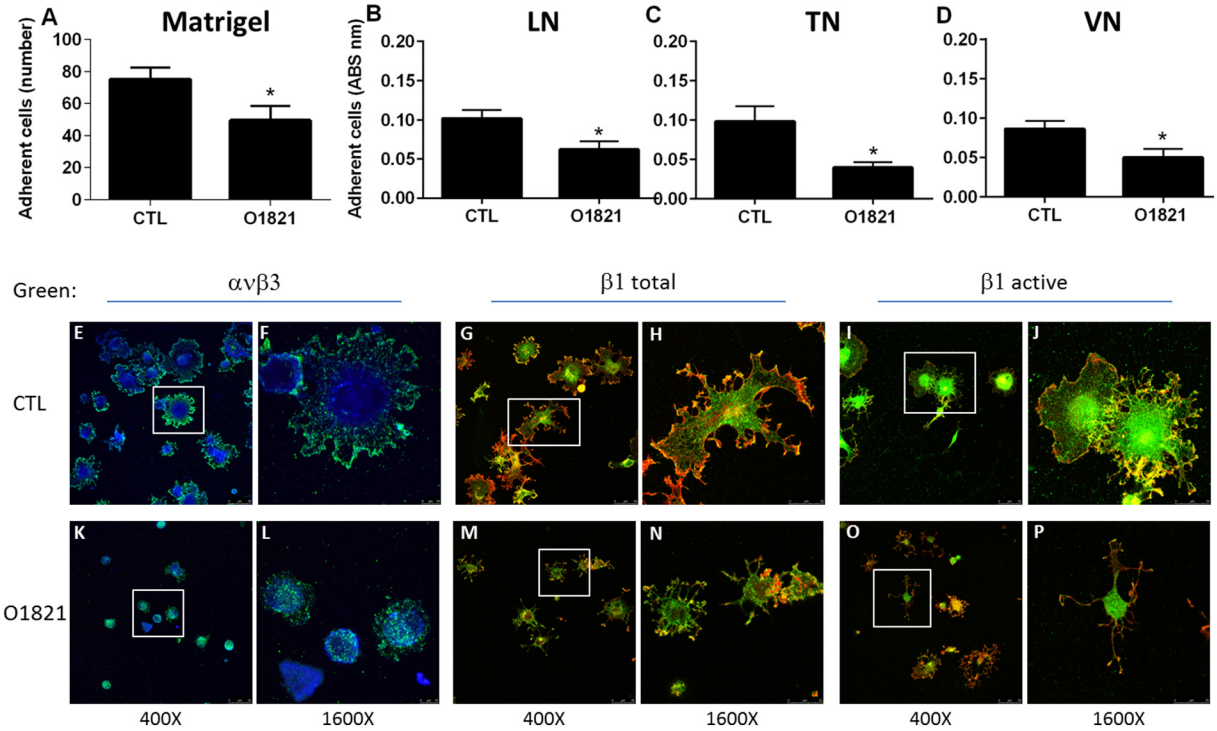

Fig. 2. O1821 decreases RA FLS adhesion to Matrigel, LN, TN and VN and affects integrin localization. RA FLS pre-treated with O1821 10 μM for 30 min and then allowed to adhere for 1 h to different substrates had (A) 30% lower adhesion to Matrigel (n = 8, ∗p = 0.05), (B) a 40% reduced adhesion to LN (n = 7, ∗p = 0.02), (C) a 60% reduced adhesion to TN (n = 7, ∗P = 0.02), and (D) a 42% reduced adhesion to VN by 42% (n = 7, ∗p = 0.03). *Mean ± SEM*; *paired t-test*. (E–J) RA FLS treated with 10% FBS or vehicle and allowed to adhere to Matrigel had increased localization of integrins to the cell periphery/plasma membrane. (K–P) RA FLS treated with O1821 10 μM for 30 min instead had reduced integrin localization to the cell periphery and predominantly cytosolic staining. (integrins: green; phalloidin: blue or red). Confocal images; E, G, I, K, M and O magnification 400×; F, H, J, L, N and P are 4× details of the respective picture on the left. (RA FLS, n = 3). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

showed little to no co-localization with lamellipodia (n = 4 different RA FLS cell lines; Fig. 1C–D).

In vehicle-treated RA FLS TRPV2 strongly localized at the cell periphery, including at the leading edge (Fig. 1E–F). However, O1821 treatment decreased TRPV2 localization to the cell periphery (Fig. 1G–H) suggesting that TRPV2 undergoes a desensitization mechanism following its activation, moving away from the plasma membrane.

2.2. TRPV2 stimulation with O1821 inhibits FLS adhesion to Matrigel and other integrin-dependent substrates

RA FLS treated with O1821 exhibited a significant 30% reduction in numbers of cells adhering to Matrigel (Fig. 2, $p < 0.05$; Supplemental Fig. 1). To determine whether TRPV2 stimulation affected integrin-mediated adhesion, FLS were cultured on different substrates. O1821 treatment decreased adhesion to LN by 40%, to TN by 60%, and to VN by 42% (n = 7 different RA FLS cell lines; $p < 0.05$; Fig. 2), but had minimal effect on cell adhesion to Coll type I, II and IV (data not shown). αν, β1 and β3 subunits are the three integrins capable of mediating the binding of all four matrix substrates affected by O1821 treatment (Table 1).

2.3. TRPV2-stimulated RA FLS displayed unusual integrin localization

Vehicle-treated RA FLS adhering to Matrigel had αν, β1 (active and total forms) and β3 subunits localized at the cell periphery (n = 3; Fig. 2E–J), while O1821-treated FLS had a predominantly cytosolic distribution of the three integrin subunits (n = 3; Fig. 2K–P). There was no difference in the total levels of integrins present in vehicle- or O1821-treated cells adhering to Matrigel (data not shown).

2.4. TRPV2 stimulation with O1821 inhibits Rac1 and RhoA activation

Integrin-mediated adhesion, invasion and actin cytoskeleton changes involve small GTPases. O1821 treatment of RA FLS decreased Rac1 activation by 27% (n = 5; $p = 0.0005$; Fig. 3A) and decreased RhoA activation by 45% (n = 3; $p = 0.002$; Fig. 3B). The addition of RhoA activators CNO3 or Calpeptin was able to bypass the inhibitory effect of O1821 on RhoA activation (Fig. 3B) and invasion (n = 4; Fig. 3C; $p < 0.05$). These observations suggest that O1821 acts is an early and upstream event on the RhoA activation pathway to suppress FLS invasion, but that effect can be bypassed by direct activation of RhoA.

2.5. O1821 inactivates Rac1-regulated and actin-binding protein Cofilin

Rac1 regulates actin filament formation by activating downstream signaling pathways important for cytoskeleton rearrangement. The actin binding protein cofilin is regulated by Rac1 and acts by severing pre-existing actin filaments from which new fibers are assembled. O1821-treated RA FLS had 20% increased levels of phosphorylated (inactive) cofilin compared with vehicle-treated cells (n = 7; Fig. 4A–B; $p = 0.03$). While levels of non-phosphorylated (active) cofilin were not changed in cells treated with O1821, protein localization shifted from the cell periphery to the cytoplasm (Fig. 4C–F). The increased levels of inactive cofilin and absence of the active form at the FLS leading edge are in line with reduced plasma membrane protrusions, lamellipodia formation and directional migration and invasion, and likely secondary to the suppression of Rac1 activation described above.

Table 1
Extracellular matrix proteins and integrins subunit.

LN	Purified human Laminin	α1β1, α2β1, α3β1, α6β1, αvβ3, α6β4
TN	Purified human Tenascin	α9β1, αvβ3
VN	Purified human Vitronectin	αvβ1, αvβ3, αvβ5, αvβ8

3. Discussion

The RA joint pathology is characterized by a hyperplastic and invasive synovial tissue that causes cartilage and bone damage. The FLS has a central role in the RA pathology and in the influx of inflammatory cells into the synovial tissues. The RA FLS produces chemokines, pro-inflammatory cytokines and MMPs. We hypothesized that better understanding the FLS behavior might lead to new and perhaps better targets for treatment in RA. We focused on the invasiveness of the FLS which has been shown to be increased in RA compared with OA [19,32,33]. The *in vitro* invasiveness of FLS correlates with radiographic damage in RA [33] and with histologic erosive changes in rodent models of RA [12]. We have previously discovered that the non-specific cation channel TRPV2 is a major suppressor of FLS invasiveness and capable of reducing the expression of MMPs [13]. However, the intracellular events mediating the TRPV2-induced suppression of FLS invasion were not known. In the present study we describe a novel mechanism where TRPV2 activation suppresses small GTPase RhoA and Rac1 to decrease RA FLS integrin-mediated adhesion and cell invasion.

Invasion through an extracellular matrix begins with cell adhesion to ECM substrates. When in contact with Matrigel *in vitro*, RA FLS rapidly adhere, undergo cytoskeleton reorganization, lamellipodia formation and then initiate the invasive process. During the transition from adhesion to invasion, TRPV2 is mainly located at the plasma membrane. Upon stimulation with O1821 TRPV2 nearly disappeared from the plasma membrane. Also, following TRPV2 stimulation there was a decrease in RA FLS adhesion to specific substrates and reduced numbers of thick actin filament and inhibition of lamellipodia formation.

TRPV2-stimulated RA FLS had reduced adhesion to Matrigel, LN, VN and TN, but adhered normally to Col type I, II and IV. Cell binding to ECM proteins is mediated by integrins, and various combinations of integrin subunits confer specificity for different substrates. αν, β3 and β1 are the integrin subunits able to bind to Matrigel, LN, VN and TN, the four substrates with reduced cell adhesion following TRPV2 stimulation in the present study [23]. Integrins cluster at focal adhesion points during cell spreading, thus initiating cell adhesion to the ECM. This first step is followed by actin cytoskeleton reorganization and by cell migration and invasion. O1821 treatment also reduced the localization of integrins αν, β1 and β3 to the plasma membrane. As a consequence, the number of adhering O1821-treated cells was not only decreased but also had a strikingly different morphology compared with vehicle-treated controls, with round shape, thin and disorganized actin filaments and almost no formation of lamellipodia. These observations suggest that TRPV2 stimulation induces re-location of integrins from the plasma membrane to the cytosol, interfering with cell attachment and preventing actin cytoskeleton changes such as linear formation of thick filaments to move efficiently, contributing to the decreased of the RA FLS invasion.

Integrin-mediated cytoskeleton rearrangement, migration and invasion involve the recruitment of several proteins and the activation of different signaling pathways [9,31] including small GTPases [26,28]. In turn, small GTPases can induce integrin activation and promote adhesion and invasion as well [5]. In our study, we demonstrated that both RhoA and Rac1 activation was reduced in RA FLS treated with O1821. RhoA is a major regulator of cellular contractility and its activation leads to the formation of actin filaments and maturation of cell–matrix adhesions [27]. RhoA activators CNO3 and Calpeptin were able to overcome the O1821-induced inhibition of RhoA activity and cell invasion, suggesting that TRPV2 acts upstream of RhoA.

Rac1 is known to regulate cancer [2,4,15,16,25] and FLS invasiveness [3]. Rac1 controls FLS cytoskeleton reorganization, cell spreading, and the development of membrane protrusions, including the formation of lamellipodia [3]. These processes are mediated by Rac1 interaction

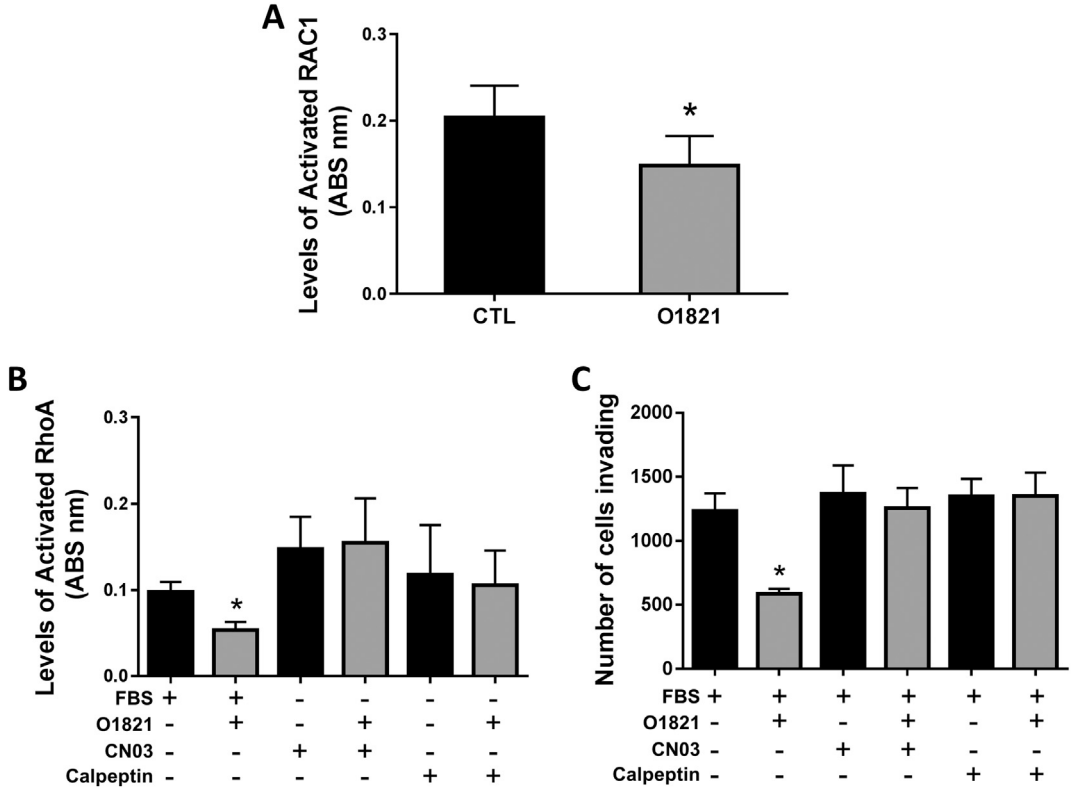

Fig. 3. O1821 reduces Rac1 and RhoA activation. O1821 10 μM reduces RA FLS (A) Rac1 activation (n = 5 * p = 0.0005), (B) RhoA activation (n = 3, * p = 0.002) and (C) cell invasion through Matrigel (n = 4, *p < 0.05,). The presence of downstream RhoA activators CN03 and Calpeptin, prevents O1821 inhibitory effect on (B) RhoA activation and on (C) RA FLS invasion through Matrigel (*Mean ± SEM; paired t-test and One-way ANOVA*).

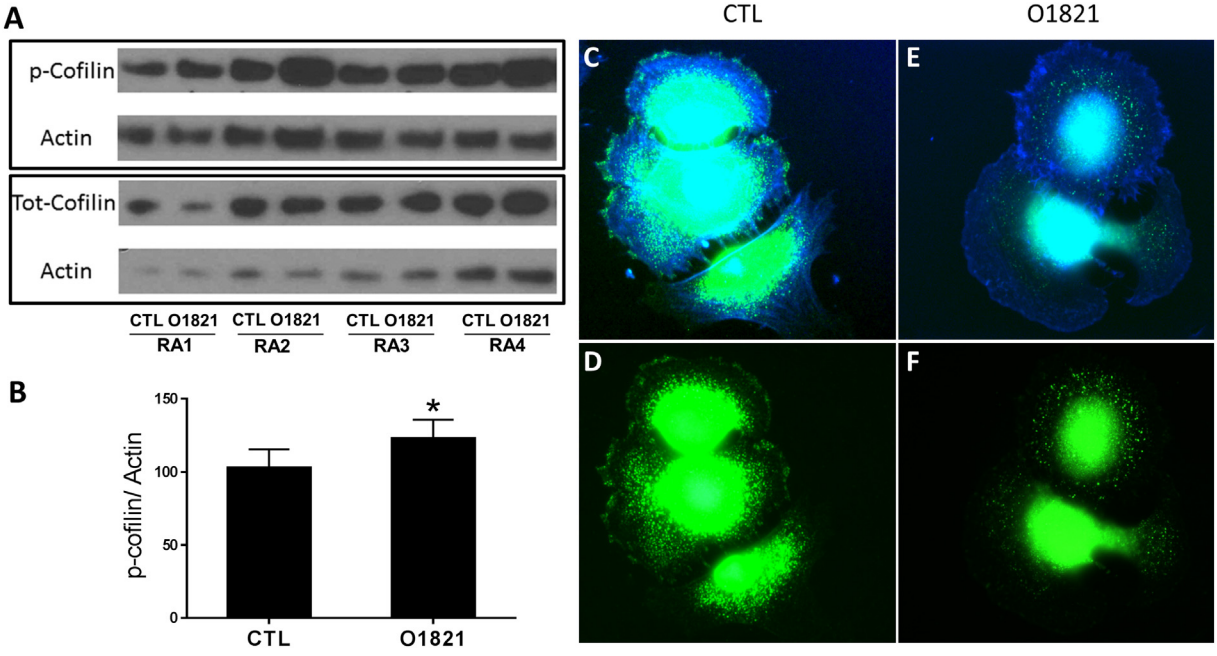

Fig. 4. O1821 increases levels of phosphorylated (inactive) cofilin and causes active cofilin movement from the cell periphery to the cytosol. (A) Western blot analysis of phosphorylated (inactive) cofilin and non-phosphorylated (active) cofilin and the corresponding gel actin bands (boxes) in RA FLS adhering to Matrigel with or without O1821 (4 representative RA FLS cell lines are shown). (B) Densitometry analysis of p-cofilin bands shows a significant increase of phosphorylated (inactive) cofilin in cells treated with O1821 (n = 7; relative ratio p-cofilin/Actin. *p = 0.03; *Mean ± SEM*; *paired t-test.*). (C–D) RA-FLS stimulated with FBS 10% and allowed to adhere to Matrigel had increased levels of non-phosphorylated (active) cofilin at the cell periphery (plasma membrane), while (E–F) O1821-treated FLS had reduced levels of cofilin at the cell periphery and increased cytoplasmatic levels. Green: cofilin, blue: phalloidin. Confocal images, magnification, 400×. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

with several proteins including the actin-binding protein cofilin [10,17]. Cofilin undergoes a dynamic process of phosphorylation and de-phosphorylation that controls cytoskeleton rearrangements essential for cell movement and invasion. De-phosphorylated (active) cofilin binding to actin promotes severance of pre-existing actin filaments, thus supplying a pool of actin monomers for de-novo actin polymerization [18]. This process initiates the formation of membrane protrusions and sets the direction of migration and invasion [35]. Phosphorylation of cofilin at ser3 inactivates the protein and inhibits the interaction with actin. TRPV2 stimulation with O1821 significantly increased levels of phosphorylated (inactive) cofillin, decreasing de-novo actin remodeling essential for cell migration and invasion. Localization of cofilin was also affected by TRPV2 stimulation with O1821. O1821 also affected the localization of active cofilin keeping it in the cytosol away from cell protrusions and lamellipodia.

Rac1 and RhoA have been previously implicated in cancer invasiveness, and tumor levels correlate with increased risk for metastasis and poor outcome [2,4,6,11,15,16,25]. While a non-specific Rac inhibitor has been developed [7], to our knowledge it has not been tested in human disease perhaps due to concerns with risk of toxicity given the multiple cellular functions of different Rac proteins. We are also not aware of any RhoA inhibitors on clinical trials [16,30]. The challenges in developing agents that act on these signaling enzymes include the lack of obvious druggable pockets and their membrane-bound activities. [30]. We have previously shown that Rac1 is a key mediator of FLS invasiveness [3] and therefore, the observations reported here suggest that TRPV2 agonists might be a novel and indirect way to suppress Rac1 and RhoA activity to suppress cell invasiveness and arthritis severity, perhaps without completely suppressing Rac1/RhoA activity in other tissues, thus minimizing possible toxicities. These observations also raise the possibility that TRPV2 agonists might have a role in the treatment of cancers to reduce invasiveness and the risk of metastasis.

In conclusion, we describe new intracellular signaling events regulated by TRPV2 that explain the suppressive effects on FLS invasiveness and in ameliorating arthritis severity. Targeting TRPV2 has the potential to become a new strategy to treat RA reduce disease severity and joint damage. Given that TRPV2 appears to be relevant mostly to mesenchyme-derived FLS and not to immune cells' functions we propose that it might be an interesting target for predominantly FLS-specific treatments with reduced risk of systemic immunosuppression or increased risk for infections.

4. Material and methods

4.1. Isolation and culture of FLS

FLS from patients with RA were obtained as previously described [13]. Briefly, freshly obtained synovial tissues were minced and incubated with a solution containing DNase (0.15 mg/ml), hyaluronidase type I–S (0.15 mg/ml), and collagenase type IA (1 mg/ml) (Sigma, St. Louis, MO) in DMEM (Invitrogen, Carlsbad, CA) for 1 h at 37 °C. Cells were washed and re-suspended in complete media containing DMEM supplemented with 10% FBS (Invitrogen), glutamine (300 ng/ml), amphotericin B (500 ng/ml) (Sigma), and gentamicin (20 μg/ml) (Invitrogen). After overnight culture, non-adherent cells were removed and adherent cells kept in culture. All experiments were performed with FLS after passage four (> 95% FLS purity).

4.2. Immunofluorescence microscopy

Immunofluorescence was performed as previously reported [14]. Briefly, cells were plated on plastic or Matrigel-coated coverslips after pre-treatment with the TRPV2-specific agonist O1821 (10 μM based on previous titrations) for 30 min. Serum-free media (SFM) or 10% FBS was then added to the plastic coverslips at the indicated time. Cells were fixed with 4% formaldehyde for 15 min at room temperature and permeabilized with PBS/Triton X-100 0.1% for 5 min. Non-specific binding was blocked with 5% nonfat milk. Cells were then stained with mouse anti-$\alpha v\beta 3$, anti-β1 (clone HUTS21 active form) or anti-β1 (clone MAR4 total form) (BD Bioscience). Alexa Fluor 488 (green) anti-mouse IgG (Invitrogen) was used as secondary antibody. Alexa Fluor 594 (red) or Alexa Fluor 350 (blue) Phalloidin (Invitrogen) were used to stain the actin filament. Images were acquired with a Leica DMi8 microscope at 600× magnification and analyzed with the Leica application suite X (LAS X) software. Confocal microscopy pictures were taken with a Leica SP5 DM and LAS X software at 400× magnification.

4.3. Adhesion assay

RA FLS were pre-treated with the TRPV2-specific agonist O1821 [24] 10 μM or vehicle for 30 min in SFM. 10^5 cells were then plated in 96-well plates pre-coated with either human Collagen (Col) type I, II or IV, Fibronectin (FN), Laminin (LN), Tenascin (TN), Vitronectin (VN) (ECM cell adhesion array kit, EMD Millipore, Burlington MA) or Matrigel (BioCoat™ Matrigel® Matrix Thin-Layer, Corning, New York, NY). After 1 h, non-adherent cells were gently aspirated and wells washed three times with 1× PBS. Adherent RA-FLS were then stained with Cell Stain solution (Millipore Sigma, St. Louis, MO) and absorbance quantified at 550 nm, and also manually counted.

4.4. Invasion assay

The *in vitro* invasiveness of FLS was assayed in a transwell system using Matrigel-coated inserts (BD Biosciences, Franklin Lakes, NJ) as previously described [12,13]. Briefly, 70–80% confluent cells were harvested by trypsin-EDTA digestion and 2×10^4 cells pre-treated with vehicle (1% DMSO) or O1821 10 μM in serum-free DMEM were placed in the upper compartment of each Matrigel-coated insert with or without RhoA activators CNO3 (1 μg/ml) or Calpeptin (10 μg/ml). The lower compartment was filled with media containing 10% FBS and the plates were incubated at 37 °C for 24 h. After 24 h the upper surface of the inserts was wiped with cotton-swabs to remove non-invading cells and the Matrigel layer. The opposite side of the insert was stained with Crystal Violet (Sigma) and the total number of cells that invaded through Matrigel counted at 50× magnification. Experiments were done in duplicate.

4.5. Rac1 and RhoA activation assay

RA FLS were plated in 6-well plates at a 90% confluence and starved on SFM for 24 h. The cells were then treated with vehicle or O1821 10 μM for 30 min. After that, FBS 10%, Calpeptin (10 μg/ml) or CNO3 (1 μg/ml) were added to both vehicle-treated and O1821-treated RA FLS. Incubation times were 30 min for FBS and Calpeptin and 2 h for CN03 (based on previous titration results). Equal amounts of cell lysates were used to quantify Rac1 or RhoA activation with G-LISA Activation Assay Kits (Cytoskeleton, Denver, CO), which measure GTP-bound Rac1 and GTP-bound RhoA (activated forms) in cells.

4.6. Statistical analysis

Data were analyzed using paired *t*-test, or with One-way ANOVA for the comparison of multiple groups. All analyses were performed using Prism software (GraphPad Software, Inc.). Differences were considered significant at a P-value of ≤ 0.05.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2019.02.051.

Authorship contributions

Research Design: Teresina Laragione and Percio S. Gulko.

Conducted Experiments: Teresina Laragione and Carolyn Harris.

Performed data analyses: Teresina Laragione, Carolyn Harris and Percio S. Gulko.

Wrote or contributed to the writing of the manuscript: Teresina Laragione and Percio S. Gulko.

Authors' conflicts of interest

PSG is an inventor in patent of TRPV2 agonists (none used in this study). TL and CH has no conflicts to disclose.

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