Sensitization of knee-innervating sensory neurons by tumor necrosis factor-α activated fibroblast-like synoviocytes: an in vitro, co-culture model of inflammatory pain.

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Abbreviations:

FB = Fast Blue, FLS = Fibroblast-like synoviocyte, IL = Interleukin, PG = Prostaglandin, COX = Cyclooxygenase, knee neurons = knee-innervating dorsal root ganglion neurons, CFA = Complete Freund’s adjuvant, CDH = Cadherin, CD = Cluster of differentiation, P = Passage, RT-PCR = Reverse transcription – polymerase chain reaction, DRG = Dorsal root ganglion, RA = Rheumatoid arthritis, OA = Osteoarthritis, MLS = Macrophage-like synoviocyte, MMP = Matrix metalloprotease, VCAM = Vascular cell adhesion molecule, TNF = Tumor necrosis factor, AIA = Antigen-induced arthritis, TRP = Transient receptor potential, ASIC = Acid-sensing ion channel, CGRP = Calcitonin gene-related peptide, ECS = Extracellular solution, PBS = Phosphate-buffered saline, Ipsi = CFA-injected side, Contra = Non-injected knee, RNA = Ribonucleic acid, DNA = Deoxyribonucleic acid, L = Lumbar, KC = Keratinocyte chemoattractant, ANOVA = Analysis of variance, AP = Action potential, RMP = Resting membrane potential, AHP = Afterhyperpolarization, HPD = Half peak duration, LIX = Lipopolysaccharide induced chemokine, MCP = Monocyte chemoattractant protein, MCSF = Macrophage colony stimulating factor, MIG = Monokine induced by gamma interferon, MIP = Macrophage inflammatory protein, RANTES = Regulated on activation, normal T-cell expressed and secreted, SDF= Stromal cell derived factor, GM-CSF = Granulocyte-macrophage colony stimulating factor, GPCR = G protein-coupled receptors.
Abstract (350 words)

Background:

Pain is a principle contributor to the global burden of arthritis with peripheral sensitization being a major cause of arthritis-related pain. Within the knee joint, distal endings of dorsal root ganglion neurons (knee neurons) interact with fibroblast-like synoviocytes (FLS) and the inflammatory mediators they secrete, which are thought to promote peripheral sensitization. Therefore, we investigated the communication between knee neurons and FLS in a co-culture system.

Methods:

Inflammation in FLS (isolated from mouse patella) was induced by stimulation with tumor necrosis factor-α (TNF-FLS). Expression of relevant genes, secretion of cytokines and functional acid response was assessed using polymerase chain reactions, inflammatory antibody array blots and Ca^{2+} imaging respectively. Electrophysiology was performed on dissociated knee neurons in mono-culture, co-culture with control FLS or TNF-FLS, or supernatant derived from TNF-FLS cultures to determine electrical excitability and sensitivity to transient receptor potential (TRP) agonists. Two group comparisons were conducted using Student’s t-tests (distributed variable) or chi-sq tests (categorical variable). ANOVA followed by Tukey’s post-hoc test was performed for comparing more than two groups.

Results:

Compared to control, TNF-FLS showed increased expression of interleukin-6 (Il-6, p < 0.01, unpaired t-test) and Il-1r1 (p < 0.05, unpaired t-test), and enhanced cytokine secretion. FLS also responded to acidic stimuli and the percentage of TNF-FLS acid responders increased in the pH range 6.0-5.0 (p < 0.001, chi-sq test). Co-culture of DRG neurons with TNF-FLS or supernatant
derived from TNF-FLS depolarized the resting membrane potential (p < 0.01, ANOVA), increased spontaneous action potential firing (p < 0.05, ANOVA) and enhanced TRPV1 function (p < 0.05, ANOVA) of knee neurons consistent with a role for FLS in the sensitization of pain sensing nerves in arthritis.

Conclusions:

TNF-FLS have a pro-nociceptive phenotype, displaying enhanced acid sensitivity and increased secretion of pro-inflammatory cytokines. Co-culture with TNF-FLS or supernatant from TNF-FLS, induces hyperexcitability of knee neurons and enhances TRPV1 function. Data from this study demonstrate the ability of FLS activated by TNF-α to promote neuronal sensitization, results that highlight the importance of both non-neuronal and neuronal cells to the development of pain in arthritis.
Joint inflammation and pain are the major clinical symptoms of arthritis. Inflammation is part of the body’s immune response to tissue damage and involves multiple cell types, including lymphocytes and synoviocytes. These non-neuronal cells are either in direct contact with, or in close proximity to, the distal endings of dorsal root ganglion (DRG) sensory neurons that innervate the joints. The physical interaction of sensory neurons with non-neuronal cells and the mediators they secrete, is thought to cause peripheral sensitization of knee-innervating nerves, which is a key pathology in the development of inflammatory pain.

The knee is a richly innervated, diarthrodial joint that is lined by the synovial membrane, which is composed of synoviocytes in its intimal lining layer and lymphocytes in its sub-lining layer (1). During inflammatory diseases such as rheumatoid arthritis (RA), the joint undergoes hyperplasia due to T lymphocyte infiltration and fibroblast-like synoviocyte (FLS) proliferation. FLS are key effector cells in RA as they become active upon stimulation by inflammatory cytokines (released by macrophage-like synoviocytes, MLS, and T-cells) and secrete matrix metalloproteases (MMP) that cause joint destruction (2). In addition, FLS can also support and maintain the ongoing inflammation in arthritic joints by themselves secreting pro-inflammatory mediators (2). Building on this, recent single-cell transcriptional analysis of patient-derived RA-FLS, and their subsequent transfer into mouse knee joints, has identified distinct FLS subgroups: “destructive” and “inflammatory” (3). Furthermore, deletion of cadherin-11 (CDH-11), a key surface marker of FLS, causes hypoplasticity in murine joints and makes them resistant to inflammatory arthritis (4), evidence further supporting a critical role of FLS in arthritic pathology.

FLS derived from rodent and human joints proliferate in vitro, unlike MLS, enabling their extensive characterization (1). Interestingly, human RA-FLS are shown to be “imprinted
aggressors” that retain inflammatory phenotype over several passages, although the effect of these activated FLS on sensory neurons remains largely unexplored (1,5). In rodents, it has been reported that FLS derived from K/BxN mice (a spontaneous, chronic arthritis model) (5) and supernatants from cultured FLS derived 3-days after induction of antigen-induced arthritis (AIA) in rats (6) have increased levels of secreted cytokines compared to control FLS. Several other studies have utilized interleukin 1β (IL-1β) or tumor necrosis factor-α (TNF-α) stimulation to robustly induce an inflammatory phenotype in FLS (7–10). In this study we tested two modes of FLS activation to understand their inflammatory phenotype. Firstly, we tested for pro-inflammatory phenotype in FLS derived from mice undergoing the commonly used acute, unilateral complete Freund’s adjuvant (CFA) model, which, as previously reported, can produce robust knee inflammation, knee neuron hyperexcitability and a concomitant decrease in digging behavior (11). Secondly, we tested TNF-α (a cytokine that is upregulated in tissues derived CFA-injected mice (12)) stimulated FLS derived from mouse knee, and determined their inflammatory phenotype to understand the potential of activated FLS to induce sensitization of sensory neurons.

Since arthritic FLS reside in a pro-inflammatory environment which is often (13) (but not always (14)) acidic, it is perhaps unsurprising that they express algogen sensing ion channels including proton sensors (15,16) and various transient receptor potential (TRP) channels (17), such as TRPV1 (transducer of noxious heat and low pH) and TRPA1 and TRPM8 (cold transducers). Experiments conducted on SW982, a human tumor derived synoviocyte cell line, and primary FLS from an RA patient, have demonstrated that a Ca^{2+} influx occurs upon application of the TRPV1 agonist capsaicin in an acidic (pH 7.1) environment and upon application of the TRPA1 and TRPM8 agonist icilin (18). In addition to Trpv1, the human FLS cell lines SW982 and MH7A also
express *Trpv4* (warmth and tonicity transducer), and both *Trpv1* and *Trpv4* are upregulated by TNF-α stimulation (19,20). Since most of these experiments were conducted on immortalized cell lines, which thus might not be physiologically relevant, there is a need to investigate TRP channel function in primary FLS. By contrast, acid sensors have been better studied in FLS derived from mouse joints, leading to the identification of acid-sensing ion channel 3 (ASIC3) as a major proton transducer. FLS from wild-type mice cultured in IL-1β show increased intracellular Ca\(^{2+}\) influx and cell death in response to acid compared to FLS derived from ASIC3\(^{-/-}\) mice, suggesting a physiological role of ASIC3 and acid-sensing in general in FLS (16,21,22). Therefore, in this study we sought to compare the algogen sensitivity of FLS before and after inducing an inflammatory phenotype using Ca\(^{2+}\) imaging.

Given the complex interaction of cells in the joint environment, co-culture has proved to be an important tool for dissecting FLS biology. Co-culture of human RA-FLS with T-cells (23–26) or macrophages/monocytes (27–29) increased the concentration of prostaglandin (PG) E\(_2\), IL-6, IL-8, MMP-1 and MMP-3 in the culture medium, along with increased expression of adhesion molecules such as vascular cell-adhesion molecule (*Vcam-1*) on FLS in a cell-mediated and soluble-factor mediated manner. Furthermore, upregulation of these pro-inflammatory mediators in culture supernatants was inhibited by anti-TNF-α (26,30) and anti-IL-6 antibodies (30). Additionally, IL-15 secreted from human RA-FLS promotes survival of B-cells (31) and natural killer cells (32) in co-culture, further demonstrating the importance of FLS in maintaining the proinflammatory environment of arthritic joints. Although co-culture techniques have revealed multiple immune interactions within the joint environment, the communication between neurons and non-neuronal cells is less well understood in the context of knee inflammation. Culturing DRG neurons with FLS derived from chronic AIA rats increased expression of receptors associated with...
nociception, namely neurokinin 1, bradykinin 2 and TRPV1 in neurons (6), validating the premise of co-culture studies to understand neuron-synoviocyte communication within the arthritic joint. However, these studies did not functionally assess modulation of DRG neuron excitability, which is a key mechanism of peripheral sensitization and hence pain. We recently showed that human osteoarthritic (OA) synovial fluid (a lubricating fluid largely secreted by FLS (1)) can cause hyperexcitability of murine sensory neurons and increase TRPV1 function (33). Therefore, in this study, we hypothesized that after induction of inflammation, mouse knee-derived FLS will increase excitability and TRP function of knee-innervating DRG neurons (knee neurons) in an FLS-DRG neuron co-culture system.
Methods

Animals

All mice used in this study were 6-12 week old C57BL/6J mice (Envigo). Unless otherwise stated, female mice were used since in humans, females are at a higher risk for inflammatory pain (34). Mice were housed in groups of up to 5 in a temperature controlled (21 °C) room with appropriate bedding materials, a red shelter and enrichment. They were on a 12-hour/light dark cycle with food and water available *ad libitum*. Experiments in this study were regulated under the Animals (Scientific Procedure) Act 1986, Amendment Regulations 2012. All protocols were approved by a UK Home Office project license granted to Dr Ewan St. John Smith (P7EBFC1B1) and reviewed by the University of Cambridge Animal Welfare and Ethical Review Body.

Knee injections

Under anesthesia (100 mg/kg ketamine and 10 mg/kg xylazine, intra-peritoneally) mice were injected intra-articularly through the patellar tendon into each knee with the retrograde tracer, fast blue (FB, 1.5 μl 2% in 0.9% saline, Polysciences) or into the left knee with 7.5 μl CFA (10 mg/ml, Chondrex). Vernier’s calipers were used to measure knee width (as before (11)) pre- and 24-hour post-CFA injection.

Isolation and culture of FLS
24-hour after CFA injection into the knee, mice were killed by cervical dislocation and decapitation. Knee joints were exposed by removing the skin, then the quadriceps muscles were resected in the middle and pulled distally to expose the patellae. Patellae were then collected by cutting through the surrounding ligaments, as described before (35), in phosphate-buffered saline (PBS) and then transferred into one well of a 24-well plate with FLS culture media containing: Dulbecco’s Modified Eagle Medium F-12 Nutrient Mixture (Ham) (Life Technologies), 25% fetal bovine serum (Sigma), 2 mM glutamine (Sigma) and 100 mg/ml penicillin/streptomycin (Life Technologies). Cells took approximately 10 days to grow to 70% confluency; medium was changed every other day. For P1, FLS were trypsinized with 1% trypsin (Sigma), re-suspended in FLS culture media and transferred into two wells of a 6-well plate. FLS from two animals were combined at P2. For subsequent passages, FLS were transferred into 60 mm dishes. Contralateral (Contra) and CFA-injected knees/cells (Ipsi) were kept separate at all stages. The cells were maintained in a humidified, 37 °C, 5% CO₂ incubator. FLS were also cultured until P5 from mice that had not undergone any knee CFA injection (control). A random selection of these dishes, from three separate biological replicates, was incubated for 24 to 48-hours (as per experimental design) in culture medium with recombinant mouse TNF-α (10 ng/ml from a stock solution of 100 µg/ml made up in 0.2 % bovine serum albumin and sterile PBS, R&D systems, aa-80325) to stimulate release of inflammatory mediators (TNF-FLS) (5).

Culture of Raw 264.7 cells
Raw 264.7 cells (EACC) were cultured in medium containing Dulbecco’s Modified Eagle Medium F-12 Nutrient Mixture (Ham) (Life Technologies), 10% fetal bovine serum (Sigma), 2 mM glutamine (Sigma) and 100 mg/ml penicillin/streptomycin (Life Technologies). Cells were maintained in a humidified 37 °C, 5% CO₂ incubator.

RNA extraction and reverse transcriptase – quantitative/ polymerase chain reaction (RT-q/PCR)

For all conditions, RNA was extracted from two 60 mm dishes (Thermo Fisher) of FLS (various passages) and from one T-25 flask (Greiner Bio-one) of Raw 264.7 cells at P3 using the RNeasy Mini Kit (Qiagen). 500 ng of the extracted RNA was used to synthesize cDNA using a High Capacity cDNA RT kit (Applied Biosystems) following the manufacturer’s guidelines, using a T100 Thermal Cycler (Bio-Rad). The resultant cDNA was diluted to a 1:5 ratio with nuclease free water and quantitative PCR (qPCR) was performed using a StepOnePlus Real Time PCR system, following the manufacturer’s guidelines on settings (Applied Biosystems) using TaqMan probes (Thermo Fisher) (See Supplementary Table 1 in Additional File 1). The fluorescence intensity of samples was captured during the last minute of each cycle. All reactions were run in triplicate with appropriate negative controls with water containing no cDNA.

For qPCR reactions, data were obtained as Ct values (the cycle number at which fluorescent signals emitted by the TaqMan probe crossed a threshold value). Only Ct values below 35 were analyzed to determine ΔCt values by subtracting the Ct of 18S ribosomal RNA from the Ct of target gene (36). ΔCt values of target genes were subtracted from average ΔCt values of their controls to calculate ΔΔCt, followed by $2^{\Delta\Delta C_t}$ to calculate fold change (37).
FLS gene expression was also assessed by RT-PCR. DreamTaq Polymerase (Thermo Fisher) was used to amplify a section within the open reading frames of various genes from 5 ng template cDNA. The sequences of designed oligonucleotides (Sigma) are listed in Supplementary Table 1 in Additional File 1. Negative controls (using water/RNA as the template) were performed for each biological sample with a randomly selected primer pairing. PCR products were resolved on 2% agarose containing 1X GelRed Nucleic Acid Stain (Biotum) and imaged with a GeneFlash Gel Documentation System (Syngene). A randomly selected subset of reactions was repeated with 2.5 ng template cDNA to ensure reproducibility. Densitometry analyses were performed using ImageJ Software (NIH) where relative expression was determined by dividing the band intensity of each gene by that of the housekeeping gene, 18S ribosomal RNA, for each biological replicate.

Cytokine antibody array

Before RNA extraction, 2 ml of culture media from P5 Contra, Ipsi, control and TNF-FLS (48-hour) were collected and stored at -80 °C until use. Culture medium was pooled from three cultures for each of the four conditions and assayed (undiluted) for the presence of 40 inflammatory mediators using Mouse Inflammatory Antibody Array Membranes (ab133999, Abcam) according to the manufacturer’s instructions. Chemiluminescence was imaged using a BioSpectrum 810 imaging system (UVP) with 3 min exposure. Location of the 40 cytokines detected by the array membranes are shown in Supplementary Table 2 in Additional file 1.

Densitometry of the spots in the array membranes was performed using ImageJ software (NIH). Briefly, the mean gray value of each spot was measured from all membranes using the same
circular region of interest. The spots of interests were then background (average of all negative control spots) subtracted and normalized to the positive control spots of the reference membrane (control FLS media). Fold change was obtained by dividing normalized intensities of the membrane of interest and the control membrane, analyte-by-analyte.

DRG neuron isolation and culture

Lumbar DRG (L2-L5, those that primarily innervate the knee) were collected from FB labelled mice 7-10 days after knee injections in ice cold dissociation media containing L-15 Medium (1X) + GlutaMAX-I (Life Technologies), supplemented with 24 mM NaHCO₃. DRG were then enzymatically digested in type 1A collagenase (Sigma) and trypsin solution (Sigma) at 37 °C, followed by mechanical trituration as described before (11). Dissociated DRG neurons were plated onto poly-D-lysine and laminin coated glass bottomed dishes (MatTek, P35GC-1.5-14-C) and cultured either on their own (mono-culture), on a layer of FLS (co-culture, see below) or in 48-hour conditioned media from TNF-FLS for 24-hours. The DRG culture medium contained L-15 Medium (1X) + GlutaMAX-I, 10% (v/v) fetal bovine serum, 24 mM NaHCO₃, 38 mM glucose, 2% (v/v) penicillin/streptomycin.

DRG neuron/FLS co-culture
For co-culture studies, FLS were plated onto MatTek dishes and cultured for 24-hours with FLS culture media (with or without TNF-α stimulation). The next day medium was removed from FLS plates, then DRG neurons were isolated as described above and plated on top of the FLS. Co-culture plates were then kept in DRG culture medium for up to 24-hours for electrophysiological recording.

Cell staining

**FLS immunocytochemistry:** FLS were plated overnight in wells of a 24-well plate, fixed with Zamboni’s fixative (38) for 10 min, permeabilized with 0.05% TritonX-100 and blocked with antibody diluent (0.2% (v/v) Triton X-100, 5% (v/v) donkey serum and 1% (v/v) bovine serum albumin in PBS) for 30 min. The cells were then incubated overnight at 4 °C in 1:100 (in antibody diluent) anti-cadherin-11 antibody (CDH-11, rabbit polyclonal, Thermo Fisher, 71-7600). Cells were washed three times with PBS-tween and incubated in the conjugated secondary antibody, anti-rabbit Alexa-568 (1:1000 in PBS, Thermo Fisher, A10042) for 1-hour at room temperature (21 °C). The secondary antibody was washed off three times with PBS-tween and the cells were incubated in the nuclear dye DAPI (1:1000 in PBS, Sigma, D9452) for 10 min. Cells were further washed with PBS-tween once and imaged in PBS using an EVOS FLoid Cell Imaging Station (Thermo Fisher) at 598 nm (for CDH-11) and 350 nm (for DAPI) wavelength of light. Cells without primary antibody did not show fluorescence.

**DRG/FLS co-culture live cell stain:** To visualize neuron/FLS co-culture, live cell imaging was performed. FLS were plated on MatTek dishes with 1:1000 (diluted in FLS culture medium) CellTracker Deep Red Dye (Thermo Fisher, C34565) and incubated for 24-hours in a humidified 37 °C, 5% CO₂ incubator. Dissociated DRG neurons (see above) were incubated in CellTracker
Green Dye (1: 1000 diluted in DRG culture media, Thermo Fisher, C7025) for 15 min at room temperature (21 °C), centrifuged (16000 g, 3 min, 5415R, Eppendorf) and re-suspended in fresh medium. The FLS dishes were then washed twice with PBS and the neuronal suspension was plated on top of the FLS monolayer and incubated overnight in the incubator. The co-culture dishes were washed with PBS and imaged the following day using an Olympus BX51 microscope and QImaging camera at 650 nm (for deep red dye) and 488 nm (for green dye) wavelength of light.

Whole-cell patch-clamp electrophysiology

Neurons were bathed in extracellular solution containing (ECS, in mM): NaCl (140), KCl (4), MgCl$_2$ (1), CaCl$_2$ (2), glucose (4) and HEPES (10) adjusted to pH 7.4 with NaOH. Only FB labelled neurons identified by their fluorescence upon excitation with a 365 nm LED (Cairn Research) were recorded. Patch pipettes of 5–10 MΩ were pulled with a P-97 Flaming/Brown puller (Sutter Instruments) from borosilicate glass capillaries and the intracellular solution used contained (in mM): KCl (110), NaCl (10), MgCl$_2$ (1), EGTA (1), HEPES (10), Na$_2$ATP (2), Na$_2$GTP (0.5) adjusted to pH 7.3 with KOH.

Action potentials (AP) were recorded in current clamp mode without current injection (to investigate spontaneous AP firing) or after step-wise injection of 80 ms current pulses from 0 – 1050 pA in 50 pA steps using a HEKA EPC-10 amplifier (Lambrecht) and the corresponding Patchmaster software. AP properties were analyzed using Fitmaster software (HEKA) or IgorPro software (Wavemetrics) as described before (11) and shown in Figure 3D (inset). Neurons were excluded from analysis if they did not fire an AP in response to current injection. For recording whole-cell voltage-clamp currents in response to TRP agonists capsaicin (1 μM from a 1 mM stock
in ethanol, Sigma-Aldrich), cinnamaldehyde (100 μM from a 1 M stock in ethanol, Alfa Aesar) and menthol (100 μM from a 1 M stock in ethanol, Alfa Aesar), a 5 s baseline was established with ECS, followed by a 5 s randomized drug application. Peak drug response was measured in Fitmaster by subtracting the average of 2 s baseline immediately preceding the drug application and the maximum peak response reached during the 5 s of drug application. Peak current density is represented in graphs by dividing this peak response by the capacitance of the neuron. Data from at least four mice were used in all conditions (with each mouse being used for at least two conditions) and at least three neurons were recorded from each mouse in each category.

Ca²⁺ imaging

FLS or neurons were incubated with the Ca²⁺ indicator, Fluo-4 AM (10 μM diluted in ECS from a 10 mM stock solution in DMSO, Invitrogen) for 30 min at room temperature (21 °C). Culture dishes were then washed and imaged using an inverted Nikon Eclipse Ti microscope. Fluo-4 fluorescence was excited using a 470 nm LED (Cairn Research) and captured with a camera (Zyla cSMOS, Andor) at 1 Hz with 50 ms (for neurons) 250 ms (for FLS) exposure time using Micro-Manager software (v1.4; NIH). Solutions were perfused in this system through a gravity-driven 12 barrel perfusion system (39). During imaging of neurons, MIP-1γ (10 and 100 ng/ml diluted in ECS from a stock concentration of 0.1 mg/ml in 0.1% bovine serum albumin/sterile PBS, Sigma) was applied for 20 s after establishing a baseline with ECS for 10 s. Neurons were allowed to recover for 5 minutes between drug applications. 50 mM KCl was used as a positive control.

During imaging of FLS, a 10 s baseline was established with ECS and then pH 4-7, and TRP agonists, 1 μM capsaicin, 100 μM cinnamaldehyde and 100 μM menthol were applied for 10 s
before a wash out period of 4 min between drug applications. TRP agonist sensitivity and acid sensitivity was assessed on separate dishes. Three biological repeats were conducted on separate days for each condition. 10 µM ionomycin was used as a positive control after all FLS Ca\(^{2+}\) imaging experiments.

Analysis was conducted as described before (33). Briefly, mean grey values were extracted by manually drawing around FLS or neurons in the ImageJ software. These values were then fed into a custom-made R-toolbox (https://github.com/amapruns/Calcium-Imaging-Analysis-with-R.git) to compute the proportion of cells responding to each drug and their corresponding magnitude of response (normalized to their peak ionomycin (FLS) or KCl (neuron) response, (ΔF/Fmax); cells not crossing threshold for positive controls were excluded from the analysis).

Statistics

Comparisons between two groups with distributed variables were performed using two-sided Student’s t-tests (paired if comparing two conditions of the same sample, unpaired otherwise) with suitable corrections and amongst three groups using one-way analysis of variance (ANOVA) followed by Tukey’s post-hoc tests. Proportions were compared for categorical data using chi-sq tests. Data are shown as mean ± SEM.
Results

1. TNF-FLS, but not FLS derived from CFA-induced inflamed knees, have a pro-inflammatory phenotype and secrete cytokines that activate DRG neurons.

Key genes have been described that act as identifiers of cultured FLS isolated by enzymatic digestion of mouse joints (5), as well as for establishing their inflammatory phenotype. In this study, we performed RT-qPCR on adherent cells (P2-P5) originating from mouse patellae that proliferated in culture (Figure 1A, B). From P2 to P3, the expression of the macrophage marker (1,5) cluster of differentiation 68 (\(Cd-68\), relative to the macrophage cell line RAW 267.4) was significantly reduced, whereas the expression of FLS markers \(Cdh-11\) and \(Cd-248\) (1,5) remained consistent from P2 to P5 (Supplementary Figure 1 in Additional file 1); the endothelial marker \(Cd-31\) was not detected (data not shown). These results suggest that from P3, cells cultured from mouse patellae were predominantly FLS and hence subsequent studies were conducted on FLS from P3-P6.

Concurrently, to establish the pro-inflammatory phenotype of FLS, we used RT-qPCR to determine the expression of the inflammatory genes \(Il-6\), \(Il-1r1\) and \(Cox-2\), as well as the constitutively expressed gene \(Cox-1\). When control FLS (P5) were stimulated with 10 ng/ml TNF-\(\alpha\) for 48-hours, there was an upregulation of \(Il-6\) (fold changes: Control, 1.5 ± 0.8 vs. TNF-FLS, 135.1 ± 31.1, \(n = 3\), \(p = 0.006\), unpaired t-test) and \(Il-1r1\) (fold changes: Control, 1.1 ± 0.2 vs. TNF-FLS, 3.4 ± 1.0, \(n = 3\), \(p = 0.04\), unpaired t-test) expression levels, but not that of \(Cox-2\) (fold changes: Control, 1.0 ± 0.2 vs. TNF-FLS, 0.8 ± 0.2, \(n = 3\), \(p = 0.2\), unpaired t-test) or \(Cox-1\) (fold changes: Control, 1.0 ± 0.2 vs. TNF-FLS, 0.8 ± 0.2, \(n = 3\), \(p = 0.18\), unpaired t-test) (Figure 1C).
However, when FLS derived from the inflamed knee (Ipsi, knee width, pre-CFA, 3.1 ± 0.09 mm vs. post-CFA, 4.1 ± 0.08 mm, n = 6, p = 0.0001, paired t-test, Supplementary Figure 1 in Additional File 1) of CFA injected mice was compared to those of the matched contralateral knee (Contra), we did not find any changes in expression levels of the genes between Contra and Ipsi FLS across P2-P5 (Figure 1C, Supplementary Figure 1 in Additional File 1).

We next tested the media (n = 3, each) isolated from control, Contra, Ipsi and TNF-FLS against a mouse inflammation antibody array (Figure 1E) to determine the levels of different secreted pro-inflammatory mediators. This demonstrated that when compared to control FLS media, TNF-FLS media showed presence of Regulated on activation, normal T-cell expressed and secreted (RANTES) and granulocyte-macrophage colony stimulating factor (GM-CSF). Additionally, TNF-FLS contained higher levels of IL-6 (p < 0.0001), keratinocyte chemoattractant (KC, mouse homolog of IL-8, p < 0.0001), lipopolysaccharide induced chemokine (LIX, p < 0.0001), stromal cell derived factor 1 (SDF-1, p < 0.0001), fractalkine (p < 0.0001), monocyte chemoattractant protein 1 (MCP-1, p < 0.0001) and macrophage inhibitory protein 1γ (MIP-1γ, p = 0.002) and lower levels of macrophage colony stimulating factor (MCSF, p = 0.003) compared to control FLS media (multiple t-tests with Holm-Sidak corrections, Figure 1Dii). TNF-α was not detected in TNF-FLS media as previously shown in TNF-α stimulated airway epithelial cells (40). Mirroring the absence of pro-inflammatory gene upregulation in Ipsi vs. Contra FLS, the spot intensity values of inflammatory cytokines between Ipsi and Contra FLS were similar (multiple t-tests with Holm-Sidak corrections, graph not shown), suggesting similar levels of secreted cytokines. However, when spot intensities were normalized to control FLS, TNF-FLS media showed increased levels of secreted IL-6 (p < 0.0001, ANOVA followed by Tukey’s multiple comparison test) and KC (p
Interestingly, MIP-1\(\gamma\) was elevated in both Ipsi and Contra FLS when compared to control and TNF-FLS (Figure 1Diii), perhaps indicating its role in acute, systemic inflammatory pathways. MIP-1\(\gamma\) has been described as having a role in hyperalgesia induced by diabetic neuropathy (41) and OA-related pain (42), and we find that MIP-1\(\gamma\) directly activates mouse DRG neurons by producing a dose-dependent \(\text{Ca}^{2+}\) influx (Supplementary Figure 1 in Additional File 1), which thus might be a potential mechanism for the \textit{in vivo} effects described above. However, because MIP-1\(\gamma\) does not have a human homolog (NCBI gene database entry #20308), and thus has limited clinical potential, we did not explore this further.

Taken together, our data suggest that TNF-\(\alpha\) stimulation of FLS upregulates expression of canonical inflammatory markers, whereas the \textit{in vivo} pathology observed 24-hours after CFA-induced knee inflammation does not correlate with a sustained pro-inflammatory phenotype of FLS isolated from patellae of CFA mice. We also identify other soluble mediators secreted by TNF-FLS that might be involved in FLS-DRG neuron communication. Therefore, given the pro-inflammatory phenotype of TNF-FLS (and the lack of such phenotype in the CFA model), we focused solely on the TNF-FLS for the rest of the study.

2. TNF-FLS have reduced sensitivity to mild acidosis, but enhanced sensitivity to more acidic pH.

During arthritis, FLS are located in an environment with abundant algogens that have been shown to signal through the modulation of TRP channels (2). Therefore we tested FLS sensitivity to a...
range of acidic pH stimuli, as well as the prototypic TRP channel agonists, capsaicin (TRPV1),
cinnamaldehyde (TRPA1) and menthol (TRPM8) using Ca\(^{2+}\) imaging. Confirming results from
other studies on acid sensing in FLS (22), we found that both control and TNF-FLS respond to a
range of pH solutions (pH 4.0 – 7.0) with an increase in intracellular Ca\(^{2+}\) (Figure 2A, B). An
increased percentage of control FLS (24.1 %) respond to pH 7.0 compared to TNF-FLS (11.0 %,
p = 0.0004, chi-sq test), but in the more acidic range an increased percentage of TNF-FLS
responded to acid than did control FLS, i.e. pH 6.0 (control, 7.2 % vs. TNF-FLS, 26.3 %, p <
0.0001, chi-sq test) and pH 5.0 (control, 14.4 % vs. TNF-FLS, 30.5 %, p < 0.0001 , chi-sq test).
At the very acidic pH of 4.0, ~ 40% (p = 0.4, chi-sq test) of FLS responded in both groups possibly
due to ceiling effect. Although the proportion of responding neurons varied, the magnitude of peak
normalized response was similar across the pH range in control (pH 7.0: 0.4 ± 0.04 F/Fmax (n =
50), pH 6.0: 0.4 ± 0.08 F/Fmax (n = 27), pH 5.0: 0.3 ± 0.03 F/Fmax (n = 46), pH 4.0: 0.3 ± 0.02
F/Fmax (n = 129)) and TNF-FLS (pH 7.0: 0.3 ± 0.06 F/Fmax (n = 21), pH 6.0: 0.4 ± 0.03 F/Fmax
(n = 50), pH 5.0: 0.3 ± 0.02 F/Fmax (n = 58), pH 4.0: 0.3 ± 0.02 F/Fmax (n = 72), Figure 2Bii).
To identify the proton sensors expressed by FLS that likely mediate the responses measured, we
conducted RT-PCR of control and TNF-FLS to determine expression of known proton-sensing G
protein-coupled receptors (GPCRs), ASICs and TRPV1. Our data show that FLS express the
proton sensing GPCRs – Gpr4, Gpr65, Gpr68 and Gpr132, as well as Asic1, Asic3 and Trpv1
(Figure 2Ci). When relative expression was assessed compared to the housekeeping gene 18S,
Gpr132 was found to be increased (p = 0.005, Figure 2Cii) in TNF-FLS implicating it as a potential
contributor to the enhanced proportion of acid responders seen in TNF-FLS (Figure 2Bi). The pro-inflamatory phenotype of TNF-FLS was confirmed by the increased Il-6 band intensity (p <
0.0001), as observed previously using qPCR (multiple t-tests with Holm-Sidak corrections, Figure 2Cii).

We also tested FLS sensitivity to capsaicin (control, 3/201, TNF-FLS, 13/252), cinnamaldehyde (control, 1/201, TNF-FLS, 11/252) and menthol (control, 0/201, TNF-FLS, 1/194) to find that only a few cells responded to each compound (data not shown). This led us to conclude that there are very few mouse primary FLS with functional TRPV1 (also corroborated by the observation of low Trpv1 gene expression, Figure 2C), TRPA1 or TRPM8 ion channels and hence we did not explore this further.

3. TNF-FLS increase knee-innervating DRG neuron excitability in co-culture.

Changes in primary sensory neuron excitability underlie peripheral sensitization which drives arthritis-related pain (43). Non-neuronal cells like FLS, which are in close proximity with distal terminals of knee neurons, can play instrumental roles in modulating sensory neuron excitability by direct cell contact and/or secretion of pro-inflammatory cytokines. To determine neuron/FLS communication in health and inflammation we compared the DRG neuronal excitability of four groups: 1) knee neuron mono-culture, 2) knee neurons co-cultured with control FLS (Figure 3A, B), 3) knee neurons with FLS that have been exposed to TNF media for 24-hour followed by DRG culture media for 24-hours and 4) knee neurons with conditioned media from FLS that have been exposed to TNF media for 48-hours to understand the role of soluble mediators in peripheral sensitization. In current-clamp mode, we observed many neurons spontaneously firing AP in groups 3 and 4. In order to statistically compare the proportions, we converted our four group data into binary categories: neuron mono-culture and neuron/control FLS co-culture were assigned to the class “healthy”, and neuron/TNF-FLS and neuron/TNF-FLS media were assigned to the class “inflamed”. 19.5% of inflamed neurons (neuron/TNF-FLS, 5/21; neuron/TNF-FLS media, 3/19)
fired spontaneous AP compared to 2.7% (neuron mono-culture, 0/19; neuron/control FLS, 1/18) of healthy neurons (p = 0.02, chi-sq test) suggesting that there is a general increase in excitability of knee neurons when exposed to an FLS-mediated inflammatory environment (Figure 3C, D).

Upon measuring the AP properties (Figure 3D inset) we found that the resting membrane potential (RMP) was more depolarized in the neuron/TNF-FLS media group compared to both neuronal mono-culture (p = 0.0004) and neuron/control FLS co-culture (p = 0.012), which highlights that secreted pro-inflammatory factors from TNF-FLS likely act upon knee neurons to increase their excitability (ANOVA followed by Tukey’s post hoc comparison, Figure 3Ei). The other measured properties were unchanged among the four groups (ANOVA followed by Tukey’s post hoc comparison, Table 1, Figure 3Eii-iv).

4. TNF-FLS increase TRPV1 function and decrease TRPA1/TRPM8 function of DRG neurons in co-culture.

DRG neurons in co-culture with inflamed FLS derived from AIA rats reportedly have increased expression of TRPV1 (6). Here we sought to investigate whether FLS can modulate knee neuron responses to TRP agonists using whole-cell patch-clamp recordings. Knee neurons in mono-culture and when co-cultured with control FLS displayed very similar responses, i.e. a mean capsaicin peak current density response of 3.5 ± 1.0 pA/pF (n = 7) and 3.2 ± 0.9 pA/pF (n = 7) respectively. By contrast, knee neurons in neuron/TNF-FLS co-culture showed a trend for larger magnitude of capsaicin responses (27.7 ± 9.3 pA/pF, n = 8), and those cultured with TNF-FLS media had a capsaicin response of 65.8 ± 25.7 pA/pF (n = 7) which was significantly larger in magnitude than neuronal mono-culture (p = 0.02) and neuron/control FLS co-culture (p = 0.02)
(ANOVA followed by Tukey’s post-hoc comparison, Figure 4Ai,ii). No difference was observed between the percentage of capsaicin responders in healthy (40.5%) vs. inflamed groups (37.5%, p = 0.8, chi-sq test, Figure 4Aiiii).

With regards to menthol (mono-culture, 2.4 ± 1.2 pA/pF, n = 7; neuron/healthy FLS, 4.8 ± 2.1 pA/pF, n = 6; neuron/TNF-FLS, 1.4 ± 1.2 pA/pF, n = 2; neuron/TNF-FLS conditioned media, 4.0 ± 1.9 pA/pF, n = 4; Figure 4Bi) and cinnamaldehyde (mono-culture, 1.9 ± 0.7 pA/pF, n = 10; neuron/healthy FLS, 2.8 ± 1.1 pA/pF, n = 4; neuron/TNF-FLS, 0.6 ± 0.3 pA/pF, n = 3, neuron/TNF-FLS conditioned media, 2.3 ± 1.5 pA/pF, n = 3; Figure 4Ci) responses, all four groups showed similar mean peak current density values (Figure 4Bii, Cii). However, in response to both the TRPA1 and TRPM8 agonists, the percentage of responding neurons was significantly less in the inflamed group compared to healthy (menthol: healthy vs. inflamed, 37.8% vs. 15%, p = 0.02, chi-sq test; cinnamaldehyde: healthy vs. inflamed, 43.2% vs. 15%, p = 0.006, chi-sq test, Figure 4Biii, Ciii). Taken together our data suggests that “inflamed” FLS can alter TRP agonist response of knee neurons in co-culture.
Discussion

In this study we demonstrate that FLS can be obtained from mouse patella via cell-outgrowth and maintained in culture. When stimulated with TNF-α, these FLS show an increase in: pro-inflammatory gene expression, secretion of pro-inflammatory cytokines and acid sensitivity in the pH 6.0-5.0 range consistent with a pro-nociceptive activated phenotype. Finally, we establish a co-culture system, which shows that FLS and knee neurons can interact, specifically, factors secreted by TNF-FLS increase knee neuron excitability and the magnitude of the response to capsaicin, whereas the proportions of cinnamaldehyde and menthol responding neurons are diminished. To the best of our knowledge, this is the first report to demonstrate FLS-mediated changes in neuronal excitability in co-culture and hence directly demonstrate how FLS can regulate articular neurons and in turn arthritis-related pain.

FLS from mouse are generally cultured by enzymatically digesting and combining excised joints of fore- and hind-limbs (5,21,22,44), which assumes similarity of FLS derived from all joints. However, a genome-wide study on DNA-methylation has shown that important differences exist between knee and hip FLS, including of genes involved in IL-6 signaling (45). By using a cell-outgrowth method to culture mouse FLS, as previously described in humans (46) and rats (6), we have avoided the biological ambiguity introduced by joint-to-joint variability. Using these FLS, we investigated the expression of inflammatory genes Il-6, Il-1r1 and Cox-2, all of which have been linked to the inflammatory phenotype of FLS. In brief: stimulating human-derived FLS with
IL-1β increases Cox-2 and IL-6 expression (46); increased IL-6 expression is seen in FLS derived from K/BxN mice and following 24-hour stimulation of healthy mouse FLS with TNF-α (5).

Lastly, supernatants from FLS derived 3-days into a rat AIA model show increased IL-6 and PGE₂ (6). Here we observed that neither the expression level of II-6, II-1r1, Cox-2, nor the level of secreted cytokines were upregulated in FLS derived from CFA-injected knee. This is possibly because the model used here is too brief to influence FLS gene expression. Indeed, evidence to support this is that FLS derived from rats with longer (3-28 days) AIA-induced knee inflammation did have higher PGE₂ and IL-6 concentrations in culture supernatants compared to control FLS (6). Alternatively, the affected area of the synovium might be too small for sufficient proliferation of “inflamed” FLS and thus they are lost over time in culture. This result suggests a limited role of FLS in the acute CFA model of knee inflammation with regard to modulation of neuronal excitability.

We reasoned that FLS need more direct and prolonged exposure to inflammatory mediators to show a robust inflammatory phenotype. TNF-α is one such cytokine that is locally upregulated within 3-hours of intra-planter CFA injection in mice (12). It is also present in high concentrations in the synovial fluid (47) and tissue of OA and RA patients (48,49); along with anti-TNF-α agents being the leading treatment of RA (50). Indeed, we show that TNF-α stimulated FLS display increased expression of II-6 and II-1r1 mRNA, with a concomitant increase in secretion of many pro-inflammatory cytokines, including IL-6 as has been reported previously in human FLS (51).

This observation is consistent with the “passive responder and imprinted aggressor model” of inflammatory FLS behavior (2), i.e. these cells can respond to pro-inflammatory environment and then themselves become effectors to drive disease pathology. The functional repercussions of this model are not well-explored and here we investigate two such possibilities: change in FLS
functionality after induction of inflammation and “inflamed” FLS-induced functional changes in nerves supplying the knee joint to drive pain.

Comparing the control and TNF-FLS response to acidic stimuli, we found a decreased percentage of TNF-FLS responding to pH 7.0, but in the pH 6.0-5.0 range the percentage of TNF-FLS responding to acidic stimulation increased. Multiple proton-sensors with sensitivity to varied pH range might underlie the apparent incongruity of TNF-FLS acid sensitivity in mild and strongly acidic environment (52). For example, increased expression of proton sensors programmed to detect highly acidic environments can come at the cost of sensors active at mildly acidic range. This concept has been alluded to previously with mouse primary FLS showing increased Asic3, but decreased Asic1, expression after IL-1β stimulation (22). The present study shows that ASIC1 and 3 may underlie acid sensitivity in FLS, as has been shown previously (21,22) and here we also show that multiple proton sensing GPCRs are expressed in FLS. Additionally, an increased level of Gpr132 in TNF-FLS lends support to the hypothesized role of GPR132 (15) in Ca2+ mobilization of FLS in response to acidification. Furthermore, TRPV1 is activated at pH below 6, which can contribute to the enhanced acid response of TNF-FLS in that range. Although we report very few FLS respond to capsaicin at physiological pH of 7.4 (as has been shown before (18)), more cells responded in the TNF condition compared to control.

In order to understand the effector role of FLS in driving nociception through peripheral sensitization, we set up a co-culture system combining FLS and knee neurons. Studying co-culture of rat FLS and DRG neurons, von Banchet et al showed using immunohistochemistry that bradykinin 2 receptor labelling (but not that of neurokinin 1 or TRPV1) was increased when DRG neurons were cultured with healthy rat FLS (6), i.e. co-culture of DRG neurons with FLS can alter expression of genes associated with nociception. Therefore, we first verified that knee neurons in
co-culture with control FLS do not show dysregulation of excitability or TRP agonist response. Then we asked whether TNF-FLS modulates knee neuron function and found using whole-cell patch clamp that 23% and 16% of knee neurons in neuron/TNF-FLS co-culture and neuron/TNF-FLS media (inflamed conditions) respectively evoked spontaneous AP compared to 6% in neuron/control FLS and 0% in neuron mono-culture (healthy conditions). This suggests that TNF-FLS increases excitability of knee neurons. von Banchet et al (6) also showed that compared to mono-culture, FLS derived from acute and chronic AIA rats induced an increase in TRPV1 protein expression in DRG neurons. However, we did not see increase in proportion of neurons responding to capsaicin between healthy and inflamed conditions, which might reflect a species difference and/or difference in knee-specific neuronal population (unlike in this study, von Banchet et al did not discriminate between knee-innervating neurons and non-knee innervating neurons); however, we did observe that capsaicin responsive neurons produced larger magnitude responses when incubated with TNF-FLS medium, see below. We also observed that cinnamaldehyde and menthol-evoked responses were decreased in the inflamed condition, suggesting functional downregulation of TRPA1 and TRPM8. Functional downregulation of TRPA1 might be explained through desensitization via increased TRPV1 function (see below) (53) or via an increase in intracellular Ca$^{2+}$ (due to increased excitability in the inflamed condition) (54). The latter reason can also be applied to explain decrease of menthol-evoked responses (55).

We also observed a tendency of a more depolarized RMP and an enhanced magnitude of capsaicin-evoked peak current density of knee neurons in inflamed conditions, albeit both only reached statistical significance in the TNF-FLS media incubated group when compared to mono-culture and neuron/control FLS. These results suggest that soluble mediators released by FLS are key players in modulating knee neuron excitability. We posit that because our experimental design
involved a media change 24-hours after TNF incubation of FLS (so as not to directly stimulate
neurons with residual TNF-α (56)) in the neuron/TNF-FLS co-culture condition, the accumulated
soluble mediator concentrations were lower compared to when knee neurons were incubated in
48-hours TNF-FLS conditioned media (no remaining TNF-α was measured in the media at this
point). Indeed we have recently established the role of soluble mediators present in OA synovial
fluid in increasing neuronal excitability and TRPV1 function (33). These soluble mediators mainly
consist of cytokines/chemokines which form complex signaling pathways with neurons (reviewed
in (57,58)). In this study, we have identified IL-6 (59), KC (60), RANTES (61), GM-CSF (62),
LIX (63), SDF-1 (61), MCP-1 (64), MIP-1γ (present study) to be upregulated in TNF-FLS media
which can also directly signal to neurons; and in the cases of IL-6, MCP-1 and GM-CSF increase
TRPV1 function (59,62,64). Although fractalkine does not directly stimulate neurons (57), a recent
study showed a correlation between levels of this cytokine in protein extracted from human OA
synovia and brain derived neurotrophic factor - tropomyosin receptor kinase B mediated signaling
in joint nerves to induce mechanical hyperalgesia (65).

Therefore, this study demonstrates an increase in knee neuron excitability and TRPV1 function
upon interaction with non-neuronal TNF-FLS.

Conclusion

Knee pain in arthritis is a major cause of disability and peripheral sensitization mediated by
enhanced excitability of nociceptive neurons play a key role in pain (43,66). Those neurons
supplying the knee joint are exposed to a pro-inflammatory environment due to cell-contact and
soluble mediator release from immune cells. One such key effector in the joint is FLS which can
respond to inflammatory mediators and also themselves secrete inflammatory soluble factors (2).

In this study we show that TNF-FLS increase mRNA expression of inflammation-linked genes *Il-6* and *Il-1r1* along with enhanced secretion of cytokines compared to control. Additionally, TNF-FLS can increase excitability of knee neurons and alter TRP channel function in these neurons in a co-culture system. Therefore, we establish a direct inflammation-pain axis through FLS and DRG neurons. This system can be adapted to investigate peripheral sensitization using FLS activated by other pro-inflammatory cytokine or arthritic synovial fluid.

**Declarations**

Ethics approval: Mice used in this study were regulated under the Animals (Scientific Procedure) Act 1986, Amendment Regulations 2012. All protocols were reviewed by the University of Cambridge Animal Welfare and Ethical Review Body.

Consent for publication: Not applicable.

Availability of data and materials: The datasets supporting the conclusions of this article will be available in University of Cambridge Apollo Repository (https://doi.org/10.17863/CAM.44367).

Competing interests: The authors declare no competing interests.

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Authors’ contributions:
S.C. designed experiments, conducted experiments, analyzed data and drafted the manuscript. L.A.P. and C.N.B. conducted and analyzed experiments and revised manuscript. G.C. designed experiments and revised the manuscript. D.C.B revised the manuscript. E. St. J. S. designed experiments and drafted the manuscript.

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Figure Legends:

Figure 1: Induction of inflammation in FLS derived from mouse knee. A) Representative picture showing exposed inside of the patella (black triangle) and the surrounding ligament and fat pad (white triangle) after midline resection and distal pull of the quadriceps muscles. Scale bar = 2 mm. B) Representative picture of FLS in culture. Scale bar = 50 µm. C) Bars represent fold change of the genes \textit{Il-6}, \textit{Il-1r1}, \textit{Cox-2} and \textit{Cox-1} from either Contra (vs. Ipsi) or control (vs. TNF). Black hatched bars = control FLS, red bars = TNF-FLS, grey bar = Contra, white bar = Ipsi. All FLS at P5. D) Images of mouse inflammatory array membranes probed against FLS conditioned medium (i) which were quantified by densitometry and represented as bar graphs showing fold change of various cytokines between control and TNF-FLS (ii, multiple t-test with Holm-Sidak correction) and among Contra, Ipsi and TNF from control FLS media (iii, ANOVA with Tukey’s post-hoc test) and spot intensity differences. Dotted rectangles highlight IL-6 spots (Di) and corresponding quantifications (Dii, iii). Only cytokines that were present in all of the compared groups are shown in graphs. * p < 0.05, ** p < 0.01, *** p < 0.001 and **** p < 0.0001. Error bars = SEM.

Figure 2: Acid sensitivity in FLS. A) Representative Ca$^{2+}$ imaging trace from an FLS responding to pH 5 and ionomycin. Bi) Bars representing proportions and magnitudes (ii) of control (black hatched) and TNF-FLS (red) responding to pH 7, 6, 5 and 4. Comparison between control vs. TNF-FLS made using chi-sq test. Data from three biological replicates in each category. Ci) Gel image of control and TNF-FLS expression of proton sensors along with the densitometry analysis of the relative band intensity compared to the 18S band (ii, multiple t-test with Holm-Sidak correction). ** p < 0.01, *** p < 0.001 and **** p < 0.0001. Error bars = SEM.
Figure 3: TNF-FLS mediated increase in excitability of knee neurons. A) Representative live-cell imaging picture showing FLS (magenta) and neuron (cyan) in co-culture. Scale bar = 50 µm. B) Representative FB labelled knee neuron (white arrow) being recorded using a patch pipette (triangular shadow), surrounded by FLS (yellow arrow). Scale bar = 50 µm. C) Pie-chart showing proportion of knee neurons that fired AP without current stimulation in healthy (neuron mono-culture + neuron/control FLS, n = 37, black) and inflamed (neuron/TNF-FLS + neuron/TNF-FLS media, n = 41, red) condition. D) Representative knee neuron incubated with TNF-FLS media firing spontaneous AP along with schematic diagram of AP properties measured (inset). E) Bar graphs showing measured RMP (i), threshold (ii), AHP peak (iii) and HPD (iv) from knee neurons in mono-culture (n = 19, white bar/black open circle), in co-culture with control FLS (n = 18, grey bar/black dotted circle), in co-culture with TNF-FLS (n = 20, light red bar/red dotted circle) and incubated in TNF-FLS media (n = 21, white bar/red open circle). * p < 0.05 and *** p < 0.001, ANOVA followed by Tukey’s post hoc test. Data from 4-5 female mice in each group. Error bars = SEM.

Figure 4: TNF-FLS mediated modulation of TRP agonist response in knee neurons. Representative traces showing capsaicin (TRPV1 agonist, Ai), cinnamaldehyde (TRPA1 agonist, Bi) and menthol- (TRPM8 agonist, Ci) evoked responses in knee neurons. Black traces obtained from knee neuron in mono-culture, red trace obtained from knee neuron incubated in TNF-FLS media. White boxes represent perfusion of extracellular solution. Bar graphs showing peak current densities of capsaicin- (Aii), cinnamaldehyde- (Bii) or menthol-evoked (Cii) currents from knee neurons in mono-culture (white bar/black open circle), in co-culture with control FLS (grey bar/black dotted circle), in co-culture with TNF-FLS (light red bar/red dotted circle) and incubated in TNF-FLS media (white bar/red open circle) Comparison between groups made using ANOVA with Tukey’s
post hoc test. Bar graphs showing percent of knee neurons that responded to capsaicin (Aiii), cinnamaldehyde (Biii) and menthol (Ciii) in healthy (neuron mono-culture + neuron/control FLS) and inflamed (neuron/TNF-FLS + neuron/TNF-FLS media) condition. Comparison made using chi-sq test. * p < 0.05. Data from 4-5 female mice in each group. Error bars = SEM.

Additional materials:

- File name: Additional file 1
- File format including the correct file extension: .docx
- Title of data: Table 1, 2 and Figure 1
- Description of data: Table 1 contains list of genes assessed in this study using qPCR and RT-PCR, Table 2 contains location of cytokines detected by the mouse inflammatory antibody array membrane. Figure 1 shows characterization of mouse primary FLS and neuronal activation of FLS secreted MIP-1γ.
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Table 1: AP properties of knee neurons. *** represents $p < 0.001$ when compared to knee neuron mono-culture, $*$ represents $p < 0.05$ when compared to neuron/control FLS co-culture.
|                                | Knee neuron mono-culture (n = 19) | Knee neuron/contro l FLS (n = 18) | Knee neuron/TNF-FLS (n = 21) | Knee neuron/TNF-FLS media (n = 20) |
|--------------------------------|-----------------------------------|----------------------------------|-----------------------------|----------------------------------|
| RMP (mV)                       | Mean -46.6 ± 2.0                  | Mean -42.8 ± 2.9                 | Mean -39.7 ± 2.3            | Mean -32.0 ± 2.3                 |
|                                | SEM 2.0                           | SEM 2.9                         | SEM 2.3                     | SEM 2.3                         |
| Threshold (pA)                 | Mean 447.4 ± 71.5                 | Mean 319.4 ± 56.3               | Mean 245.2 ± 49.4           | Mean 315.0 ± 70.0               |
|                                | SEM 71.5                          | SEM 56.3                        | SEM 49.4                    | SEM 70.0                        |
| Half peak duration (HPD, ms)   | Mean 2.2 ± 0.4                    | Mean 2.3 ± 0.2                  | Mean 2.4 ± 0.5              | Mean 2.4 ± 0.6                  |
|                                | SEM 0.4                           | SEM 0.2                         | SEM 0.5                     | SEM 0.6                         |
| Afterhyperpolarization amplitude (AHP peak, mV) | Mean 13.7 ± 2.6                  | Mean 19.6 ± 2.7                 | Mean 16.1 ± 2.9             | Mean 21.6 ± 3.3                 |
|                                | SEM 2.6                           | SEM 2.7                         | SEM 2.9                     | SEM 3.3                         |
Figure 1

A

B

C

D

Di

Contra

Ipsi

Dii

iii

Control

TNF

Contra

Ipsi

Fold change

Control

TNF

Contra

Ipsi

Normalized spot intensity (A.U.)

Fold change from Control

Contra

Ipsi

TNF

Control
Figure 2

A

Bi

ii

Ci

ii

Expression relative to 18S

Control

TNF

bp

Gpr4

Gpr65

Gpr132

Ase1

Ase2

Ase3

Ase4

Tnp1

m-ccl

18S

Expression relative to 18S

Control

TNF
Figure 3

A

B

C

D

E
Table 1: Genes of interest analyzed in this study.

| Gene   | TaqMan Assay ID/sequence | Role                                |
|--------|--------------------------|-------------------------------------|
| **Taqman Probes**                      |                             |                                     |
| *18S*  | Mm03928990_g1            | Housekeeping                      |
| *Cd-68* | Mm03047343_m1          | Macrophage marker                 |
| *Cdh-11* | Mm00515466_m1          | Intimal synovial fibroblast marker |
| *Cd-248* | Mm00547485_s1          | Synovial fibroblast marker        |
| *Cd-31*  | Mm01242576_m1          | Endothelial marker                |
| *Il-6*   | Mm00446190_m1          | Inflammation                      |
| *Il-1r1* | Mm00434237_m1          | Inflammation                      |
| *Cox-1*  | Mm04225243_g1          | Constitutively active gene        |
| *Cox-2*  | Mm03294838_g1          | Inflammation                      |

**RT-PCR primers**

| Gene   | Fwd/Rev sequence | Product size |
|--------|------------------|--------------|
| *18S*  | Fwd: CCGGTACAGTGAAACTGCGA  
Rev: ATCTAGAGTCAACCAAGCCGC | 230 bp       |
| *Gpr4* | Fwd: ATTCAGCACCCGCTCTTCCAT  
Rev: CAGGGCCAGACGTGGATCT | 236 bp       |
| *Gpr65* | Fwd: CAACATGGATCTTTTATGCG  
Rev: ATGTAGTGAAGAAAACGCT | 195 bp       |
| *Gpr68* | Fwd: TTCTCCTTCCTCCTCAAGCAG  
Rev: GGCTGAGTGAGCTGGTTA | 234 bp       |
| *Gpr132* | Fwd: CCCACTCTGCCTGTCCAT  
Rev: CAGGAAGATGGTGACGACC | 161 bp       |
| *Asic1* | Fwd: ACACATCTCAACTCCGGCCAA  
Rev: TGCTCCTGCAAGACACAAA | 250 bp       |
| Gene  | Fwd: Primer Sequence | Rev: Primer Sequence | Product size | Function                        |
|-------|----------------------|---------------------|-------------|---------------------------------|
| Asic2 | TGCTGCCCTACTTTGGTGACA| CGGAGTGGTTTGGCATTGTG| 194 bp      | Acid-sensing ion channel        |
| Asic3 | AGAAGGAGCTCTCAAAGGC| AGGTAACAGGTACGGTGGGA| 158 bp      | Acid-sensing ion channel        |
| Asic4 | AGCGGCTAACTTATCTGCC| CAAGGGAGTCCAGTGTTGGG| 234 bp      | Acid-sensing ion channel        |
| Trpv1 | GACACCATTGCTCTGCTCT | GCCTGGACATCTGCTCCATT| 176 bp      | Heat and proton transducing ion channel |
| Il6   | AGCCAGAGTCCTTCAGAGA | TGGTCTTTGGTCCTTAGCCAC| 226 bp      | Inflammation                    |

Fwd = Forward primer sequence, Rev = reverse primer sequence.
Supplementary Table 2: Location of cytokines detected by the mouse inflammatory antibody array membrane.

|   | A     | B     | C     | D     | E     | F     | G     | H     | I     | J     | K     | L     |
|---|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| 1 | Pos   | Pos   | Neg   | Neg   | Blank | BLC   | CD30 L| Eotaxin| Eotaxin-2| Fas Ligand| Fractalkine| GCSF   |
| 2 | Pos   | Pos   | Neg   | Neg   | Blank | BLC   | CD30 L| Eotaxin| Eotaxin-2| Fas Ligand| Fractalkine| GCSF   |
| 3 | GM-CSF| IFNγ  | IL-1α | IL-1β | IL-2  | IL-7  | IL-4  | IL-6   | IL-9   | IL-10  | IL-12 p40/p70| IL-12 p70|
| 4 | GM-CSF| IFNγ  | IL-1α | IL-1β | IL-2  | IL-7  | IL-4  | IL-6   | IL-9   | IL-10  | IL-12 p40/p70| IL-12 p70|
| 5 | IL-13 | IL-17 | I-TAC | KC    | Leptin| LIX   | MCP-1 | M-CSF  | MIG    | MIP-1α | MIP-1γ   |
| 6 | IL-13 | IL-17 | I-TAC | KC    | Leptin| LIX   | MCP-1 | M-CSF  | MIG    | MIP-1α | MIP-1γ   |
| 7 | RANTES| SDF-1 | TCA-3 | TECK  | TIMP-1| TIMP-2| TNF-α | sTNF R | sTNF R | Blank  | Blank    | Pos     |
| 8 | RANTES| SDF-1 | TCA-3 | TECK  | TIMP-1| TIMP-2| TNF-α | sTNF R | sTNF R | Blank  | Blank    | Pos     |

BLC = B lymphocyte chemoattractant, GCSF = granulocyte colony stimulating factor, GM-CSF = granulocyte-macrophage colony stimulating factor, IFN = interferon, IL= interleukin, I-TAC = interferon inducible T-cell alpha chemoattractant, KC = Keratinocyte chemoattractant (chemokine ligand 1), LIX = lipopolysaccharide induced chemokine, MCP = monocyte chemoattractant protein, M-CSF = macrophage colony stimulating factor, MIG = monokine induced by gamma interferon, MIP = macrophage inflammatory protein, RANTES = Regulated on activation, normal T-cell expressed and secreted, SDF= stromal cell derived factor, TCA = T-cell activation gene, TECK = thymus expressed chemokine, TIMP = Tissue inhibitor of metalloproteinase, TNF(R) = tumor necrosis factor (receptor), Pos = Positive spot, Neg = negative spot.
Supplementary Figure 1

Characterization of FLS

A

\( \text{Cd-68} \)

Bi

\( \text{Cdhl-11} \)

ii

\( \text{Cd-248} \)

Expression of inflammation-related genes in FLS derived from CFA-injected knee joint

Ci

Contra Ipsi

ii

\( \text{Il-6} \)

\( \text{Il-1\text{r1}} \)

Cox-2

Cox-1

MIP-1\( \gamma \) activation of DRG neurons

D

\( \frac{F}{F_{\text{max}}} \) (A.U.)

\( \% \text{ responder} \)

\( \frac{F}{F_{\text{max}}} \) (A.U.)

100 s

***

0.0

0.5

1.0

MIP-1\( \gamma \)

10 - 100

10 - 100

MIP-1\( \gamma \) (ng/ml)

MIP-1\( \gamma \) (ng/ml)
Supplementary Figure 1: Characterization of mouse primary FLS and neuronal activation of FLS secreted MIP-1γ. A) Bars showing reduction in mRNA expression of the macrophage marker Cd-68 from P2 to P3 expressed as fold change from macrophage cell line Raw 264.7 cells. ** p <0.01, ratio paired t-test. Bi) Expression of FLS marker Cdh-11 across P2-P5 in ipsi and contra FLS normalized to housekeeping gene 18S (left) and a representative image of CDH-11 (red) and DAPI (blue) stained FLS in culture. ii) Expression of FLS marker Cd-68 across P2-P5 in ipsi and contra FLS normalized to housekeeping gene 18S. Ci) Mouse knee width in Contra (grey circles) and Ipsi (open circles) limbs before and after injection of CFA (n = 6, paired t-test. ii) Bars represent fold change of the genes Il-6, Il-1r1, Cox-2 and Cox-1 from Contra vs. Ipsi. Black bars = P2, grey bars = P3. D) Intracellular Ca²⁺ influx in lumbar DRG neurons in response to 20 s application of 100 ng/ml MIP-1γ, followed by percentage of neurons responding to 10 and 100 ng/ml of MIP-1γ and their respective peak response. > 500 neurons imaged from three male mice. *** p < 0.001, chi-sq test. Error bars represent SEM.