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Chronic Inflammation and Pain in a TNFR (p55/p75-/-) Dual Deficient Murine Model

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Abstract

Many aspects of tissue damage following acute or chronic inflammatory reactions can be directly attributed to the concomitant biosynthesis and release of inducible early pro-inflammatory cytokine tumor necrosis factor alpha (TNFα). Conversely, systemic inflammation is impacted by consequences of tissue damage. Dysregulated TNFα contributes to numerous pathophysiological conditions including inflammatory bowel disease (IBD) and arthritis. Inflammatory stimuli trigger proteolytic cleavage and shedding of extracellular domains of TNFα receptors giving rise to two soluble fragments (p55 sTNFR1 and p75 sTNFR2) that block further binding, activity and synthesis of TNFα. We hypothesized that absence of sTNFR inhibitory feedback control would result in accumulated high levels of TNFα and other inflammatory factors promoting the cardinal signs of chronic inflammation and pain.

The present study reports a translational murine model of chronic arthritis precipitated by two consecutive inflammatory insults. The “double hit” procedures provoke a chronic inflammatory response and pain related behaviors in mice that are dually deficient in p55 (TNFR1) and p75 (TNFR2). The inflammation and pain related behaviors are transient in similarly treated wild type (WT) mice. The complete Freund's adjuvant (CFA) method was used initially to induce knee joint inflammation, tactile mechanical and heat hypersensitivity, and gait disturbance. After these transient effects of the insult were resolved, a recrudescence persisting at least through 23 weeks was promoted by gastrointestinal (GI) insult with dilute intra-colonic mustard oil (MO) only in the mutant mice and was reversed by a P2X7 antagonist. Serum Proteome Profiling analysis revealed high levels of serum inflammatory factors TNFα, RANTES, CXCL9 (MIG), CXCL10 (IP-10), and CCL2 (MCP-1).

In conclusion, these data suggest that impaired signaling of TNFα due to deficit of the two protective soluble p55 and p75 sTNFR inhibitory factors plays a pivotal role in re-activation of the immune response to GI insult that can produce recrudescence of inflammatory injury and a chronic pain state through promotion of high levels of serum inflammatory factors.

Keywords

TNFα; recrudescence; autoimmune; arthritis; P2X7

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Introduction

Despite current clinical treatments (*i.e.* anti-TNFα monoclonal antibodies) that effectively diminish inflammation and pain by reducing TNFα and other cytokines, the fact remains that the inflammatory response and pain will likely re-emerge in most patients with autoimmune disease. Therefore, the disease is not completely abrogated by current therapies, suggesting unresolved processes or salvage pathways still active that can re-kindle the inflammatory reaction. Consequently, understanding molecular pathways and neuroregulatory modulation of inflammatory responses will assist in development of adjunct therapy to provide permanent remission of disease processes for more complete abrogation of pain and disability for patients with IBD and other autoimmune conditions.

The etiology of reactive clinical arthritis has been attributed to combined genetic and microenvironmental factors, most notably gastrointestinal (GI) pathogens. Toivanen (2003)[1] proposed that even normal GI flora and their degradation components harbor sufficient arthrogenic ability. Activated gut immune cells were shown to home to knee joints of patients suffering from rheumatoid arthritis (RA) never treated for GI disorders[1]. This clinical trial and others provide evidence that combined genetic polymorphisms and environmental factors such as GI bacterial infections or injuries to the joints play a critical role in the etiology of arthritis. Bacterial pathogens and fragments are frequently found in synovial fluid extracted from inflamed knees of patients, though less so in long term cases. Animal models in which two consecutive inflammatory insults are provided to enhance the immune response are often referred to as “double hit” models and will be used in this study to provoke a chronic inflammatory state.

The “double-hit” hypothesis for inflammatory pathology evolved many years ago as an explanation for transition of the innate immune response into chronic conditions such as arthritis. This hypothesis is based on the ability of soluble non-antibody proteins released from inflammatory cells to prime the immune response so that a subsequent insult elicits an amplified immune response[2]. Based on this theory and modern gene association studies, we hypothesized that (1) a known genetic polymorphism reportedly inducing high levels of TNFα combined with (2) inflammatory “double hits” would initiate chronic arthritis for study of mechanisms precipitating chronic inflammatory pain. We used mice with genetic deletion of TNFR1 and TNFR2 (TNFR1/R2-/-). Without soluble receptor (sTNFR1/2) inhibitory feedback TNFα levels would accumulate. Inflammatory “double hit” approaches were utilized to aggravate neurogenic mechanisms that would then facilitate the transition to chronic joint inflammation.

In preliminary studies, three methods of experimental arthritis were tested followed weeks later by a gastrointestinal “second hit” with colonic infusion of diluted mustard oil (MO, allylisothiocyanate) for all groups. Arthritis was induced with either (1) the antigen induced autoimmune (AIA) method using incomplete Freund's adjuvant (IFA) and methylated bovine serum albumin (mBSA)[3], (2) complete Freund's adjuvant (CFA)[4], or (3) the collagen-induced (CIA)[5] method. These agents typically evoke only transient hyperalgesic responses in WT mice. MO also promotes transient hypersensitivity responses “referred’ to the footpad[6].

Compelling data are presented here finding that a colonic MO “second hit” evokes recrudescence of CFA induced knee joint inflammation that becomes chronic in mice with genetic deletions of TNFR1 and TNFR2. The wild type background, B6129SF2/J mice, recovered fully from all of the insults. Data not shown found that TNFR1/2-/- mice are resistant to the CIA model as previously published[5] and thus did not respond with pain related behavior or inflammation. Recrudescence was minimal with the AIA model. This

suggests necessity of a pathogen evoked response to provide transition to a chronic inflammatory state. We hypothesize that recrudescence in this model occurs in the TNFR1/R2-/- mice in response to pathogen activated upregulation of TNFα serum levels that enhance inflammation and hypersensitivity that then continue in the absence of sTNFR feedback inhibition.

Methods

Animals

All animal procedures were approved by the University of Kentucky IACUC committee. Mice were monitored daily for continued weight gain and general health. Health Status and Procedures were documented daily on the UK IACUC SOP-102 Post-Operative Evaluation form. Experiments were performed using dually deficient (TNFR1/R2-/-) (20-25 g; Jackson Laboratory) and matched wild type (WT) background mice (B6129SF2/J) inbred at the University of Kentucky animal facilities. Mice were housed in individual cages with a 10h/14h dark/light reversed cycle and allowed access to food and water *ad libitum* except during testing and dosing. A flowchart for the experimental design is provided in Fig. 1.

Induction of Chronic Arthritis: "First Hit" Knee Joint Insults

Induction of CFA Monoarthritis—Mice were briefly anaesthetized with 2% isoflourane inhalation. The left knee joint cavity was injected with CFA (1mg/ml, *i.e.* 50 μg heat killed, desiccated *Mycobacterium tuberculosis* H37 (DIFCO Laboratories, Detroit, MI) in 50 μl of sterile 1:1 saline:peanut oil) solution. The CFA method was effective in providing chronic knee joint inflammation and pain related behavioral changes in both the WT and the TNFR1/R2-/- mice. Two other inflammatory methods (AIA, CIA) were tested but the results not reported in this study because the inflammation induced was transient in the case of adjuvant induced arthritis or did not occur as in the case of collagen induced arthritis as reported previously[5] by others. Several additional controls that did not evoke inflammation included vehicle or injection of IFA alone. Recrudescence did not occur in mice given the "first" or "second hit" only.

Pain-related Behavioral Assessments after Knee Joint CFA

Baseline testing of footpad nociceptive responses to mechanical and heat stimuli with von Frey fiber and paw withdrawal latency (PWL) was determined prior to induction of inflammation[9-10]. These tests are standards in the field of pain research (411,000+ references). Reflex testing for "referred" secondary mechanical hyperalgesia/allodynia with von Frey fibers was developed by Max von Frey, who in 1896 identified "pain spots" on human skin. Mechanical nociceptive thresholds were assayed using von Frey filaments according to the "up down" algorithm described before[10]. Paw withdrawal response latencies to noxious thermal stimulation were measured using the method of Hargreaves et al. [8]. Withdrawal responses from heat proceeded as in our previous studies and as described in the literature[9,11]. Pain-related behavior was assessed weekly throughout the study by an observer blinded to group assignment. Gait disturbances (curling toes, limping, guarding, etc) were tallied by an observer blinded to treatment group as in our previous studies[12].

Assessment of Secondary Mechanical Allodynia by Testing Paw Withdrawal Threshold—Mice were placed into clear cylindrical plastic enclosures (7 × 4 × 4 cm) on the metal meshed (3 × 3 mm) platform (36 × 29 × 21.5 cm). A mechanical withdrawal threshold testing was done on the plantar surface of both hind paws using a set 8 of von Frey monofilaments [(4.74) 6.0 g; (4.31) 2.0 g; (4.08) 1.0 g; (3.61) 0.4 g; (3.22) 0.16 g; (2.83)0.07; (2.36) 0.02 g; (1.65) 0.008 g]. The von Frey filaments were applied perpendicularly to the plantar surface with sufficient force to bend the monofilament slightly

and held for about 5 seconds. A positive response was defined as an abrupt withdrawal (flick response) of the foot during stimulation or immediately after the removal of stimulus. Up/down method was used, *i.e.* whenever there was a negative or positive response, the next stronger or weaker filament was applied, respectively. Testing proceeded in this manner until four fibers had been applied after the first one caused a withdrawal response, allowing the estimation of the 50% mechanical withdrawal threshold using a curve-fitting algorithm.

Assessment of Secondary Thermal Hyperalgesia—In this assay, mice were placed on a glass platform (2 mm thickness of glass) in a clear plastic enclosure similar to those described above. After 30 min of acclimation, a beam of focused light (5 × 5 mm) was directed through the glass onto the plantar surface of the hind paw. A 15 sec cutoff was used to prevent tissue damage. Prior to the start of the experiment, the light beam intensity was adjusted to 50°C to provide approximately 10 sec baseline latency. Three measurements were made per animal per test session separated by at least 5 min. A shortened PWL response in animals with knee joint inflammation was interpreted as indicative of secondary thermal hyperalgesia. The PWL tests were performed before knee joint injection as baseline, on day 3 and once a week after CFA injection.

Evaluation of the pain-related posture—The abnormal posture of each animal with an affected hind limb was given a single score using a subjective pain-related behavioral scale (spontaneous pain rating score 0-5) *i.e.* 0- normal; 1- curling of the toes, 2- eversion of the paw; 3- partial weight bearing; 4- non-weight bearing and guarding; and 5- avoidance of any contact with the hindlimb[12].

Induction of Gastrointestinal Irritation: “Second Hit” Colonic Mustard Oil Insult

The “second hit” exposed the mice to their own GI pathogens and inflammatory mediators by infusing diluted MO into the colon in week 8 after CFA.

Intracolonic Infusion of MO—Each mouse was acclimated by placed in the recording chamber 10 min before infusion of MO. Petroleum jelly (Vaseline) was applied in the perianal area to avoid the stimulation of somatic areas by contact with the irritant chemical. Mice were given vehicle or the second inflammatory insult with colonic MO infusion (50 μl, 0.5% in peanut oil, allylisothiocyanate, Sigma-Aldrich, St. Louis, MO). The colonic infusion with MO or vehicle was administered from a syringe connected to a 24 cm soft polyethylene PE 90 tubing (1.27 mm diameter) marked at 4 cm with colored tape to indicate the correct depth for insertion into the colon (and to prevent further entry). The tubing tip was flame melted to form a smooth bulb at the tip and pierced repeatedly along its length for even release of MO in the colon. Immediately after the infusion, the animal was placed in an observation cage for a 25 min recording session to determine if pain related behaviors were displayed.

Spontaneous Visceral Pain Assessment—Animals were placed in the observation chamber 10 min before the colon infusion. Immediately after the infusion, the animal was placed back in the observation chamber for a 25 min recording session. The observation chamber is a 28 × 17.5 × 12.5 cm see-through plastic home cage with one mirrored side located in an isolated room with constant “white noise”. A digital camcorder located 0.5 meter from the chamber with an unobstructed view, was used to record animal spontaneous visceral pain related behaviors. The camcorder was linked to a computer recording program for offline data analysis (Logitech Image Studio). The chamber was washed with a detergent/disinfectant and dried between animals. Postures defined as visceral pain-related behavior in this study included licking of the lower abdomen, stretching the abdomen or

hindlimb, lowering the abdomen against the floor and abdomen retractions or arching the back. Recordings were analyzed by an observer blinded to group.

Serum Sample Collection and Measurement

At the end of the study, animals were deeply anesthetized with an overdose of isoflurane inhalant prior to heart puncture to collect blood through an open thorax[13].

Cytokine/chemokine profiles—Blood samples were collected in microtainer tubes stored on ice and centrifuged for 5 min (5000 × g). Serum was assayed immediately or stored at -80°C. Cytokine/chemokine profiles of serum samples were determined using Proteomic Profiler kits (Mouse Cytokine Array Panel A array Kit #ARY006, R&D Systems) following manufacture's protocol with modifications. Values were analyzed with NIH Image J and expressed as relative densities (arbitrary unit) of dot blot.

Statistical analysis

All results are expressed as mean and standard error of mean (+/-SEM) unless otherwise stated. Data were analyzed using two-way ANOVA, followed by Bonferroni post *hoc* test using GraphPad Prism Software, (San Diego, CA), or paired t-test where indicated. Statistical significance was set at $p \leq 0.05$.

Results

The timeline for the model induction, treatments, and behavioral testing to determine evidence of hypersensitivity are diagramed in Fig. 1 flowchart. Behavioral testing indicated that pain-related postural changes, secondary mechanical allodynia, and thermal hyperalgesia were present along with the knee joint inflammation. A lowered mechanical threshold and a shortened thermal PWL tested on the footpad indicated hypersensitivity (Fig. 2). Hypersensitivity reached a peak at 3 days after knee joint injection of CFA and persisted over 3 weeks.

Pain-Related Behavioral Assessments for CFA-Induced Arthritis “First Hit”

Tactile Mechanical Sensitivity—Baseline values for behavioral testing of tactile mechanical sensitivity in both TNFR1/R2-/- mice and WT mice were equivalent. The baseline 50% paw withdraw threshold to von Frey fiber stimuli for both the WT background B6129SF2/J strain mice and TNFR1/R2-/- was 1.28-1.87 g (1.68±0.38 g average) (Fig. 2a). The 50% paw mechanical threshold lowered to 0.15±0.04 g on day 3 after CFA injection, was 0.20±0.05 g on day 7, and persisted for 3 weeks. Mechanical threshold decrease persisted for 3 weeks after CFA injection in WT mice (0.25±0.11g); 4 weeks for KO mice (0.25±0.034g). The mice were all well recovered by week 5 (1.24±0.05 g for WT and 1.50±0.05 g for KO mice).

Heat Sensitivity—The average baseline paw withdrawal latency (PWL) for withdrawal from radiant heat applied to the footpad was 10.23±0.24 sec for all mice (Fig. 2b). The PWL was shortened by about 3 seconds in WT mice with CFA induced arthritis (7.94±0.32 sec) on day 7, remained lowered at week 2 (7.87±0.8 sec), and returned back to baseline at 3 weeks (10.07±0.6 sec) equivalent to responses of wild type mice. In contrast, the PWL on the affected legs of TNF R1/R2-/- mice was 6.85±0.38 sec on day 7 and remained shortened (8.8±0.55 sec) for at least 4-5 weeks. PWL for mice with CFA induced arthritis (both wild type and KO) compared with saline control group or to their own baseline levels (10.24±0.8 sec for WT; 10.02±0.88 sec for KO) was significantly reduced, ($p<0.001$) and ($P<0.01$, two way ANOVA test).

Pain-Related Postural Scores—One day after induction of the CFA knee joint inflammation, all arthritic mice displayed gait disturbance and abnormal pain-related posture, consisting of guarding, curling of the toes, eversion of the foot, and decreased weight bearing on the affected limb and lasted about one week. The pain related posture score for each animal at baseline was recorded as zero. Changes after CFA are reflected in increased spontaneous pain rating scores, with a median of 3.0 for CFA arthritic mice persisting for 7days (Table 1).

Sensitization Induced by Colonic Mustard Oil Infusion after GI "Second Hit"

After the transient CFA-induced tactile sensitization and gait disturbances were resolved, visceral sensitization and pain-related behaviors were promoted with a GI inflammatory "second hit" insult in mice given dilute colonic mustard oil (MO) at week 8. The footpad hypersensitivity was transient for the WT mice. A recrudescence of footpad hypersensitivity occurred in the TNFR1/R2-/- mice. Joint inflammation and gait disturbance reappeared in 30% of the TNFR1/R2-/- mice. Secondary mechanical and heat hyperalgesia (decreased mechanical threshold and shortened PWL) were maintained chronically after the GI insult in the CFA injected limb in TNFR1/R2-/- mice through the remaining experimental period ending at 23 weeks. Specific details are provided below.

Visceral Sensitization—Both WT mice and KO mice developed spontaneous visceral pain related behaviors immediately after colon mustard oil infusion. Abnormal behaviors included increased facial grooming, increased body grooming, frequent lower abdomen licking, and abdomen retraction. (Fig. 3). The spontaneous pain activities lasted for 25–30 min. Subsequently, the animals would remain in a corner of the chamber.

Mechanical and Heat Sensitization—A lowered mechanical threshold and shortened PWL involving both footpads was evident 30 min after the colon MO infusion "second hit" in both groups (Fig. 2). The tactile referred hypersensitization after the visceral insult was evident on both hindpaws, *i.e.* 50% paw mechanical threshold (0.38±0.17 g for WT; 0.15±0.14 g for KO) and shortened PWL to heat stimulus (8.04±0.19 sec for WT; 7.20±0.32 sec for KO). The lowered mechanical threshold persisted for 2 weeks on both feet (0.17±0.046 g and 0.22±0.080 g, respectively) in WT mice, and 3 weeks in KO mice. Thereafter, the mechanical threshold for WT mice returned back to baseline levels (1.09±0.16 g and 1.11±0.15 g).

Lowered Mechanical Threshold and Heat Paw Withdrawal Latency Persists After "Second Hit" GI Insult in TNFR1/R2-/- mice

Mechanical Sensitivity—The footpad mechanical sensitivity threshold for the leg with the initially inflamed knee joint in TNFR1/R2-/- mice remained lowered (~0.25±0.08 g) chronically for the remainder of the experiment (23 weeks). The hypersensitive responses in the TNFR1/R2-/- mice were significantly reduced compared to the WT mice (Fig. 2a).

Heat Sensitivity—The significantly shortened PWL in the CFA injected leg remained chronically throughout the 3-4 month experimental period (Fig. 2b). A shortened PWL (6.95 ±0.3 sec - 8.04±0.6 sec) appeared on the uninjected feet of KO mice for 4-5 weeks. After MO infusion, there was not a significant change in the PWL for the WT group (9.78±0.4 sec).

Postural Changes—Spontaneous pain related postures (guarding of the injected leg) re-developed in 30% of the mice 2 weeks after the "second hit".

P2X7 Antagonist Reverses Chronic Arthritic Symptoms

At the 20 week time point, an i.t. injection of a highly potent, selective P2X7 antagonist, A438079 (50 μg/5 μl/per mice) was given to the TNFR1/R2-/- mice with chronic hypersensitivity. Pain-related behaviors, secondary mechanical allodynia (0.09±0.24 g), and thermal hyperalgesia (6.85±0.2 sec) were reversed on the injured leg of TNFR1/R2-/- mice. The reversal effect of A438079 began within 0.5 hour (0.63±0.12 g), reached a peak at 1 hour (1.08±0.24 g), and persisted for at least 3 hours (0.90±0.15 g for 50% mechanical threshold and 9.3±0.3 sec for PWL) (Fig. 4a, b).

Cytokine/Chemokine Proteome Profiles

High levels of pro-inflammatory cytokine TNFα and other inflammatory mediators were prominent at 23 weeks (5 months), including RANTES, CCL2 (MCP-1), CXCL9 (MIG), CXCL10 (IP-10). Relative dot blot densities of these mediators are shown in Table 2.

Discussion

The data indicate that chronic arthritis develops in TNFR1/R2-/- mice with CFA knee joint injections when followed by the gastrointestinal “second hit” provided by MO inflammation, while WT mice recover. Our studies determined that mice with genetic alteration of both TNFRs have maladaptive high levels of TNFα and other specific inflammatory mediators present along with tactile mechanical and heat hypersensitivity referred to the footpad. A selective antagonist for P2X7 ion channels acutely reduced the pain-related behaviors and mobility issues precipitated by chronic knee joint inflammation.

These studies demonstrate that combined genetic deletions of TNFR1 and TNFR2 receptors facilitate emergence of an altered immune state with chronic joint inflammation, hypersensitivity, and gait disturbance in animals exposed to an inflammatory “double hit” regimen. The chronic inflammatory state was induced with injection of CFA followed weeks later by a mild colitic insult with MO. We have noted previously that intracolonic MO promotes ulceration and evidence of cFos activation in the spinal cord with recovery within 4-8 hours[14]. The CFA arthritis is a well established *in vivo* model with some features of human chronic inflammatory disease and rheumatoid pathology, *i.e.* increased nociceptive responses, edema, and gait disturbances[3,15] (Arthritis Foundation, http://www.arthritis.org). In preliminary studies, the TNFR1/R2-/- mice were resistant to induction of a chronic pain state with the collagen + CFA (CIA) method, and the IFA + mBSA (AIA) method produced only transient effects (data not shown). The severity and chronic persistence of the pain related behaviors was significant in mice with CFA induced arthritis.

Further investigation of the cytokine/chemokine profile determined that high levels of serum TNFα and other inflammatory mediators [RANTES, CCL2 (MCP-1), CXCL9 (MIG), CXCL10 (IP-10)] were prominent at 5 months in TNFR1/R2-/- mice with chronic arthritis compared to the recovered WT mice. Gene mutations that produce increases in TNFα initiate cascades of other inflammatory cell-to-cell mediators including RANTES (Regulated upon Activation Normally T-cell Expressed and Secreted), MIP-1α (macrophage inflammatory protein-1alpha) and IL-8. This has been demonstrated in TNFα-treated primary cultures of synovial fibroblasts obtained from patients with inflamed joints[16-17]. Inflammatory stimuli provoke elevations of TNFα in both serum and synovial fluid of adults with arthritis[18]. Locally in the joint, inflammation occurs when TNFα binds and activates its receptors causing synoviocytes to proliferate and thicken the synovial lining (pannus). Proliferation of the synoviocyte pannus layer further amplifies local production of inflammatory mediators[19]. Dysregulation of TNFα and sTNFR1/R2 has been shown by others to contribute to joint pain and bone destruction[20-21]. High levels of TNFα in synovial

fluid are correlated with increased pain experienced by RA patients in response to mechanical and thermal stimuli[22]. TNFα itself sensitizes neuronal network provoking pain[23]. High levels of TNFα are reported in numerous other pathophysiological conditions, including, cerebrospinal fluid (CSF) of patients with complex regional pain syndrome[24], in Schwann cells from patients with painful neuropathies[25], in blood of patients with long-term pain[26], in LPS induced systemic inflammatory syndrome[27] and in CSF of headache patients[28]. Dysregulated TNF also contributes to inflammatory bowel disorder (IBD)[13,29-30], but functional involvement of TNFRs in IBD is not fully discovered. The mechanism for the high levels of TNFα that promotes the chronic pain related behavior in the absence of regulating TNFR fragments is unknown and under investigation. To date there are no other known pathways through which TNF can act. However, biochemical studies are typically done in control animals rather than animals with ongoing persistent pain related behaviors that likely have undergone plastic changes.

Translational Significance

These data supported the concept that maladaptative genetic mutation conditions that promote chronic overexpression of TNFα can contribute to the establishment of chronic inflammatory hypersensitivity, and gait disturbance. The data also imply that aberrations in TNFR1 and TNFR2 function are factors relevant to chronic arthropathies and pain in patients with polymorphic TNFR defects. It is speculated that the TNFR1/R2-/- mice develop chronic hypersensitivity through complex interactions involving local immune cells, high levels of circulating inflammatory mediators as a result of the TNFR1/R2 genetic deletions, and the neurogenic responses of the peripheral and/or central nervous system since administration of a selective P2X7 antagonist abrogated the mechanical hypersensitivity in these mice. Holmes and colleagues[31] reported that TNFR1 but not TNFR2 is found in nerve fibers innervating the spinal cord dorsal horn of WT mice. TNFR2 immunoreactivity is reportedly present in the central canal ependymal cells, motor neurons, and vascular endothelial cells. A recent study has shown in mice with single deletions of TNFR1 or TNFR2, that both TNFR1 and TNFR2 play distinct roles in regulating different phases of inflammation induced sensitization[32]. TNFR1 is ubiquitously expressed on the surface of most cell types. TNFR2 is primarily found on lymphocytes[33].

Literature review finds no single mechanism or pharmacological agent can effectively reduce chronic pain and inflammation long term. Previous studies using two consecutive immune challenges to prime pain behavioral responses serve as reminders of the complexity of the immune response which involves activation of the innate immune response of cells residing within tissues as well as the adaptive immune response[34]. However, activation of microglia, the local immune cells resident in the spinal cord, is a likely contributor to the increased vulnerability to a “second hit” similar to macrophage priming peripherally[33]. Since the P2X7 channels are present on microglia the reduction of the behavioral hypersensitivity by the P2X7 antagonist implies that this mechanism of microglial priming may be involved. The reversal of pain-related behaviors in TNFR1/R2-/- mice by the P2X7 antagonist indicates involvement of this ion channel in the arthritis model likely through sensitization of the peripheral nerves and/or the spinal cord dorsal horn ganglia. P2X7 has been found in neuronal fibers of patients with neuropathic pain[35]. It is also known that P2X7 is elevated in spinal cord microglia and in synoviocytes after LPS initiating release of IL-1β[36–37]. In addition, P2X7 stimulation of keratinocytes with ATP *in vitro* has been shown to increase CCL2 chemokine expression acutely[38].

The data provide experimental evidence that combined genetic polymorphisms and environmental factors such as GI bacterial infections or injuries to the joints play a critical role in the etiology of chronic arthritis as proposed by Toivanen (2003)[1]. The double hit model has provided us with an effective experimental tool for study of the transition from

acute to chronic inflammatory pain states. Understanding molecular pathways and neuroregulatory modulation of inflammatory responses will assist in development of adjunct therapy to provide permanent remission of disease processes for more complete abrogation of pain and disability in patients.

Conclusions

Induction of CFA knee joint inflammation followed by a colitic “double hit” with MO evokes a recrudescence of the initial knee joint inflammatory response in a mouse strain genetically deficient in the TNFR1/R2 subunits promoting chronic elevations of TNFα levels. Chronic hypersensitivity and gait disturbance persist through at least 23 weeks of trials. High levels of serum TNFα, and other inflammatory mediators, such as RANTES, CCL2 (MCP-1), CXCL9 (MIG), CXCL10 (IP-10) are also prominent in this chronic inflammatory and pain model. Pain-related behaviors, gait disturbances, remain chronically (>5 months) in TNFR1/R2-/- mice while WT background mice recover. Other non-pathogen activated methods of knee joint inflammation (AIA, CIA) did not produce chronic behavioral hypersensitivity responses in preliminary studies. This suggests that combined genetic polymorphisms and environmental factors such as GI bacterial infections or injuries to the joints play a critical role in the etiology of chronic arthritis.

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Abbreviations

AIA	Antigen induced Arthritis
CCL2 (MCP-1)	Chemokine (C-C motif) ligand 2
CFA	complete Freund's adjuvant
CIA	Collagen-induced arthritis
CXCL9 (MIG)	Chemokine (C-X-C motif) ligand 9
CXCL10 (IP-10)	Chemokine (C-X-C motif) ligand 10
DRG	Dorsal root ganglia
IACUC SOP	Institution Animal Care and Use Committee Standard Operating Procedure
IFA	Incomplete Freund's adjuvant
mBSA	Methylated bovine serum albumin
MIP-1α	Macrophage inflammatory protein-1alpha
MO	Mustard oil
PFA	Paraformaldehyde in phosphate buffered saline
PWL	Paw withdrawal latency
sTNF-R	Soluble TNF receptor
TNFα	Tumor necrotic factor
RA	Reactive arthritis
RANTES	Regulated upon Activation Normally T-cell Expressed and Secreted

WT	Wild type

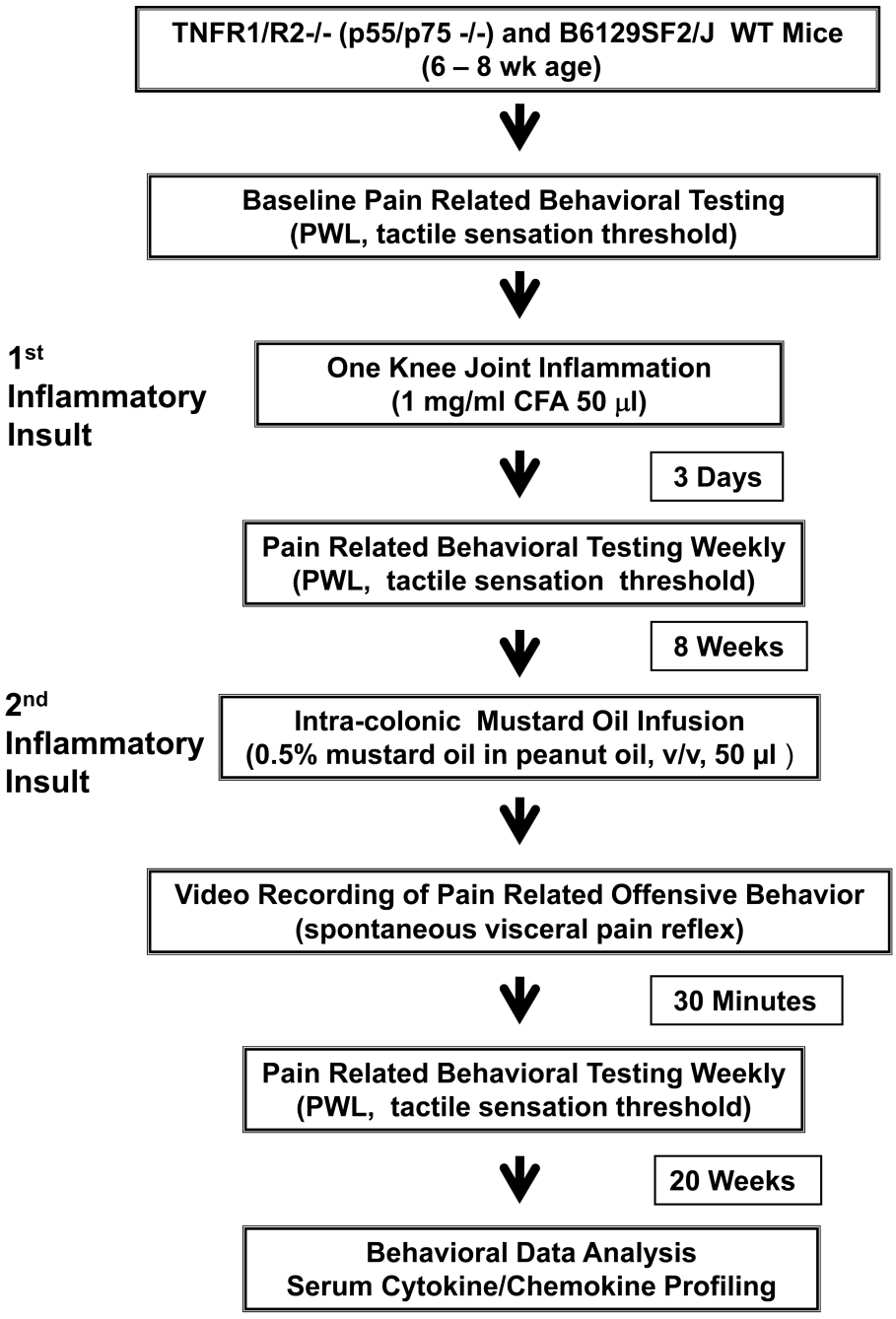

Figure 1. Experimental Design

The experimental timeline for inductions, behavioral testing and antagonist treatment, is shown for the “double hit” chronic inflammation model.

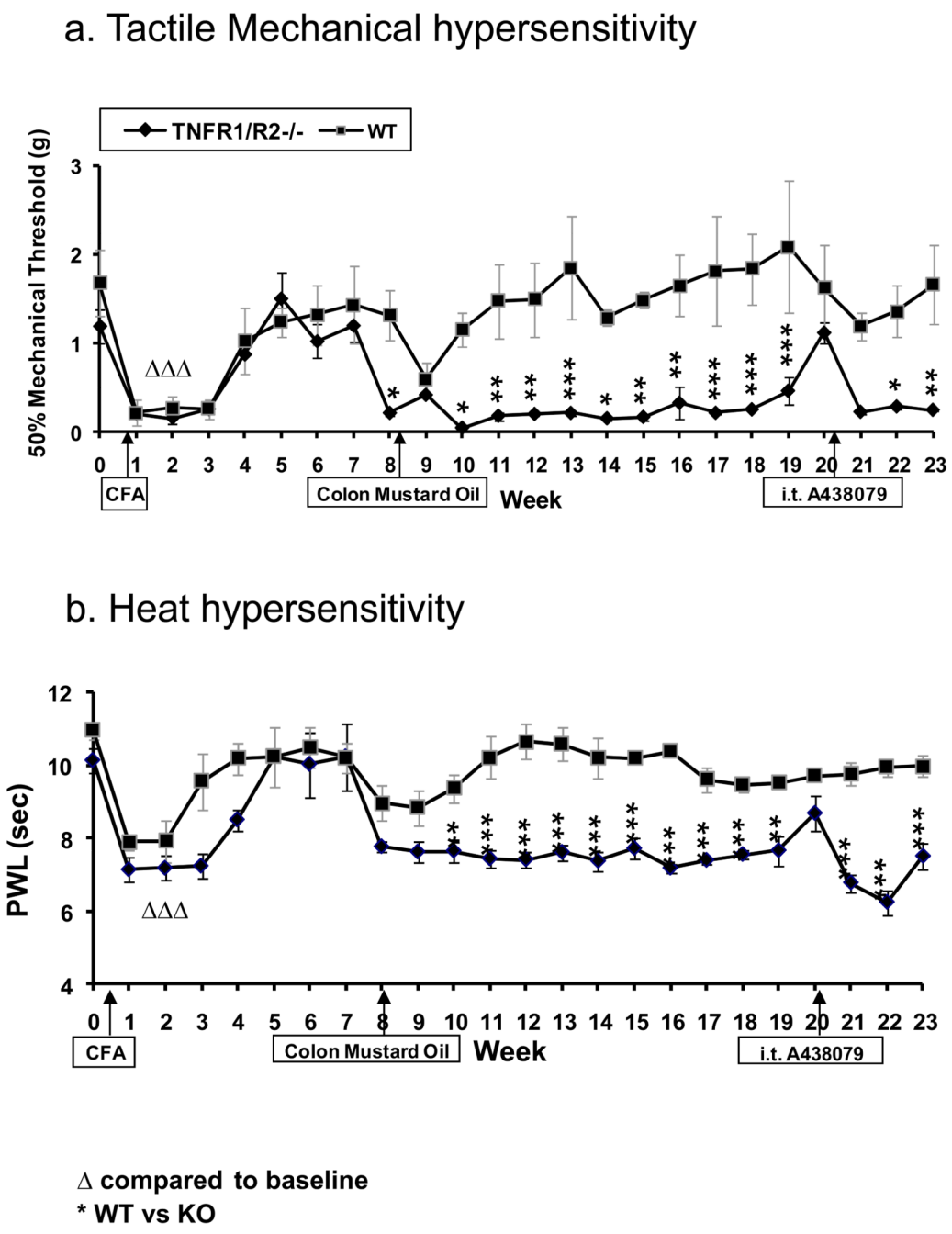

Figure 2. Development of Chronic Arthritis

Baseline **(a)** 50% paw mechanical threshold and **(b)** paw withdrawal latency (PWL) responses are the same for both TNFR1/R2-/- (p55/p75-/-) and WT mice. After knee joint CFA injection (1st arrow), pain related behaviors develop, peak at 7 – 21 days, and then dissipate. Pain related behaviors re-occur after intra-colonic MO (2nd arrow), but do not regress after the "second hit" in p55/p75 deficient mice. Sensitization, guarding and limping persist at least though the subsequent 23 weeks of testing in the TNFR1R2-/- mice. A highly potent, selective P2X7 receptor antagonist (i.t. A438079) effectively attenuates the

secondary thermal hyperalgesia and mechanical allodynia for about 3 hrs at the 20 week time point (3rd Arrow). * p≤0.05; **p≤0.01); ***p≤0.001).

Fig 3. a

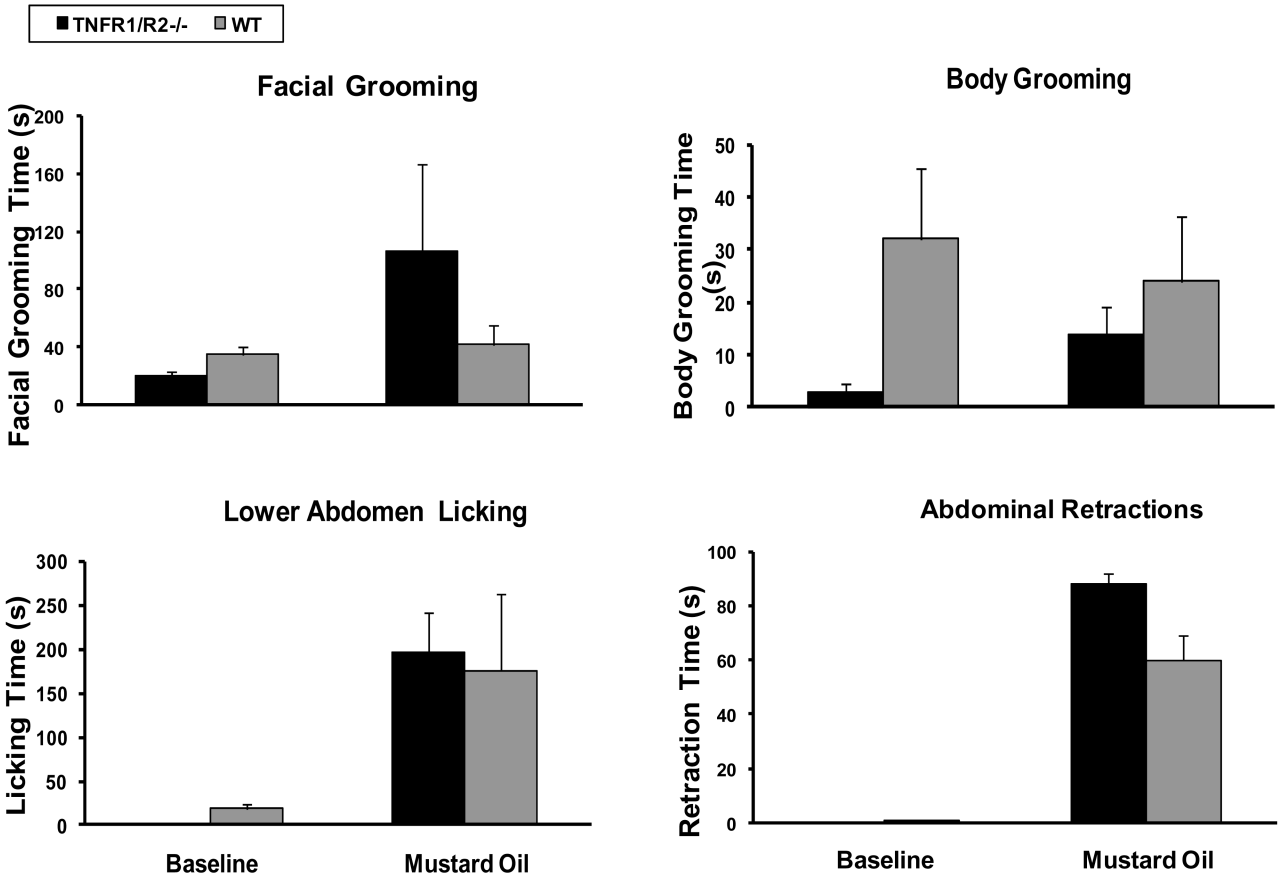

Fig 3 b

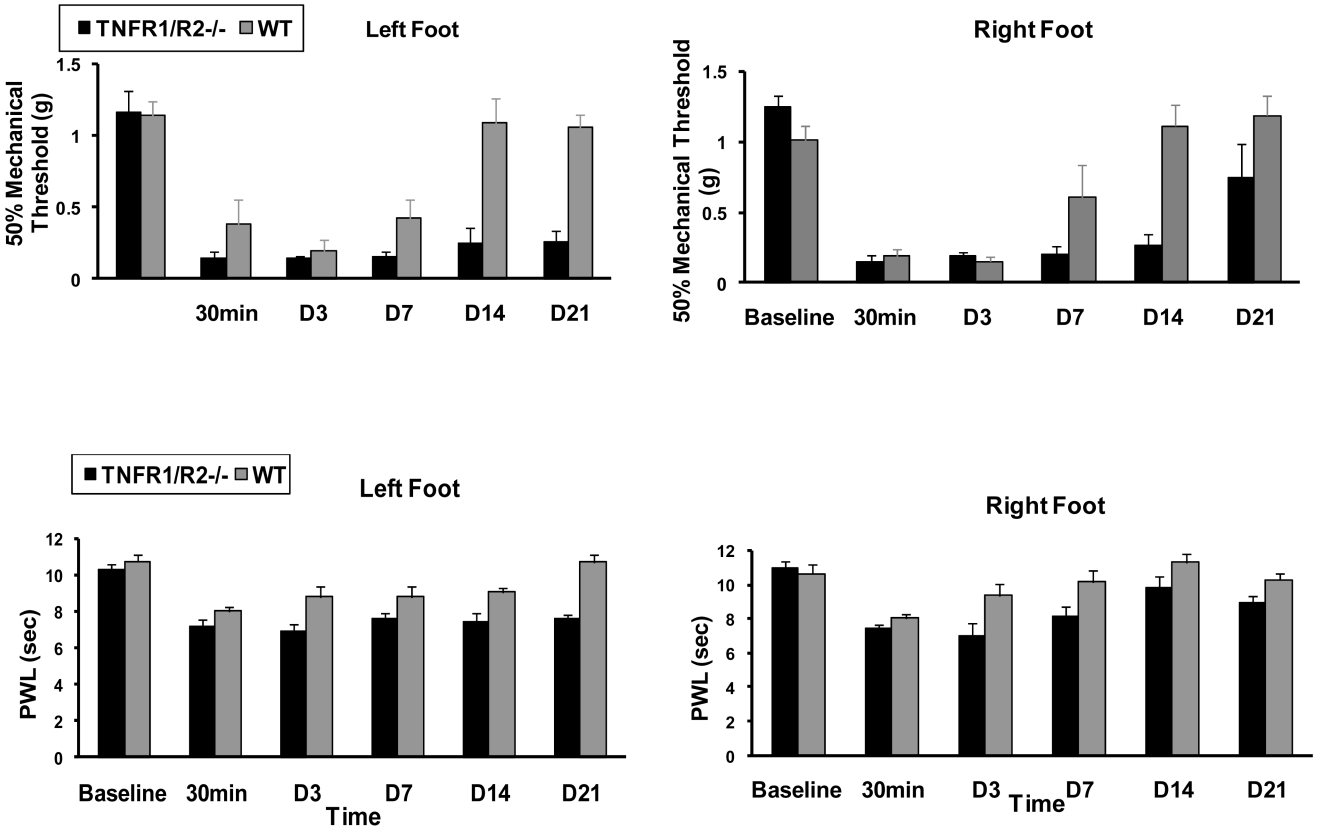

Figure 3. Visceral Pain Development in Both TNFR1/R2-/- and Wild Type Mice after MO Colonic Infusion

Spontaneous visceral pain behaviors develop immediately after MO colonic infusion in both TNFR1/R2-/- and wild type mice. Increases in lower body licking and abdominal retractions are accompanied by facial and body grooming.

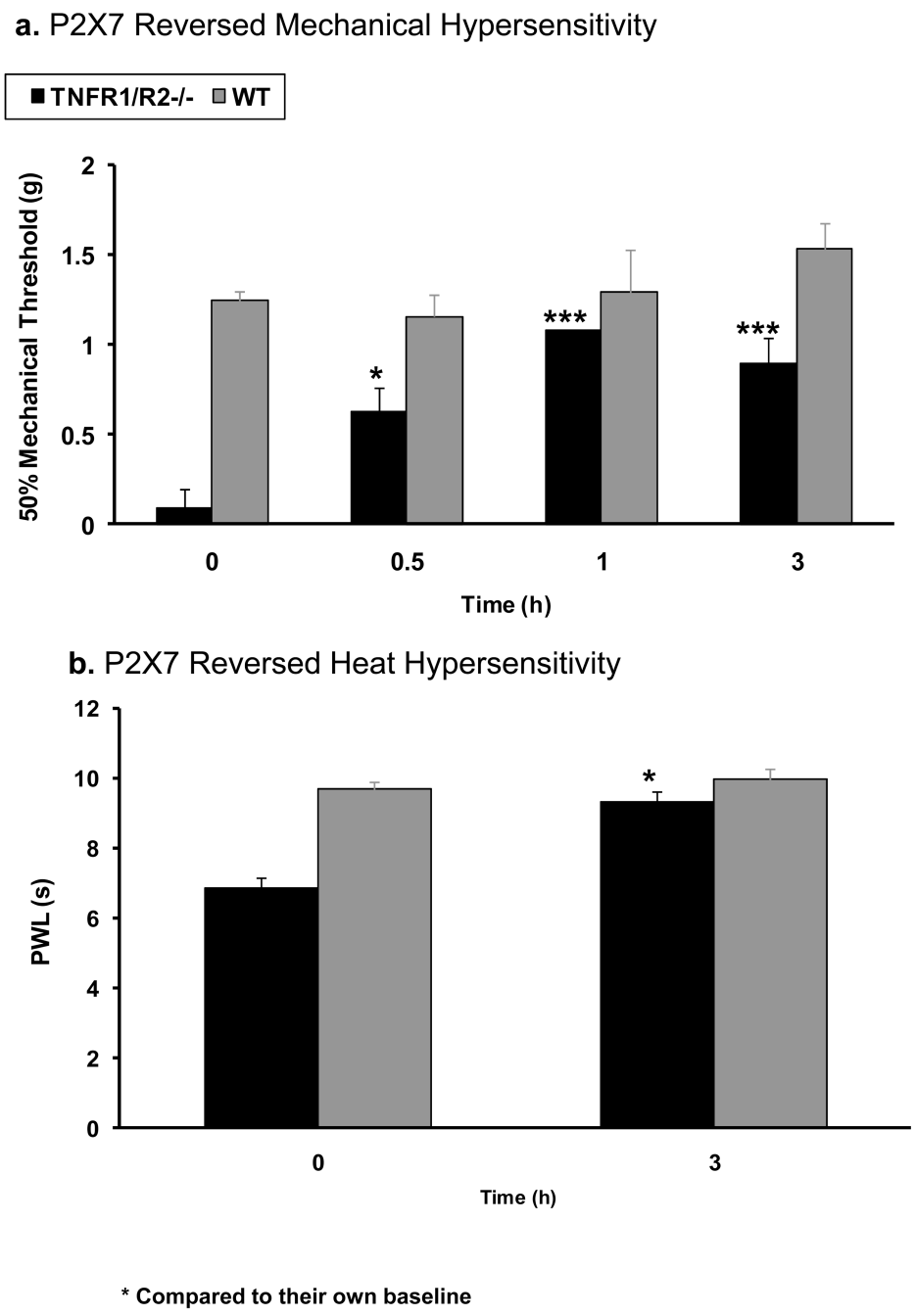

Figure 4. Chronic Arthritis Induced Hypersensitization is Reversed by P2X7 Antagonist

Intrathecal injection of a selective P2X7 receptor antagonist, A438079 (50μg/5μl/mice) reverses the tactile hypersensitivity characterized as secondary thermal hyperalgesia and mechanical allodynia in TNFR1/R2-/- mice with chronic arthritis. Sensitization begins within 0.5 hour and persists for over 3 hours. Significant differences compared to the baseline values (two-way ANOVA. * $p \leq 0.05$; *** $p \leq 0.001$).

Table 1

Spontaneous pain rating score (0-5) evaluated in CFA arthritis mice of both KO and WT. All mice displayed spontaneous pain postures 3 days after CFA knee joint injection and persisting for 1 to 2 weeks. Spontaneous pain-related behavioral scale (0-5) [12]: 0- normal; 1- curling of the toes, 2- eversion of the paw; 3- partial weight bearing; 4- non-weight bearing and guarding; and 5- avoidance of any contact with the hindlimb.

Spontaneous Pain Rating Score (MEDIAN)					
Group\ time point	**Week 0**	**Day 3**	**Week 1**	**Week 2**	**Week 3**
WT (n=6)	0	3	1.5	0	0
KO (n=6)	0	3	2.67	1.3	0.3
		ns	ns	ns	ns

Table 2

Cytokine/chemokine profile analyses at 23 weeks found high levels of TNFα and several other inflammatory mediators in TNFR1/R2-/- mice with arthritis contributing to the chronic inflammatory hypersensitivity. Relative levels in serum of TNFR1/R2-/- mice were compared to wild type mice and expressed as relative dot blot intensity (arbitrary units, a.u.).

	TNFR1/R2-/- (a.u.)	WT (a.u.)	Fold Increases TNFR1/R2-/- vs. Controls
TNFα	5911.39±851.78	705.94±49.6	8.37 *
CCL5 (RANTES)	4192.16±1000.73	1780.85±158.94	2.35 *
CCL2 (MCP-1)	4263±289.09	2091.52±310.0	2.04 *
CXCL10 (IP-10)	4922.79±652.51	2394.71±628.45	2.06 *
CXCL1	5587.85±628.34	3526.53±190.43	1.58 *
CXCL9 (MIG)	4840.78±1594.37	1946.71±1946.71	2.49

* indicates $p \leq 0.05$.