IL-33/ST2 induces neutrophil-dependent reactive oxygen species production and mediates gout pain

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Abstract

Objective: Gout, induced by monosodium urate (MSU) crystal deposition in joint tissues, provokes severe pain and impacts life quality of patients. However, the mechanisms underlying gout pain are still incompletely understood. Methods: We established a mouse gout model by intra-articularly injection of MSU crystals into the ankle joint of wild type and genetic knockout mice. RNA-sequencing, in vivo molecular imaging, Ca^{2+} imaging, reactive oxygen species (ROS) generation, neutrophil influx and nocifensive behavioral assays, etc. were used. Results: We found interleukin-33 (IL-33) was among the top up-regulated cytokines in the inflamed ankle. Neutralizing or genetic deletion of IL-33 or its receptor ST2 (suppression of tumorigenicity) significantly ameliorated pain hypersensitivities and inflammation. Mechanistically, IL-33 was largely released from infiltrated macrophages in inflamed ankle upon MSU stimulation. IL-33 promoted neutrophil influx and triggered neutrophil-dependent ROS production via ST2 during gout, which in turn, activated transient receptor potential ankyrin 1 (TRPA1) channel in dorsal root ganglion (DRG) neurons and produced nociception. Further, TRPA1 channel activity was significantly enhanced in DRG neurons that innervate the inflamed ankle via ST2 dependent mechanism, which results in exaggerated nociceptive response to
endogenous ROS products during gout. **Conclusions:** We demonstrated a previous unidentified role of IL-33/ST2 in mediating pain hypersensitivity and inflammation in a mouse gout model through promoting neutrophil-dependent ROS production and TRPA1 channel activation. Targeting IL-33/ST2 may represent a novel therapeutic approach to ameliorate gout pain and inflammation.

**Keywords:** Gout; arthritis; TRPA1; reactive oxygen species; cytokine; neutrophil;
Introduction

Gout is caused by monosodium urate (MSU) crystal accumulation in the joints and periarticular tissues [1]. It is the most common inflammatory arthritis worldwide [1]. Unfortunately, the incidence of gout is still increasing due to life style/dietary changes and the aging population [2]. Patients with gout suffer from intense joint inflammation and excruciating arthritic pain, which severely reduced the patients’ life quality [3]. Gout attack is usually treated with colchicine, nonsteroidal anti-inflammatory drugs (NSAIDs) or corticosteroids [4-6]. In spite of that, gout is still difficult to treat due to the many unwanted adverse effects of these conventional therapies [3]. Currently, interleukin-1β (IL-1β) neutralizing monoclonal antibody and synthetic IL-1R antagonists are available alternative options [7, 8]. But patients will have to be closely watched for intolerabilities or side effects [4, 9].

MSU provokes the activation of innate immune system and thereby, elicits strong inflammatory response in the joint and periarticular tissues [5, 10]. Infiltrated macrophages engulf MSU via phagocytosis and subsequently release an array of inflammatory mediators [11]. Some of these mediators may possibly interact with the peripheral nociceptors to elicit gout pain. In addition, our recent work, together with others, demonstrated that endogenous ROS products are critically involved in mediating gout pain and inflammation [12, 13]. However, it still remains elusive how ROS is generated in the inflamed tissues in the context of gout and mediates gout pain.

It was recently reported that serum IL-33 expression was increased in gout patients compared to healthy controls and positively correlated with the inflammatory indicator C-reactive protein [14,
IL-33, a member of the IL-1 family, binds to the receptor complex, consisting of IL-33 specific ST2 and IL-1 receptor accessory protein IL-1RAcp, to elicit biological functions in inflammation, autoimmune response and homeostasis [16, 17]. Recent evidence suggests that IL-33 can act as an important pain mediator in certain pain conditions, including joint pain, bone cancer pain, muscle pain and neuropathic pain [12, 18, 19]. Our recent work further identified IL-33 engages with ST2 in peripheral sensory neurons to exert sensory neuron hyperexcitability [20, 21]. These studies all demonstrate a pivotal role for IL-33/ST2 in mediating nociception and sensory neuron activities.

In the present study, we aimed to explore the potential pronociceptive or inflammatory mediators in the inflamed ankle from MSU-induced mouse gout arthritis model via RNA-Seq expression analysis. We further found that IL-33/ST2 mediates pain hypersensitivity and inflammation in the mouse gout model through promoting neutrophil-dependent ROS production and TRPA1 channel activation.

Results

1. **MSU-induced gout arthritis triggers the release of IL-33 from the inflamed ankle tissues.**

We first established the mouse model of gout arthritis by intra-articular (i.a.) injection of MSU (0.5 mg/20 μL) into mouse ankle (MSU group) as we previously described [12]. Control animal received PBS injection (20 μl PBS, i.a., Veh group). MSU injection elicited obvious ankle edema, which showed up 2 h after injection and persisted until 72 h, compared with Veh group (Figure 1A&B). Pathology analysis indicated strong inflammatory cell infiltration into the ankle of
MSU-treated mice (Figure 1C). Moreover, MSU-injected mice developed robust mechanical allodynia, manifested by reduction of paw withdraw threshold (PWT), which peaked at 24 h and lasted until 48 h (Figure 1D). To explore potential endogenous pain mediators in MSU-induced gout arthritis, we performed RNA-Seq to examine gene expression profiles in ankle tissues isolated from MSU-injected and vehicle-injected mice. Ankle tissues were collected 24 h after MSU or PBS injection and high quality RNA was extracted for RNA-Seq analysis (Figure S1A&B). A total of 1722 genes, which take up 8.9% of total genes (19323), was found to be significantly up- or down-regulated (DEGs, $|\log_2$(Fold Change)| ≥ 1.0; q≤0.001) in ankle tissues of MSU-injected mice vs. vehicle-injected mice (Figure 1E). The 594 upregulated and 1128 downregulated DEGs were illustrated in scatter plot (Figure 1F).

Among these DEGs, we especially focused on inflammatory cytokines or chemokines that were upregulated. Figure 1G showed the top 10 inflammatory cytokines or chemokines identified to be significantly up-regulated. Among these genes, certain well-known inflammatory mediators, such as Il-6, Il-1β and Cxcl3, were significantly increased in MSU group. We were particular interested in Il-33, which has not previously been implicated in gout pain, but implicated in some other pain conditions, including neuropathic, cancer and muscle pain, etc. [18, 19] Therefore, we set to examine the possible involvement of IL-33 in mediating the pain response of the mouse gout model.

qPCR confirmed that Il-33 gene were significantly increased in ankle tissues of MUS group (Figure 2A). St2, the gene encoding the specific receptor for IL-33, was significantly upregulated
as well (Figure 2B). IL-1RAcP, which forms IL-33 receptor complex with ST2, was not changed (Figure 2C). ELISA confirmed a corresponding increase of IL-33 protein concentration in ankle tissues from MSU group mice 24 h after model establishment (Figure 2D). Western blot further indicated IL-33 protein expression was significantly increased in ankle tissues of MSU group 8, 24 and 48 h after model establishment, which peaked at 24 h time point (Figure 2E). ST2 protein expression in ankle tissue was also significantly increased 24 h after MSU injection (Figure 2F). In contrast, IL-33 or ST2 protein expression was not significantly changed in Veh group at all time points (Figure 2E&F).

2. **IL-33/ST2 signaling is involved in mediating the pain hypersensitivity and inflammatory response of the mouse gout model.**

We set to study whether IL-33 was involved in mechanical allodynia of the mouse gout model. An IL-33 neutralizing antibody (5 μg/mouse, i.p.) or isotype control IgG (goat IgG, 5 μg/mouse, i.p.) was injected into mice 1 h before and 8, 23 h after model establishment (Figure 3A). IL-33 neutralizing antibody significantly reduced ankle edema and mechanical allodynia of MSU group mice at 24 and 28 h time points (Figure 3B&C). IL-33 neutralizing antibody gradually lost its effect 8 h after its last application (Figure 3B&C). We further examined the effects of IL-33 using IL-33 deficient (Il-33−/−) mice. Similarly, Il-33−/− mice exhibited reduced ankle edema and mechanical allodynia compared with wild type (WT) control group after model establishment (Figure 3D&F). Area under the curve (AUC) indicated accumulated reduction in mechanical allodynia and ankle edema in Il-33−/−+MSU group vs. WT+MSU group (Figure 3E&G). We then studied whether ST2 was also involved in the mechanical allodynia of mouse gout model using
St2 deficient (St2^−/−) mice. When MSU was injected, St2^−/− mice (St2^−/−+MSU) exhibited significantly reduced mechanical allodynia and ankle edema compared with WT (WT+MSU) group (Figure 3H&J). Area under the curve (AUC) indicated accumulated reduction in mechanical allodynia and ankle edema in St2^−/−+MSU group compared with WT+MSU group (Figure 3I&K). Additionally, naïve Il-33^−/− and St2^−/− mice did not exhibit any deficits in general locomotor activities in open field test and body weight vs. WT mice (Figure S2A-D).

We then studied the behavioral effects of exogenously injecting IL-33 into gout model mice. 24 h after gout model establishment, recombinant mouse IL-33 (rIL-33) was injected into the ankle (3-300 ng/10 μl, i.a., Figure S3A) of the gout model mouse. 30 or 300 ng rIL-33 injection further enhanced the ankle edema and exacerbated the mechanical allodynia of the gouty mice at both 24.75 and 28 h time points compared with vehicle (BSA)-injected gouty mice, whereas 3 ng rIL-33 was not effective (Figure S3B&C). We further tested whether IL-33’s effect is mediated via ST2. We found that co-injection of ST2 neutralizing antibody with rIL-33 largely abolished rIL-33’s effect on ankle edema and 50% PWTs in MSU-treated WT mice (Figure S3D&E). Furthermore, rIL-33 injection (300 ng) into MSU-treated St2^−/− mice did not further induce ankle edema and mechanical allodynia compared with BSA (vehicle) (Figure S3F), which is consistent with the results derived from ST2 neutralizing antibody. Therefore, these data suggest that exogenous IL-33 acts on ST2 to exacerbate inflammation and pain hypersensitivities in gout model mice.

We further examined the expression of some key inflammatory cytokines and chemokines in ankle
tissues via Bio-Plex multiplex immunoassays. As shown in Table 1, MSU treatment triggered upregulation of a number of cytokines and chemokines in ankle tissues of WT mice. Compared with WT mice, St2−/− mice showed significantly reduced expression of IL-1β, IL-6, IL-13, CCL11, G-CSF, CXCL1, CCL2 and CCL3 proteins in ankle tissues (Table 1), suggesting St2−/− mice showed generally attenuated inflammatory response during gout.

3. Macrophage is among one of the cellular mechanisms for IL-33 overproduction in gout condition.

IL-33 expression can be induced in macrophages by the bacterial endotoxin, lipopolysaccharide (LPS) [22-24]. As macrophages accumulate in the inflamed tissues to react to MSU deposition by uptake of the crystals via phagocytosis [7], we set to test whether MSU could trigger IL-33 expression in macrophages. We first tested the effect of MSU on macrophage cell line RAW264.7 in vitro. As a positive control, incubation with LPS (1 μg/ml) induced obvious upregulation of **Il-33** gene expression in RAW264.7 cells compared with vehicle (PBS) incubated cells (Figure 4A), a result consistent with previous studies [22]. MSU (0.5 or 1.0 mg/ml) incubation triggered a significant upregulation of **Il-33** gene expression in RAW264.7 cells (Figure 4A). ELISA test further confirmed that MSU incubation promoted IL-33 protein expression in RAW264.7 cells (Figure 4B). We then tested the effect of MSU on mouse macrophages. Thioglycollate-elicited macrophages (TPMs) were harvested from mice and exhibited over 90% macrophages purity examined by flow cytometry (Figure S4A&B). MSU (1 mg/ml) or LPS incubation (1 μg/ml) resulted in a significant increase in IL-33 protein expression in TMPs (Figure 4C).
We further examined the effect of in vivo macrophages depletion using clodronate-containing liposomes on IL-33 release in gout (Figure 4D). Clodronate (1 mg/100 μl, i.p.) robustly reduced the macrophages detected by F4/80 staining in the spleen of naïve mice compared with vehicle (liposome) treatment (Figure S5A&B), demonstrating the effectiveness of clodronate on macrophage depletion. MSU-treated mice showed obvious macrophage accumulation in periarticular ankle tissues, which was significantly reduced by clodronate treatment (Figure 4E&F). The ankle tissues were then collected and subjected to IL-33 protein detection. Clodronate-induced macrophage depletion significantly reduced the upregulation of IL-33 protein expression in ankle tissues from MSU-treated mice (Figure 4G). Clodronate-induced macrophage depletion further reduced ankle edema and mechanical allodynia of MSU-treated mice (Figure 4H-K). Based upon the above data, we reasoned that macrophage might be among one of the cellular mechanisms for IL-33 overproduction during gout.

4. IL-33/ST2 signaling participates in oxidative stress modulation and promotes ROS production in gout.

We next explored the mechanisms underlying how IL-33/ST2 mediates the pain response of mouse gout model. We and others have demonstrated that endogenous ROS products resulted from oxidative stress played an important role in mediating gout pain and inflammation [12, 13, 25]. Therefore, we began to explore whether IL-33/ST2 could have any effect on endogenous ROS production and oxidative stress level under gout condition. We found that H₂O₂ level was significantly increased in ankle tissues from WT+MSU group mice vs. WT+Veh group mice, whereas St2⁻/⁻+MSU group mice showed significantly reduced H₂O₂ level vs. WT+MSU group.
mice (Figure 5A). Furthermore, the levels of some lipid peroxidation products, such as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), were both significantly increased in ankle tissues from WT+MSU group mice vs. WT+Veh group mice. In comparison, St2−/−+MSU group mice showed significantly reduced levels of MDA and 4-HNE (Figure 5B, E&F). We also evaluated the activities of antioxidant enzymes superoxidase dismutase (SOD) and glutathione peroxidase (GSH-Px). The levels of SOD and GSH-Px were dramatically decreased in ankle tissues of WT+MSU group mice, whereas St2−/−+MSU group mice showed significantly improved levels compared with WT+MSU group (Figure 5C&D). We further performed a noninvasive in vivo imaging of ROS productions in mouse ankle using the chemiluminescent probe L-012 [26]. WT+MSU group displayed significantly increased L-012 chemiluminescent signals in the ankle compared with MSU+Veh group, a sign of increased ROS production in vivo. In contrast, St2−/−+MSU group showed significantly reduced L-012 chemiluminescent signals vs. WT+MSU group (Figure 5G&H).

We then tested the effect of exogenously applied rIL-33 on oxidative stress in ankle tissues of the mouse gout model. Injection of rIL-33 (300 ng/mouse) into MSU-treated mice (MSU+rIL-33 group) promoted a further reduction of antioxidant enzymes SOD and GSH-Px activities and a further increase in MDA level in ankle tissues vs. MSU-treated mice injected with BSA (MSU+BSA group, Figure 5I, J&K). Furthermore, rIL-33-induced MDA level increase in ankle tissues of gout model mice was abrogated by co-administration with ST2 neutralizing antibody or in St2−/− mice (Figure 5K&L). Collectively, the above results demonstrated that IL-33/ST2 promotes oxidative stress and ROS production in gout.
5. **IL-33/ST2 signaling drives neutrophil influx to produce oxidative stress in gout condition.**

We then explored the mechanisms underlying how IL-33/ST2 promotes oxidative stress and ROS production. Neutrophils played an important role in mediating gout pain [27]. Neutrophil exerts respiratory burst that rapidly releases ROS products [28-30]. We found that myeloperoxidase (MPO) activity, a quantitative measurement of neutrophil congregation [27], was significantly increased in the inflamed ankles of MSU group mice, whereas MPO activity was significantly attenuated in St2⁻/⁻ mice (Figure 6A). Besides, injecting rIL-33 into the inflamed ankles of MSU group mice resulted in further increase in MPO activity (Figure 6B). We further established the mouse MSU air pouch gout model, which allows for convenient collection of adequate tissue exudates for cell infiltration analysis. Cytopathological analysis indicated that MSU injection into air pouches elicited an increase in the numbers of total cells, including neutrophils, lymphocytes and monocytes, among which neutrophils are the predominant cell type infiltrated. The numbers of total cells and neutrophils were both largely reduced in St2⁻/⁻ mice (Figure S7).

We continued to examine whether neutrophil was the cellular source for oxidative stress and ROS production in gout condition. We used fucoidin, a potent selectin inhibitor that reduces the aggregation of neutrophils, to reduce neutrophil influx [27]. Fucoidin (20 mg/kg) was applied intravenously twice into MSU-treated mice (Figure 6C). Fucoidin treatment almost completely inhibited neutrophil influx into the ankle tissue after MSU injection, as indicated by a change in MPO activity (Figure 6D). Moreover, fucoidin significantly reduced ROS production
and improved antioxidant enzyme activities in ankle tissues of MSU-treated mice (Figure 6E-H). Fucoidin treatment also attenuated the ankle edema and mechanical allodynia of MSU-treated mice (Figure 6I&J). These data suggest that IL-33/ST2 drives neutrophil influx and triggers neutrophil-dependent ROS production, resulting in mechanical allodynia of gout model mice.

6. **TRPA1 channel activity is increased in DRG neurons of gout model mice via ST2 dependent mechanism, resulting in enhanced nociceptive response to endogenous ROS products.**

Endogenous ROS products, including H$_2$O$_2$ and 4-HNE, activate TRPA1 in peripheral sensory neurons to produce pain. Evidence suggests that MSU-induced gout arthritis increases TRPA1 expression in joint tissues [25]. Thereby, we continued to explore whether TRPA1 channel activity was functionally enhanced in peripheral sensory neurons that innervate the ankles of MSU-treated mice. Ipsilateral L3-5 DRG neurons were acutely harvested from MSU- or PBS (Veh)-treated mice and loaded with Fura-2 for Ca$^{2+}$ imaging (Figure 7A). The percentage of DRG neurons responding to endogenous TRPA1 agonist H$_2$O$_2$ (500 nM) was significantly higher in MSU group vs. Veh group (Figure 7A&B). In addition, the amplitude of Ca$^{2+}$ responses elicited by H$_2$O$_2$ was significantly higher in MSU group vs. Veh group (Figure 7C&D). We also observed that the enhanced TRPA1 channel activity is reduced in DRG neurons from St2$^{-/-}$ mice (Figure 7A-D). The *in vitro* results were further corroborated by *in vivo* behavioral tests. Injecting H$_2$O$_2$ (100 μg/site) into ipsilateral hind paws of MSU group mice resulted in more robust nocifensive behavior than Veh group mice (Figure 7E&F). The enhanced nocifensive response to H$_2$O$_2$ was eliminated by co-injecting mice with TRPA1 specific antagonist HC-030031 (10 μg/site, Figure 7E&F).
Furthermore, H₂O₂ injection into MSU-treated ST2⁻/⁻ mice resulted in significantly reduced nociceptive response vs. MSU-treated WT mice (Figure 7E&F), a result consistent with the in vitro Ca²⁺ imaging results. Therefore, both in vivo and in vitro data suggests that TRPA1 channel activity is enhanced in DRG neurons of gout model mice via ST2 dependent mechanism, resulting in exaggerated nociceptive response to endogenous ROS products during gout.

Discussion

In this study, we first screened the potential inflammatory cytokines or chemokines in the inflamed ankle tissue from a mouse gout arthritis model via RNA-Seq. We found that IL-33 was among the top up-regulated cytokines in the inflamed ankle. Neutralizing or genetic deleting IL-33 and its receptor ST2 significantly ameliorated pain hypersensitivities and inflammation in the mouse gout model. IL-33 promoted neutrophil influx and triggered neutrophil-dependent ROS production in the inflamed ankle, which activated TRPA1 channel in DRG neurons and produces nociception. Meanwhile, TRPA1 channel activity was significantly enhanced in DRG neurons that innervate the inflamed ankle, resulting in exaggerated nociceptive response to endogenous ROS products. These data all suggest a pivotal role of IL-33/ST2 in mediating gout pain and inflammation.

IL-33 can be released by several cell types, including keratinocytes, epithelial cells, fibroblasts and macrophages, etc. upon trauma, infection or inflammation [16, 31, 32]. During gout, macrophages are the cells that accumulate in the inflamed tissues to react to MSU deposition by uptake of the crystals via phagocytosis [11]. We found that both macrophage cell line RAW264.7
and cultured mouse peritoneal macrophages react to MSU challenge by producing IL-33 \textit{in vitro}.

\textit{In vivo} depletion of macrophages with clodronate significantly reduced IL-33 protein upregulation in ankle tissues of gout arthritis mice and further attenuated pain hypersensitivity and inflammation. This is by far the first evidence showing that macrophage reacts with MSU to produce IL-33. Therefore, our results suggest that macrophage may be among one of the cellular sources for IL-33 overproduction during gout. However, the participation of other cell types, such as fibroblasts, epithelial cells, etc. in IL-33 production under gout condition cannot be rule out and needs to be further tested.

Gout is accompanied with oxidative stress that provokes ROS production in local inflamed tissues [12]. H$_2$O$_2$ has been found to be significantly increased in joint tissues from the mouse gout model and triggers hyperalgesia and inflammation via activating peripheral TRPA1 channel [13, 25]. Our recent work identified that reducing ROS production by eucalyptol or ROS scavengers significantly reduced pain and inflammation in the mouse gout model, demonstrating an important role of ROS in mediating gout pain and inflammation [12]. However, the exact mechanism of how oxidative stress is generated and modulated under gout condition is still not fully understood. In our study, we found that St2$^{-/-}$ mice displayed significantly reduced oxidative stress, ROS production and pain hypersensitivity in gout condition, whereas exogenously injected IL-33 further promoted oxidative stress, ROS production and exacerbated pain hypersensitivity in gout condition via ST2 dependent mechanism. \textit{In vivo} ROS imaging further indicated that ROS production in gout is related with ST2. These results in all suggest a pivotal role of IL-33/ST2 in modulating oxidative stress during gout.
Neutrophil influx into joint tissue is considered as the pathological hallmark of gout, which makes important contributions to gout pathogenesis [27]. Here, we observed that St2–/– mice showed much reduced neutrophil influx, whereas exogenously injected rIL-33 further promoted neutrophil influx into the inflamed ankle. This result suggests that IL-33/ST2 is involved in mediating neutrophil influx in gout. We found that St2–/– mice showed much reduced tissue contents of CXCL1, CCL3 and IL-1β, which are neutrophil chemotactants crucial for neutrophil recruitment [33]. This result indicates that CXCL1, CCL3 and IL-1β are produced via ST2 dependent mechanism in gout condition. IL-33 can act via ST2 expressed in macrophages to produce CXCL1, CCL3 and IL-1β during inflammation [33, 34]. We reason here that IL-33, after being released from macrophages, may act in an autocrine manner to induce CXCL1, CCL3 and IL-1β production via ST2 in macrophages during gout. These cytokines or chemokines may then induce chemotaxis of neutrophils. Meanwhile, excellent previous studies have demonstrated in murine models and humans, IL-33 can directly promote neutrophil influx by acting on ST2 receptors expressed on neutrophils [33, 35]. Thus, IL-33 may directly promote neutrophil influx by acting on ST2 expressed in neutrophils during gout via the paracrine mechanism. On the basis of these findings, we propose that IL-33 may act through ST2 via above mentioned autocrine or paracrine or both mechanisms to attract neutrophils in gout condition. This proposed mode of action is a reminiscent of IL-33-mediated neutrophil migration in rheumatoid arthritis, in which both autocrine and paracrine mechanisms as mentioned above are involved [33]. Nonetheless, the exact cellular interactions that IL-33/ST2 mediated to promote neutrophil influx under gout condition remain to be further studied.
A recent study by Shang et al. reported that IL-33 attenuates inflammation in a MSU-induced mouse peritonitis model [15]. They observed IL-33 treatment significantly reduced neutrophil influx in MSU-induced mouse peritonitis model. Moreover, IL-33 treatment reduced the inflammatory cytokine IL-1β and IL-6 production, while promoted anti-inflammatory cytokine IL-10, IL-5 and IL-13 production in peritoneal cavity gavage fluids. They further found that IL-33 recruited a subset of myeloid-derived suppressor cells, namely CD11b^Gr-1^infF4/80^+^ cells, to suppress MSU-induced IL-1β production in peritoneal lavage fluids. These results are in contrast to our current observations. However, it is well established that IL-33 possesses unique biological function, which is about its seemingly contradictory pro-inflammatory vs. anti-inflammatory properties under different physical/pathological conditions [16]. For example, in peritoneal cavity, IL-33 has been reported to promote the recruitment of IL-10-producing regulatory B cells to attenuate mucosal inflammatory responses [36]. In contrast, in arthritis and some other conditions, IL-33 serves as a pro-inflammatory cytokine and exacerbates inflammation and pain via promoting the production of TNF-α, CXCL1, IL-1β and PGE2 through ST2 in plantar skin tissues [37-39]. IL-33 also substantially potentiates substance P-induced TNF-α and IL-1β production from mast cells to enhance inflammation [40, 41]. In addition, our recent work found that IL-33 released by keratinocytes can activate peripheral sensory neurons via promoting Ca^{2+} influx through TRPA1/TRPV1 channels in ST2 dependent mechanism, resulting in potentiation of skin inflammation and pruritus in a mouse allergic contact dermatitis model [20]. The mouse gout model we used here is totally different from Shang et al., in which MSU was intra-articularly injected into the ankle of mice for one single time, whereas Shang et al. repetitively injected MSU...
into the peritoneal cavity for a total of 4 times during 4 days’ period. It is believed that the biological effects of IL-33 is highly tissue specific and influenced by the microenvironment where it is produced [16]. Given the fact that ankle cavity possesses totally different morphological structure, cell distributions and immune responses vs. peritoneal cavity, therefore, the different observations we had with Shang et al., may likely be attributed to different animal models we used. Nonetheless, the immunological mechanisms underlying the complex dichotomous functions of IL-33 still need to be further investigated.

TRPA1 is a target of endogenous ROS and lipid peroxidative products, such as OxPAPC, 4-HNE and H₂O₂ [42-45]. TRPA1 is expressed by a subpopulation of peptidergic nociceptors and contributes to pain and neurogenic inflammation [43]. Previous studies identified H₂O₂ level is significantly increased in the inflamed ankle tissue of gout model mice [25]. Our study further identified that, in addition to H₂O₂, the lipid peroxidation product 4-HNE was also increased in the inflamed ankle tissues of gout model mice. It is reported that TRPA1 expression is upregulated in the inflamed joint tissues of gout model mice [25]. We further demonstrated that TRPA1 channel activity is significantly enhanced in DRG neurons that innervate the inflamed ankle from gout model mice. This finding was further corroborated by the enhanced nocifensive response to the endogenous TRPA1 agonist H₂O₂ exhibited by gout model mice. We also observed that DRG neurons from St2⁻/⁻ mice showed much reduced TRPA1 activity upon H₂O₂ challenge. Certain inflammatory cytokines, such as IL-1β and IL-6 can promote TRPA1 expression in DRG neurons or chondrocytes [46, 47]. IL-1β and IL-6 were both upregulated in the inflamed ankle tissue of gout model mice but much reduced in St2⁻/⁻ mice. Therefore, the enhanced TRPA1 channel
functional activity we observed may be due to channel expression upregulation mediated by IL-1β, IL-6 or other inflammatory mediators released during gout.

**Conclusions**

In all, our study demonstrated a previous unidentified role of IL-33/ST2 in mediating pain hypersensitivities and inflammation in a mouse gout model through promoting neutrophil-dependent ROS production and TRPA1 channel activation. Our study suggests that targeting IL-33/ST2 signaling may represent a novel therapeutic approach to ameliorate pain and inflammation in gout.

**Methods**

*Animals*

Male C57BL/6j or BALB/c mice (8-10 weeks; 20-25 g) were purchased from Shanghai Laboratory Animal Center, Chinese Academy of Sciences. St2 knockout (St2−/−) mice in the background of BALB/c were kindly provided by Dr. Andrew McKenzie at the MRC Laboratory of Molecular Biology, Cambridge, United Kingdom. Il-33 knockout (Il-33−/−) mice in the background of C57BL/6j were kindly provided by Dr. Hiroshi Kiyonari at the Laboratory for Animal Resources and Genetic Engineering, Center for Developmental Biology, Institute of Physical and Chemical Research, Kobe, Japan. All animals were housed in the Laboratory Animal Center of Zhejiang Chinese Medical University accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) under standard environmental conditions (12 h light–dark cycle and 24 °C) with access to food and water provided *ad libitum*. The mice
were randomly allocated and five mice were housed per cage. For group size estimation, we make sure that the data subjected to statistical analysis in our study has a group size \( n \geq 5 \), according to our previous experience and studies using similar experimental protocols. We only included male mice in our study to circumvent hormonal female cycle possible interferences in behavioral analysis. Besides, gout has been considered as a male predominant disease and it occurs much more frequently in men than women [48].

**MSU-induced gout arthritis model establishment**

Gout arthritis was induced by intra-articularly (i. a.) injection of MSU crystals (0.5 mg) suspended in 20 µl endotoxin-free phosphate buffered saline (PBS) into the tibio-tarsal joint (ankle) of mice under isoflurane anesthesia as we described before [12]. Control group mice received an i. a. injection of 20 µL sterile PBS. A successful gout arthritis model establishment was judged by obvious swelling and mechanical hyperalgesia 2 h after MSU injection [13].

**Evaluation of ankle joint mechanical pain hypersensitivity**

Mice were individually placed in transparent Plexiglas chambers on an elevated mesh floor and were habituated for 30 min before test. The mechanical hyperalgesia was determined using a series of von Frey filaments (0.04 to 4 g) applied perpendicularly to the heel area of the hind paw in ascending order beginning with the finest fiber. The minimum force that caused the mouse to withdraw its hind paw away from the filament was considered as the withdrawal threshold. For each mouse, a von Frey hair was applied 5 times at 10 s intervals. The threshold was determined when paw withdrawal was observed in more than three out of five applications as we previously
described [49]. The behavior tests are conducted by an experimenter blinded to experimental conditions.

**RNA-Seq and data processing**

24 h post-injection of MSU or vehicle, mice were euthanized. The injected ankles were then collected, diced and stored in RNAlater (Thermal Fisher Scientific, Waltham, MA, USA). Total RNA from vehicle and MSU group was extracted using Trizol reagent (Thermal Fisher Scientific, Waltham, MA, USA). RNA quality and purity were validated by TapeStation (Agilent, Santa Clara, CA, USA) and NanoDrop (Thermo Fisher Scientific, Waltham, MA, USA). Only RNA samples showing RNA Integrity Number \( \geq 8.0 \) and \( A_{260}/A_{230} \geq 1.5 \) were used for RNA-Seq. The samples were sequenced by BGISEQ-500 from BGI Group (Shenzhen, China). Raw sequencing reads were aligned to mouse genome (mm10). Differential expression analyses were performed with R and Bioconductor packages of edgeR and limma voom as reported in our previous studies [50, 51]. The threshold required for the genes to be considered significantly changed was as follows: q-value \( \leq 0.001 \) and absolute value of \( |\log_2 (\text{Fold Change})| \geq 1.0 \).

**Myeloperoxidase (MPO) activity measurement**

Neutrophil recruitment in ankle joint was evaluated via quantification of the enzyme MPO activity using a commercial MPO detection kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) as we described [12]. Briefly, mice were terminally anesthetized and the ankle joint was homogenized and centrifuged at 10,000 rpm at 4 °C for 15 min. 10 µL of the supernatant was transferred into PBS (pH 6.0) containing 0.17 mg/ml 3, 3’, 5, 5’-tetramethylbenzidine and 0.0005%
H₂O₂. MPO catalyzed the redox reaction of H₂O₂ and 3, 3’, 5, 5’-tetramethylbenzidine and produced yellow-colored compounds, through whose absorbance at 460 nm was determined. MPO activity was calculated and expressed as U/mg protein. One unit of MPO activity was defined as the quantity of enzyme that degraded 1 μmol H₂O₂ at 37 °C per g wet tissue.

Air pouch model

Mice were anesthetized with isoflurane. The back skin was shaved and 3 ml of sterile air was subcutaneously injected (s.c.) into the back to establish air pouch as described [52]. 3 days after the first injection, an additional 3 ml sterile air was injected into the pouch again. 3 days later, 3 mg of MSU crystals in 1 ml PBS, or 1 ml of PBS alone, was injected into the air pouches, respectively. 6 h after injection, mice were euthanized and exudates were collected and centrifuged at 500 g for 5 min at room temperature. Total cells were then counted with a hemocytometer and spun onto cytoslides and stained with Diff-Quick reagent. Differential cell counts were achieved by microscopic counting using standard morphological and staining criteria as we previously described [53].

Cell culture

Raw 264.7 cells: Raw 264.7 macrophage cell line was purchased from Shanghai Academy of Life Sciences (Shanghai, China) and cultured in DMEM (Hycolon, USA) supplemented with 10% fetal bovine serum (FBS, BI, Israel), 100 units/mL penicillin and 100 μg/mL streptomycin at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. DRG neurons: Mice were sacrificed 24 h after MSU injection. Ipsilateral L3-5 dorsal root ganglia (DRGs) were harvested and dissociated using
collagenase type 1 and dispase (Gibco, Thermo Fisher Scientific, USA) as described previously [54, 55]. DRG neurons were cultured in DMEM plus 10% FBS, 100 units/mL penicillin and 100 μg/mL streptomycin on round coverslips coated with poly-D-lysine (Sigma, MO, USA) and mouse laminin (Invitrogen, CA, USA) in 24-well chamber.

Ca^{2+} imaging

DRG neurons were used 4 h after dissociation. Cells were loaded with Fura 2-AM (10 μM, Invitrogen) for 45 min in a loading buffer containing: NaCl 140, KCl 5, CaCl₂ 2, MgCl₂ 2, HEPES 10 (pH 7.4 adjusted with NaOH). Cells were subsequently washed 3 times and imaged in the loading buffer. Ratiometric Ca^{2+} imaging was performed on a Nikon ECLIPSE Ti-S (Japan) microscope with a Polychrome V monochromator (Till Photonics, USA) and an Orca Flash 4.0 CCD camera (Hamamatsu, Japan). Images were captured and processed with MetaFluor software (Molecular Devices, CA, USA). Ratiometric images were obtained with exposures of 0.5 ms at 340 nm and 0.3 ms at 380 nm excitation wavelengths. Representative Ca^{2+} imaging images were generated using ImageJ software. A cell was considered responsive if the peak Ca^{2+} response is above 20% of the baseline according to our previous publications [56, 57].

In vivo ROS imaging

24 h after MSU injection into the right ankle, mice were injected (i.v.) with the fluorescent ROS indicator dye L-012 (Tocris, USA, 25 mg/kg), as described previously [58]. Mice were anesthetized with isoflurane, then placed on the stage of an IVIS Lumina LT in vivo imaging system (PerkinElmer, Waltham, MA, USA) and the fluorescence imaging was performed 5 min
post injection. The luminescence signal intensities were quantified with Living Image software (PerkinElmer, USA).

*Ethnic statement*

This study was approved by the Laboratory Animal Management and Welfare Ethical Review Committee of Zhejiang Chinese Medical University (Permission Number: ZSLL-2017-183).

*Statistical analysis*

Statistical analysis was conducted using SPSS 19.0 (IBM Corp., Armonk, NY, USA). Results were expressed as mean ± SEM. Student’s *t*-test was used for comparisons between two groups. One-way or two-way ANOVA followed by Tukey’s post hoc test was used for comparison among groups ≥ 3. Comparison is considered significantly different if *p* < 0.05.

*Author contributions*

CY, BL (Boyu Liu), YL (Yuanyuan Li), XL, JW, RC, YT, QS, PW, XS, YL (Yi Liang) conducted the experiments, collected and analyzed the data; HZ and WM provided reagents. JF and BL (Boyi Liu) designed and supervised the study. BL (Boyi Liu) wrote the manuscript.

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Figure legend

Figure 1 RNA-Seq expression analysis of mRNA changes in inflamed ankle tissues of MSU-induced mouse gout model. (A) Representative photos showing mouse ankle injected with PBS (Veh group) or MSU (MSU group). The pictures were taken 24 h after injection. (B) Time course of the % increase in ankle diameter after model establishment. n = 6 mice/group, **p<0.01 vs. Veh group. (C) Representative photos of ankle H&E staining from Veh and MSU group. The right panels show enlarged area as indicated in the left. (D) Time course of PWT changes in the right hind paw after model establishment. n = 6 mice/group, *p<0.05 and **p<0.01 vs. Veh group. (E) Heat map illustration of the DEGs derived from MSU group vs. Veh group. n = 3 mice/group. (F) Volcano plot showing gene expression profiles in MSU group vs. Veh group. Red and blue spots indicate up- and down-regulated DEGs, respectively, whereas grey spots indicate non-DEGs. (G) Top 10 most highly up-regulated inflammatory cytokines or chemokines in MSU group vs. Veh group.

Figure 2 The expressions of IL-33 and ST2 proteins are up-regulated in inflamed ankle tissues of mouse gout model. (A-C) qPCR analysis of Il-33, St2 and Il-1RAcP gene expression
fold changes in MSU group vs. Veh group (PBS). Ankle tissues were harvested 24 h after model establishment. n = 6-8 mice/group. (D) ELISA result of IL-33 protein changes in MSU group vs. Veh group. n = 5 mice/group. **p<0.01, NS: no significance. (E) IL-33 protein expression in ankle tissues examined by Western blotting at 0, 8, 24 and 48 h time points after MSU or Veh group establishment. Upper panel shows representative photos of IL-33 and β-actin protein expression. Lower panel indicates the summarized IL-33 expression normalized to β-actin in MSU and Veh group. n = 6 mice/group. (F) ST2 protein expression in ankle tissues examined by Western blotting at 0, 8, 24 and 48 h time points after MSU or Veh group establishment. Upper panel shows representative photos of ST2 and β-actin protein expression. Lower panel indicates the summarized ST2 expression normalized to β-actin in MSU and Veh group. n = 6 mice/group.

**Figure 3** IL-33/ST2 is involved in mediating pain hypersensitivity and inflammatory response of the mouse gout model. (A) Schematic picture depicting the time points of IL-33 neutralizing antibody (IL-33 Ab) or isotype control IgG application (i.p.) and behavioral/ankle edema tests. MSU or PBS (Veh) was injected into the ankle (i.a.) at 0 h time point. (B&C) The comparison of % increase of ankle diameter (B) or PWT (C) 1, 4, 8 and 24 h after IL-33 Ab or isotype control IgG application. (D) Time course showing PWT changes in WT and Il33-/- mice after MSU or PBS injection. (E) Summary of the normalized area under the curve as in (D), in which WT+Veh group was taken as 100%. n = 6 mice/group. (F) Time course showing % increase in ankle diameter in WT and Il33-/- mice after MSU or PBS injection. (G) Summary of the normalized area under the curve as in (F), in which WT+Veh group was taken as 100%. n = 7 mice/group. (H) Time course showing PWT changes in WT and St2-/- mice after MSU or PBS injection. (I) Summary of the normalized area under the curve as in (H), in which WT+Veh group was taken as 100%. n = 7 mice/group. (J) Time course showing % increase in ankle diameter in WT and St2-/- mice after MSU or PBS injection. (K) Summary of the normalized area under the curve as in (J), in which WT+Veh group was taken as 100%. n = 7 mice/group. *p<0.05 and **p<0.01 vs. WT+Veh group. #p<0.05 and ##p<0.01 vs. WT+MSU group. One-way ANOVA followed by Tukey post hoc test was used for statistical analysis in panels (A-D). One-way ANOVA followed by Tukey post hoc test was used for statistical analysis in panels (E-F).

**Figure 4** Macrophage is a cellular mechanism for IL-33 overproduction during gout. (A) Upper panel: Schematic protocol illustrating time points for MSU/LPS incubation and RAW264.7 cell harvest. Lower panel: Expression of Il-33 gene in RAW264.7 cells after vehicle (Veh, PBS), MSU (0.5 or 1 mg/ml) or LPS (1 μg/ml) incubation by qPCR. (B) IL-33 protein expression changes in RAW264.7 cells after vehicle (PBS), MSU (0.5 mg/ml) or LPS (1 μg/ml) incubation by ELISA. (C) IL-33 protein expression changes in macrophages after vehicle, MSU (0.5 or 1 mg/ml) or LPS (1 μg/ml) incubation by ELISA. n = 5-6 replicates/group. (D) Protocol illustrating in vivo depleting macrophages in mice via treating with clodronate-laden liposome (i.p.). Liposome (Lipo) was used as vehicle control. (E) Immunofluorescence staining of periarticular tissues using F4/80 to detect macrophages. Right panels show enlarged fields depicted as white
boxes on the left. (F) F4/80 positively stained cell counts per observation field. n = 4 mice/group. (G) Western blot of IL-33 protein expression in ankle tissues after macrophage depletion. Upper panel: representative images of IL-33 and β-actin protein expression in Veh+Lipo, MSU+Lipo and MSU+Clodro groups. Lower panel: summarized IL-33 expression normalized to β-actin. (H) Time course of the % increase in ankle diameter after MSU injection. (I) Summary of the normalized AUC as in (H), in which Veh+Lipo group was taken as 100%. (J) Time course of PWT changes after MSU injection. (K) Summary of the normalized AUC as in (J), in which Veh+Lipo group was taken as 100%. n = 7 mice/group. *p<0.05 and **p<0.01 vs. Veh+Lipo group. #p<0.05 and ##p<0.01 vs. MSU+Lipo group. NS: no significance. One-way ANOVA followed by Tukey post hoc test was used for statistical analysis in panels (A, B, C, F, G, I&K). Two-way ANOVA followed by Tukey post hoc test was used for statistical analysis in panels (H&J).

**Figure 5 IL-33/ST2 participates in oxidative stress modulation and promotes ROS production in gout condition.** (A-D) Summarized data showing H2O2 content (A), MDA content (B), SOD activity (C) and GSH-Px activity (D) determined in WT or ST2-/- mice ankle tissues 24 h after vehicle (Veh, PBS) or MSU injection. (E) 4-HNE and β-actin expression in mouse ankle tissues determined by Western blot. (F) Summarized data of 4-HNE expression normalized to β-actin. (G) *In vivo* ROS imaging showing ROS contents in the ankle area using L-012 in different groups. (H) Summarized total fluorescence flux of L-012 in the ankle area. (I) SOD activity (I) and GSH-Px activity (J) in ankle tissues 4 h after BSA or rIL-33 (300 ng) injection. (K) MDA level in ankle tissues 4 h after BSA or rIL-33 injection. ST2 neutralizing antibody (50 μg) or isotype IgG was co-injected with rIL-33. (L) MDA level in ankle tissues 4 h after BSA or rIL-33 injection in WT or St2-/- mice. *p<0.05 and **p<0.01. NS: no significance. One-way ANOVA followed by Tukey post hoc test was used for statistical analysis.

**Figure 6 IL-33/ST2 promotes neutrophil influx into inflamed tissue to generate oxidative stress in gout condition.** (A) MPO activity determined in WT or ST2-/- mice ankle tissues 24 h after vehicle (Veh, PBS) or MSU injection. n = 5-9 mice/group. (B) MPO activity determined in ankle tissue of gout model mice after exogenously injection with BSA or rIL-33. (C) Schematic protocol for blocking neutrophil influx via fucoidine (20 mg/kg in 20 μl injecting volume, i.v.). (D-H) MPO activity (D), H2O2 (E), SOD activity (F), GSH-Px activity (G) and MDA content (H) measured in fucoidine- or vehicle (PBS)-treated gout model mice. n = 5-9 mice/group. (I) % increase in ankle diameter of gout model mice 1 or 3 h after fucoidine or PBS treatment. (J) PWT of gout model mice 1 or 3 h after fucoidine or PBS treatment. n = 5-8 mice/group. *p<0.05 and **p<0.01. One-way ANOVA followed by Tukey post hoc test was used for statistical analysis.

**Figure 7 TRPA1 channel activity is increased in DRG neurons of gout model mice via ST2 dependent manner, which enhances the nociceptive response to endogenous ROS products in gout condition.** (A) Pseudo color images from Fura-2-based ratiometric Ca2+ imaging indicating the Ca2+ responses from ipsilateral L3-5 DRG neurons in response to the endogenous TRPA1 agonist H2O2 (500 μM) in Veh (PBS) or MSU group mice. 40 mM KCl was perfused at the end of each recording to determine all live DRG neurons in observation field. (B) Summarized % of H2O2 positive responding DRG neurons in each observation field from Veh or MSU group of mice. n = 6-7 tests/group. Each group contains 150~200 neurons derived from 3~4 mice. (C)
Comparison of averaged Ca\(^{2+}\) responses induced by 500 \(\mu\)M H\(_2\)O\(_2\) between Veh and MSU group. Ca\(^{2+}\) traces are overlaid for comparison. \(n > 40\) cells/group. 
(D) Summarized data showing \(\Delta\) increase in peak 340/380 ratio before and after H\(_2\)O\(_2\) application. \(n > 60\) neurons/group derived from 3-4 mice/group. 
(E) Time course of the nocifensive behaviors of Veh and MSU group mice after H\(_2\)O\(_2\)/HC-030031 or corresponding vehicle injection. Veh: PBS, Veh1: PBS, Veh2: 1% DMSO (in PBS), HC-03: HC-030031. 
(F) Total time summary of all time points in panel (E). **\(p<0.01\). One-way ANOVA followed by Tukey post hoc test was used for statistical analysis.

Table 1. The expression (pg/mg protein) of some inflammatory cytokines or chemokines in ankle tissues of wild type and ST2\(^{−/−}\) mice treated with vehicle (Veh, PBS) or MSU, measured by Bio-Plex multiplex immunoassays. \(n=5\) mice/group. **\(p<0.01\) vs. WT+Veh, \#\(p<0.05\), ##\(p<0.01\) vs. WT+MSU. One-way ANOVA followed by Tukey’s post hoc test was used for statistical analysis.

| Cytokines | WT+Veh | WT+MSU | St2\(^{−/−}\)+Veh | St2\(^{−/−}\)+MSU |
|-----------|--------|--------|-------------------|-------------------|
| IL-1\(\alpha\) | 220.0±6.2 | 275.6±12.7 | 191.4±8.16 | 189.8±28.3** |
| IL-1\(\beta\) | 1.9±0.2 | 4.2±0.2** | 1.1±0.3 | 2.9±0.2** |
| IL-2 | 3.1±0.4 | 4.3±0.2** | 2.7±0.2 | 3.4±0.2 |
| IL-5 | 0.9±0.1 | 1.3±0.1** | 0.7±0.1 | 1.3±0.1 |
| IL-6 | 1.3±0.2 | 7.9±0.8** | 1.3±0.3 | 4.9±0.2** |
| IL-10 | 8.9±0.9 | 9.8±0.3 | 9.5±0.9 | 9.1±0.7 |
| IL-12 | 14.3±0.9 | 20.6±1.4** | 11.5±0.6 | 18.8±0.8 |
| IL-13 | 9.8±1.4 | 24.9±1.3** | 9.1±2.7 | 16.9±0.4** |
| CCL11 | 48.3±3.6 | 116.5±15.6** | 48.9±4.2 | 52.0±3.7** |
| G-CSF | 6.2±1.3 | 30.7±2.8** | 6.9±1.6 | 19.8±1.3** |
| CXCL1 | 17.4±2.4 | 76.7±7.7*** | 13.7±2.2 | 27.8±3.0** |
| CCL2 | 228.6±51.3 | 1423.5±85.5** | 202.5±91.6 | 598.1±47.1** |
| CCL3 | 16.9±3.6 | 83.8±9.2** | 12.5±3.4 | 39.3±2.8** |
| CCL5 | 154.7±30.2 | 287.0±76.8** | 113.8±27.3 | 169.4±34.7 |
**A**

![Image of MSU and Veh groups with arrows](image)

**B**

% increase in diameter over time for MSU and Veh groups.

**C**

Histological images of Veh and MSU groups.

**D**

Graph showing PWT (g) over time for Veh and MSU groups.

**E**

Heatmap representing upregulated and downregulated genes in Veh and MSU groups.

**F**

Volcano plot showing log2 fold change and -log10(q-value) for upregulated and downregulated genes.

**G**

| Symbol | Gene ID  | Log2 Ratio (MSU/Veh) | q-value       |
|--------|----------|-----------------------|---------------|
| IL24   | 93672    | 3.74                  | 3.67E-20      |
| Cxcl3  | 330122   | 2.10                  | 2.51E-143     |
| Cxcl5  | 20311    | 1.84                  | 0             |
| II10   | 16153    | 1.81                  | 6.58E-10      |
| II33   | 77125    | 1.79                  | 0             |
| II119  | 215257   | 1.50                  | 7.87E-244     |
| Ccl19  | 24047    | 1.29                  | 8.90E-40      |
| Cxcl2  | 20310    | 1.29                  | 8.90E-42      |
| IL6    | 16193    | 1.28                  | 1.02E-22      |
| IL1b   | 16176    | 1.26                  | 1.79E-222     |
**A** Raw 264.7

MSU or LPS → Collect cells for qPCR

0h → 6h

- Veh
- MSU (0.1 mg)
- MSU (0.5 mg)
- MSU (1 mg)
- LPS

**B** Raw 264.7

MSU or LPS → Collect cells for Elisa

0h → 6h

- Veh
- MSU (0.5 mg)
- LPS

**C** TPMs

MSU or LPS → Collect TPMs for Elisa

0h → 6h

- Veh
- MSU (1 mg)
- LPS

**D**

-24h → Clodronate or liposome (i.p.)

0h → MSU (i.a.)

24h → PWTs/Edema Collect tissue

**E**

Veh+Lipo

MSU+Lipo

MSU+Clodro

**F**

Cells count/field (x 10⁴)

**G**

IL-33

β-actin

**H**

% increase in diameter

Clodronate MSU

- Veh+Lipo
- MSU+Lipo
- MSU+Clodro

**I**

Normalized AUC (%)

**J**

PWT (g)

Clodronate MSU

- Veh+Lipo
- MSU+Lipo
- MSU+Clodro

**K**

Normalized AUC (%)

- Veh+Lipo
- MSU+Lipo
- MSU+Clodro
A

WT+Veh

Control

+H₂O₂

+KCl

WT+MSU

Control

+H₂O₂

+KCl

ST2⁺-MSU

Control

+H₂O₂

+KCl

L3-5 DRG neuron culture

Ratiometric Ca²⁺ imaging

B

% H₂O₂ positive responding neuron

**

WT Veh

WT MSU

ST2⁺ MSU

C

H₂O₂

KCl

Ratio (340/380)

0 200 400 600

Time (s)

WT Veh

WT MSU

ST2⁺ MSU

Δ Change in R

WT+Veh

WT+MSU

ST2⁺-MSU

D

Δ Change in R₃₄₀/₃₈₀

WT Veh

WT MSU

ST2⁺ MSU

E

WT+Veh+Veh1+Veh2

WT+MSU+H₂O₂+Veh2

WT+Veh+H₂O₂+Veh2

WT+MSU+H₂O₂+HC-03

ST2⁺+MSU+H₂O₂+Veh2

F

Nocifensive behaviors

Time (s)

WT Veh

WT MSU

WT ST2⁺

MSU

H₂O₂

HC-03

- 0 1 2 3 4 5 Nocifensive behaviors

Time (min)

- 0 2 4 6 8 10

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Supplementary Materials

Title page

Title
IL-33/ST2 induces neutrophil-dependent reactive oxygen species production and mediates gout pain

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Abstract

Objective: Gout, induced by monosodium urate (MSU) crystal deposition in joint tissues, provokes severe pain and impacts life quality of patients. However, the mechanisms underlying gout pain are still incompletely understood. Methods: We established a mouse gout model by intra-articularly injection of MSU crystals into the ankle joint of wild type and gentic knockout mice. RNA-sequencing, in vivo molecular imaging, Ca²⁺ imaging, reactive oxygen
species (ROS) generation, neutrophil influx and nocifensive behavioral assay, etc. were used.

**Results:** We found interleukin-33 (IL-33) was among the top up-regulated cytokines in the inflamed ankle. Neutralizing or genetic deletion of IL-33 or its receptor ST2 (suppression of tumorigenity) significantly ameliorated pain hypersensitivities and inflammation. Mechanistically, IL-33 was largely released from infiltrated macrophages in inflamed ankle upon MSU stimulation. IL-33 promoted neutrophil influx and triggered neutrophil-dependent ROS production via ST2 during gout, which in turn, activated transient receptor potential ankyrin 1 (TRPA1) channel in dorsal root ganglion (DRG) neurons and produced nociception. Further, TRPA1 channel activity was significantly enhanced in DRG neurons that innervate the inflamed ankle via ST2 dependent mechanism, which results in exaggerated nociceptive response to endogenous ROS products during gout. **Conclusions:** We therefore demonstrated a previous unidentified role of IL-33/ST2 in mediating pain hypersensitivity and inflammation in a mouse gout model through promoting neutrophil-dependent ROS production and TRPA1 channel activation. Targeting IL-33/ST2 may represent a novel therapeutic approach to ameliorate gout pain and inflammation.

**Keywords:** Gout; arthritis; TRPA1; reactive oxygen species; cytokine; neutrophil;
Graphical abstract

IL-33 produced by macrophages upon MSU stimulation promoted neutrophil influx and triggered neutrophil-dependent ROS and lipid peroxidation products generation via ST2, which activated TRPA1 in DRG neurons and produced gout pain.
Supplementary methods and materials

Chemicals

MSU and DMSO were purchased from Sigma (St. Louis, MO, USA). \( \text{H}_2\text{O}_2 \) and LPS were purchased from Solarbio Life Sciences (Beijing, China). Fucoidin was purchased from APExBIO Technology (Houston, TX, USA). Clodronate and liposome controls were purchased from Liposoma BV (Amsterdam, The Netherlands). Fura-2 AM was purchased from Abcam (Cambridge, UK). HC-030031 and L-012 were purchased from Tocris (Minneapolis, MN, USA).

Ankle edema test

Ankle edema was evaluated as an % increase in ankle diameter measured with a digital caliper and was calculated as follows as previously described [1, 2]: % increase in ankle diameter = \((L_{\text{after}} - L_{\text{basal}}) / L_{\text{basal}} \times 100\%\). The experimenter was blind to experimental conditions. The same experimenter carried out all edema tests to avoid inter-observer variability.

\( \text{H}_2\text{O}_2 \)-induced nocifensive behavior assay

Mice were placed into transparent chambers and habituated for 30 minutes before testing. Chemicals were injected into the hind paw of mice using 1-mL syringe and 30-gauge needle as follows: \( \text{H}_2\text{O}_2 \) (100 µg/site) was co-injected with HC-030031 (10 µg/site) or vehicle in a total volume of 10 µl dissolved in PBS. Nocifensive behavior (licking, flinching, or biting of injected paw) was recorded with a videorecorder for 10 min and quantified thereafter. All behavior tests are conducted by an experimenter blinded to experimental conditions.
The open field test

The test consists of a non-transparent Plexiglass enclosure (40 cm × 40 cm × 30 cm) placed in a sound-proof experimental room. Animals were habituated to experimental room conditions for 30 min before test. To initiate testing, the mice were placed in the center of the field individually, and the total distance they traveled in the field was monitored by the camera above the field during 5 min testing time. The movement was then analyzed by Anymaze software from Stoelting (Wood Dale, IL, USA) and compared thereafter.

ELISA test

Ankles joint samples were harvested after treatment, weighed and immediately frozen in liquid nitrogen. Tissues were homogenized with Bullet Blender (BBX24, NextAdvance Inc. NY, USA) in 50 mM Tris-base (PH 7.4) and 150 mM NaCl added with protease inhibitors (Roche, Switzerland) and 0.2% Triton X-100 (Sigma, MO, USA) at 4 ℃ for 20 min at full speed as we described previously. The supernatants were centrifuged at 12, 000g for 12 min at 4 ℃. The protein concentrations were determined by BCA assay (Thermo Fisher Scientific, Waltham, MA, USA). The supernatants were tested by ELISA in duplicates for IL-33 using a commercial kit (R&D Systems, Minneapolis, MN, USA), according to the manufacture’s instruction.

Bio-Plex multiplex immunoassays

The concentrations of some key inflammatory cytokines or chemokines in supernatant of homogenized ankle tissues of mice were detected using Bio-Plex multiplex immunoassay (RayBiotech, Norcross, GA, USA). Briefly, a total of 50 μl antibody-conjugated beads were added
to the assay plate. The samples were diluted in 1:24. 50 μl of diluted samples, standards, the blank, and controls were added to the plate, respectively. The plate was then incubated in the dark with shaking at 850 rpm for 30 min and then washed 3 times with 100 μl washing buffer. A total of 25 μl biotinylated antibody and 50 μl streptavidin-phycoerythrin were added to the plate, respectively, with shaking and washing procedure mentioned above. The readout was carried out using Bio-Plex protein array reader and Bio-Plex Manager 6.0 was used for data acquisition and analysis.

**Thioglycollate-elicited macrophages (TPMs) culture**

TPMs were generated by injecting the mice (i.p.) with 2.5 ml of 4 % Brewer’s thioglycollate medium (Sigma, USA) as described before [3]. Mice were euthanized 72 h after injection. Peritoneal cavity cells were harvested by lavage, and cells were washed and plated in 6-well chamber. Cells were cultured in DMEM plus 10% FBS, 100 units/mL penicillin and 100 μg/mL streptomycin. Cells were incubated overnight and adherent cells were then stimulated with MSU or LPS.

**Macrophage depletion with clodronate**

200 μl of a clodronate-laden liposome suspension (5 mg/ml) was injected (i.p.) 48 h before behavioral test. Liposomes lacking clodronate served as vehicle control. The efficiency of macrophage depletion was evaluated by immunostaining the spleen and periarticular ankle tissues with F4/80 antibody.
**Neutrophil aggregation blocking with fucoidine**

Mice were treated with fucoidin (20 mg/kg in 20 μl) or corresponding vehicle (PBS) by intravenous injection via the tail vein at 8 h and 23.5 h after MSU injection. The efficiency of neutrophil aggregation blockage was evaluated by MPO assay.

**IL-33/ST2 neutralizing antibody application**

For blocking IL-33 and ST2 signaling, mouse IL-33–neutralizing and ST2-neutralizing antibodies (catalog numbers: AF3626 and MAB10041; R&D Systems) and normal goat or rat isotype control IgGs (catalog numbers: I5256 and I4131; Sigma-Aldrich) were dissolved in sterile PBS. For blocking IL-33, IL-33 neutralizing antibody (10 μg/mouse) was treated at 0 h, 8 h and 23 h time points, for a total of three times, after MSU injection. In cases where ST2-neutralizing antibody was co-applied with rIL-33, ST2 neutralizing antibody (50 μg/mouse) was pre-mixed with rIL-33 (300 ng/mouse) and co-injected with rIL-33 into the ankle joint of mice. The effective dosages of IL-33- and ST2-neutralizing antibodies were derived from our previous study [4].

**Determination of oxidant/anti-oxidant status**

Ankle joint tissues from mice of each group were collected 24 h after MSU injection. The samples were then homogenized, followed by centrifugation at 3,000 rpm for 15 min. Supernatant was collected for superoxide dismutase (SOD), reduced glutathione (GSH) and malondialdehyde (MDA) assay as we described before [1]:

Detection of SOD activity: SOD activity was detected based on the inhibition of nitro blue tetrazolium reduction using the xanthine/xanthine oxidase system as a superoxide generator with a
commercially available kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Absorbance was determined with a microplate reader at 560 nm. The results were averaged and expressed as U/mg of sample protein.

Detection of MDA level: Lipid peroxidation product MDA level was determined based upon the concentration of thiobarbituric acid-reactive substances using a commercially available kit (Beyotime, Shanghai, China). Absorbance was determined using a microplate reader at 532 nm. MDA concentration was calculated using the absorbance coefficient according to manufacturer’s instruction and expressed as U/mg of sample protein.

Detection of GSH-Px level: GSH-Px level was measured using 5, 5′-dithiobis-(2-nitrobenzoic acid) recycling method with a commercially available kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Absorbance was monitored using a microplate reader at 412 nm. The results were deduced from comparison with that of the standard solution of GSH and expressed as μmol/mg of sample protein.

*Immunofluorescent staining*

Mice were deeply anesthetized with isoflurane and were perfused through the ascending aorta with 0.9% saline followed by 4% paraformaldehyde in 0.1 M PBS. After perfusion, the spleen and periarticular tissues were removed and post-fixed in the same fixative for 4-6 h (4 °C) before transferring to 30% sucrose for 72 h for dehydration. Tissues were serially cut into 8 μm thickness section on a frozen microtome (CryoStar NX50, Thermo Fisher, CA, USA) and processed for immunofluorescence. The sections were first blocked with 5% donkey serum in PBS (with 0.3% Triton X-100, blocking buffer) for 1 h at 37 °C and then incubated overnight at 4 °C with the
following primary antibodies diluted in blocking buffer: rat anti F4/80 (1:500, Abcam). The sections were then incubated 1 hr at 37 °C with mixture of corresponding secondary antibodies. Fluorescence images were captured by Nikon A1R laser scanning confocal microscope. The sections were examined and analyzed in a blinded manner. 3 images were randomly selected per mouse tissue and averaged and compared as described in our previous study [5].

*Western blotting*

After measuring protein concentrations, the samples were heated for 5 min with 5X loading buffer at 100 °C and loaded onto SDS-PAGE. The proteins were electrophoretically transferred onto polyvinylidene fluoride membranes. After being blocked with 5% nonfatty milk in Tris-buffer saline containing 0.1% Tween-20 for 1h at room temperature, the membranes were then probed with following primary antibodies overnight at 4 °C. These antibodies included goat anti-IL33 (1:500, R&D Systems), goat anti-ST2 (1:1000, R&D Systems), rabbit anti-4HNE (1:500, Abcam) and mouse anti-β-actin (1:5000, Abcam). The proteins were then detected by horseradish peroxidase-conjugated and corresponding secondary antibodies (1:5000, CST). The results of protein expression are normalized to the density of β-actin. Fold change in control/vehicle group was expressed as 100% quantification.

*Real-time quantitative PCR (qPCR)*

At 24 h after MSU injection, the mice were euthanized and ankle joint samples were collected. Total RNA from each group was extracted in 1 mL Trizol reagent and centrifuged at 12,000 rpm at 4 °C for 10 min. The extracted total RNA from ankle was reverse-transcribed into cDNA using
random haxamers primers with Prime ScriptTM RT reagent Kit (TaKaRa Bio Inc, China). Each reaction was performed in triplicates and normalized to β-actin gene expression. qPCR was performed in CFX96 Instrument Sequence Detection System (Bio-Rad, Berkeley, CA, USA) using Fast Start Universal SYBR Green Master Kit (TaKaRa Bio Inc, China) with 25 µL reaction system. The CT value of each well was determined using the CFX96 Real-Time System Software and the average of the triplicates was calculated. The relative quantification was determined by the △△CT method as described before [6]. The sequences of all primers used were shown in Table S1.
Supplementary figures and legends:

**Figure S1 High quality ankle tissue RNA obtained for RNA-Seq.** (A) Ankle tissue RNA electrophoresis image. Left lane shows the marker and right lane shows the RNA sample. (B) RNA integrity number (RIN) determined by TapeStation.
Figure S2 St2<sup>-/-</sup> and II33<sup>-/-</sup> mice showed normal general locomotor activities in open field test and body weight. (A&B) Open field test of naïve St2<sup>-/-</sup> (A) and II33<sup>-/-</sup> (B) mice compared with WT control mice. Upper panel: typical movement traces of the mice. Lower panel: Summarized total distance the mice traveled. (C&D) Comparison of body weight of St2<sup>-/-</sup> (C) or II33<sup>-/-</sup> (D) mice with WT control mice. NS: no significance. Student’s t test is used for statistical analysis.
Figure S3 Exogenously injected recombinant IL-33 exacerbates pain hypersensitivities and ankle edema of gout model mice via ST2 dependent mechanism. (A) Schematic protocol for gout model establishment and time points for i.a. injection of rIL-33/BSA (or in combination with ST2 neutralizing antibody/Iso-type IgG). Ankle diameter and PWTs were observed 45 min and 4 h after rIL-33/BSA injection. (B) % increase in ankle diameter after 3, 30 or 300 ng rIL-33 or BSA injection. (C) PWT changes after 3, 30 or 300 ng rIL-33 or BSA injection. (D) % increase in ankle diameter after IL-33 (300 ng) combined with ST2 neutralizing antibody (50 μg) or iso-type IgG injection. (E) PWT changes after IL-33 (300 ng) combined with ST2 neutralizing antibody (50 μg) or iso-type IgG injection. (F) % increase in ankle diameter after IL-33 or BSA injection into WT or St2−/− mice. (G) PWT changes after IL-33 or BSA injection into WT or St2−/− mice. n = 5-7 mice/group. *p<0.05 and **p<0.01. NS: no significance. One-way ANOVA followed by Tukey post hoc test was used for statistical analysis.
Figure S4 Thioglycollate-elicited macrophage collection and identification. (A) TPMs observed under transmitted light microscope. (B) FACS plots showing the percentage of F4/80+/CD11b+ macrophages in peritoneal fluids after thioglycollate treatment.
Figure S5 Validation of clodronate-induced macrophage depletion. (A) Schematic protocol for the time points of clodronate or liposome application and spleen tissue collection. (B) Representative immunofluorescence images showing spleen section stained with macrophage marker F4/80 in clodronate or liposome-treated group. (C) Summarized % normalized fluorescence intensity of (B). n = 7 mice/group. Scale bar indicate 50 μm. **p<0.01. Student's t test is used for statistical analysis.
Figure S6 Analysis of infiltrated cells in tissue exudates from the mouse air pouch model.

Upper panel: Protocol for establishing the mouse air pouch model. On Day 0 and 3, 5 ml sterile air was injected subcutaneously (s.c.) into the back of the mouse. On Day 6, MSU (3 mg/ml, in 1 ml injection volume) or PBS (1 ml) was injected into the air pouches. 6 h later, the animals were sacrificed and tissue exudates were collected for analysis. Lower panel: Cell counts (total, neutrophils, lymphocytes and macrophages) from tissue exudates of the air pouch model from WT and St2−/− mice. n = 5-6 mice/group. *p<0.01. One-way ANOVA followed by Tukey post hoc test was used for statistical analysis.
**Table S1.** Primers sequence used for qPCR evaluation of gene expression changes in the present study

| Gene name | Gene ID | Primer sequence (5’ to 3’) | Amplicon size (bp) |
|-----------|---------|----------------------------|--------------------|
| β-actin   | 11461   | F:5’-GTGCTATGTGGCTCTAGACTTCG-3’ R:5’-ATGCCAAGGATCCATACCC-3’ | 174                |
| Il-33     | 77125   | F:5’-CAGAAGACCAAAGAATTCTGCC-3’ R:5’-CATGCTTTGATCCGATTTTAG-3’ | 130                |
| St2       | 17082   | F:5’-TGACACCTTACAAACACCCCGA-3’ R:5’-AGGTCTCTCCATAAATGCACA-3’ | 178                |
| Il-1rap   | 16180   | F:5’-CACCAAGGTACACAGTAACT-3’ R:5’-CAGCCAGTAAACATGGTAAACC-3’ | 123                |

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