Spinal cord oligodendrocyte-derived alarmin IL-33 mediates neuropathic pain

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ABSTRACT   Neuropathic pain from injury to the peripheral and CNS represents a major health care issue. We have investigated the role of IL-33/IL-33 receptor (ST2) signaling in experimental models of neuropathic pain in mice. Chronic constriction injury (CCI) of the sciatic nerve induced IL-33 production in the spinal cord. IL-33/citrine reporter mice revealed that oligodendrocytes are the main cells expressing IL-33 within the spinal cord together with a minor expression by neurons, microglia. and astrocytes. CCI-induced mechanical hyperalgesia was reduced in IL-33R (ST2)$^{-/-}$ mice compared with wild-type (WT) mice. Intrathecal treatment of WT mice with soluble IL-33 receptor (IL-33 decoy receptor) markedly reduced CCI-induced hyperalgesia. Consistent with these observations, intrathecal injection of IL-33 enhanced CCI hyperalgesia and induced hyperalgesia in naive mice. IL-33–mediated hyperalgesia during CCI was dependent on a reciprocal relationship with TNF-α and IL-1β. IL-33–induced hyperalgesia was markedly attenuated by inhibitors of PI3K, mammalian target of rapamycin, MAPKs (p38, ERK, and JNK), NF-κB, and also by the inhibitors of glial cells (microglia and astrocytes). Furthermore, targeting these signaling pathways and cells inhibited IL-33–induced TNF-α and IL-1β production in the spinal cord. Our study, therefore, reveals an important role of oligodendrocyte-derived IL-33 in neuropathic pain.—Zarpelon, A. C., Rodrigues, F. C., Lopes, A. H., Souza, G. R., Carvalho, T. T., Pinto, L. G., Xu, D., Ferreira, S. H., Alves-Filho, J. C., McInnes, I. B., Ryffel, B., Quesniaux, V. F. J., Reverchon, F., Mortaud, S., Menuet, A., Liew, F. Y., Cunha, F. Q., Cunha, T. M., Verri, Jr., W. A. Spinal cord oligodendrocyte-derived alarmin IL-33 mediates neuropathic pain. *FASEB J*. 30, 54–65 (2016). www.fasebj.org

Neuropathic pain results from an injury of the peripheral nervous system or CNS by diseases such as multiple sclerosis, cancer, diabetes, herpes zoster, or trauma of nerves (1). It affects ~6% of the population worldwide (2) with symptoms ranging from paresthesia, spontaneous pain, allodynia to hyperalgesia. The treatment of neuropathic pain is still a challenge because only 40–60% of patients have some degree of relief with treatment (3, 4).

Healthy peripheral nerves are surrounded by numerous cells (Schwann cells, fibroblasts, endothelial cells, macrophages, mast cells, and glial cells), which regulate the responses to tissue and nerve injury (5, 6). Nerve injury results in pain behavior accompanied by an increase of proinflammatory cytokines, contributing to axonal damage (7). Cytokine production and action in neuropathic pain are related to the activation of PI3K, mammalian target of rapamycin (mTOR), MAPKs, and NF-κB (6, 8). Furthermore, in response to damage to peripheral nerves, spinal nerves, and spinal cord, both

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Abbreviations: BSA, bovine serum albumin; CCI, chronic constriction injury; cit, citrine; GFAP, glial fibrillary acidic protein; Iba-1, ionized calcium-binding adapter molecule 1; IL-1ra, IL-1 receptor antagonist; IL-1RAcP, IL-1 receptor accessory protein; mTOR, mammalian target of rapamycin; NeuN, feminizing locus on X-3; OLIG2, oligodendrocyte transcription factor; p38, p38

microglia and astrocytes in the spinal cord are activated, and their inhibition diminishes nociceptive behavior (8–10).

IL-33 or IL-1F11 is the latest member of the IL-1 family of cytokines, which includes IL-1α, IL-1β, IL-18, and IL-1 receptor antagonist (IL-1ra) (11). IL-33 requires the expression of both IL-33 receptor (ST2) and IL-1 receptor accessory protein (IL-1RAcP) to function as a classic cytokine (12). ST2 exists in transmembrane and soluble forms, which function as signaling receptors and scavenger receptors, respectively (13).

IL-33/ST2 signaling has pleiotropic roles in diseases and can be considered a target as well as a treatment depending on cytokine milieu and disease context (14). IL-33/ST2 signaling is important in antigen challenge-induced cutaneous and articular mechanical hyperalgesia (15). In addition to adaptive immune responses, IL-33/ST2 signaling mediates the hyperalgesia in the carrageenan innate inflammation model (16) and overt pain-like behavior induced by acetic acid, phenyl-p-benzoquinone, and formalin (17, 18). IL-33/ST2 signaling is also important in bone cancer pain (19). A common mechanism described is that IL-33/ST2-induced pain depends on the further production of cytokines (15, 16, 19). However, the molecular signaling pathways activated by IL-33/ST2 and its cellular targets in neuropathic pain remain to be determined. In the present study, we demonstrated that spinal cord oligodendrocyte-derived IL-33 triggers ST2 signaling mediating chronic constriction injury (CCI)-induced neuropathic pain by activating spinal cord cytokine production, PI3K/PKB, mTOR, MAPK, and NF-κB signaling pathways, and glial cells.

MATERIALS AND METHODS

Animals

BALB/c [wild-type (WT)], ST2$^{-/-}$ (BALB/c background), IL-33/citrine (cit) reporter mice (IL-33$^{cit/+}$ C57BL/6 background), C57BL/6 (WT), and TNFR1$^{-/-}$ (TNF receptor R1; C57BL/6 background) mice were used. TNFR1$^{-/-}$ mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA), and ST2$^{-/-}$ and IL-33$^{cit/+}$ mice were obtained from Dr. Andrew McKenzie (Laboratory of Molecular Biology, Cambridge University, Cambridge, United Kingdom) (20). All mice (male adults) were bred and maintained in the Ribeirao Preto Medical School at the University of Sao Paulo, Brazil, except IL-33$^{cit/+}$ mice, which were bred in the Transgenose Institute animal facility (Centre National de la Recherche Scientifique, Orléans, France). Animal care and handling procedures were in accordance with the International Association for the Study of Pain guidelines and with the approval of the Ribeirao Preto Medical School Ethics Committee of the University of Sao Paulo and Universidade Estadual de Londrina. Mice were housed in temperature-controlled rooms (22–25°C), with access to water and food *ad libitum.*

Reagents

The following materials were obtained from the sources indicated. Wortmannin, rapamycin, PD98059, SP600125, SB202190, pyrrolidine dithiocarbamate (PDTC), minocycline, fluorocitrate, and α-aminoadipate were obtained from Sigma-Aldrich (St. Louis, MO, USA). Recombinant mouse TNF-α and human IL-1β were acquired from the National Institute for Biologic Standards and Control (South Mimms, United Kingdom). Recombinant human IL-33 and soluble IL-33 receptor (sST2) were produced as previously described (21, 22).

Model of CCI

Mice were anesthetized with ketamine and xylazine (10 μl/10 g) followed by trichotomy in the surgery area. The incision was performed in the rear leg, and the sciatic nerve was exposed with a glass rod. One moderate constriction injury was performed around the sciatic nerve with a chrome suture according to the method described by Bennett and Xie (23). For the sham-surgery controls, mice underwent the same procedure without the nerve constriction. Spinal nerve ligation (SNL) was produced by removing the L5 transverse process exposing the L4 and L5 spinal nerves, then the L5 spinal nerve was isolated and tightly ligated (24).

Electronic pressure-meter test

The mechanical hyperalgesia test (25) consisted of evoking a hindpaw flexion reflex with a handheld force transducer (electronic anesthesiometer; IITC Life Science Inc., Woodland Hills, CA, USA) adapted with a 0.5 mm^2 polypropylene tip. The end point was the removal of the paw followed by clear flinching movements. The intensity of the pressure was automatically recorded after the paw withdrawal, and the value for the response was obtained by averaging 3 measurements. Mice were tested before and after treatments. Results are expressed as δ (Δ) withdrawal threshold (in grams) calculated by subtracting the mean measurements at indicated time points from the basal mean measurements. Withdrawal threshold was 8.6 ± 0.5 g (mean ± SEM; $n = 40$) before injection of the hyperalgesic agents or surgery. Experimenters were blinded to the treatments.

Cytokine measurement

Mice were terminally anesthetized with isoflurane at indicated time points after CCI, and spinal cord L4–L6 samples were collected and homogenized in 300 μl buffer containing protease inhibitors. TNF-α, IL-1β, and IL-33 concentrations were determined by ELISA (all from eBioscience, San Diego, CA, USA) using paired antibodies as instructed by the manufacturer.

Western blot assay

Mice were terminally anesthetized on an isoflurane chamber, and the L4–L6 segments of the spinal cord were dissected out immediately and homogenized in RIPA buffer containing protease and phosphatase inhibitors. The lysates were then homogenized and centrifuged (0.5 g for 10 min at 4°C). The protein extracts were separated by SDS-PAGE and transferred onto a PVDF transfer membrane (for mTOR analysis) or nitrocellulose membrane for the other analysis (GE

Healthcare-Amersham, Pittsburgh, PA, USA). Membranes were then incubated in blocking buffer [5% nonfat milk in Tris-buffered saline with Tween 20 or 1% bovine serum albumin (BSA)] for different times for each antibody at 4°C and incubated overnight at 4°C in the presence of primary antibody. All antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA), except for p-p38 (p38 MAPK), total p38, and IL-33 from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and Iba-1 (ionized calcium-binding adapter molecule 1) from Wako BioProducts (Richmond, VA, USA); all catalog numbers are indicated below. The antibodies and Western blot conditions were IL-33 (sc98660, dilution 1:100) on 10% gel and blocked with 5% nonfat milk; phospho p38 (sc7975, 1:100) and total p38 (sc535, 1:100) on 10% gel and blocked with 5% BSA; phospho JNK (#9251, 1:200) and total JNK (#9252, 1:300) on 10% gel and blocked with 5% BSA; phospho ERK (#9101, 1:500) and total ERK (#9102, 1:500) on 10% gel and blocked with 5% BSA; phospho IκBα (#2859, 1:200) on 10% gel and blocked with 5% nonfat milk; total IκBα (sc371, 1:200) on 10% gel and blocked with 5% BSA; phospho PKB (#9271, 1:300) and total PKB (#9272, 1:200) on 10% gel and blocked with 5% BSA; PI3K (#4252, 1:200) on 10% gel and blocked with 5% nonfat milk; and β-actin (#4970), glial fibrillary acidic protein (GFAP; #3670, 1:500), and Iba-1 (#016-20.001, 1:300) on 12 and 15% gel, respectively, and blocked with 5% nonfat milk. For evaluation of mTOR activity, 2 phosphorylated mTOR antibodies were used: Ser2448 (#2971, 1:500) and Ser2481 (#2974, 1:500) as well as total mTOR (#2972, 1:500) on 10% gel and blocked with 5% BSA. The molecular masses of protein were confirmed by Precision Plus Protein Standards (Bio-Rad, Hercules, CA, USA). After washing in PBS with Tween 20, the membrane was incubated with secondary antibody for 2 h at room temperature. Protein was visualized by chemiluminescence with ECL detection reagent (GE Healthcare-Amersham). The membranes were reprobed with antibody against β-actin or total protein of interest for use as loading control in addition to loading the same amount of protein.

Spinal cord immunofluorescence

Animals were terminally anesthetized with a ketamine (50 mg/ml)/ xylazine (20 mg/ml) combination and perfused through the ascending aorta with saline followed by 4% paraformaldehyde. After the perfusion, L4–L6 segments of the spinal cord were dissected out and postfixed, and then they were replaced overnight with 30% sacarose. The spinal cord segments were embedded in optimum cutting temperature, and 10 μm sections were cut in a cryostat and processed for immunofluorescence. All of the sections were blocked and incubated with a mixture of primary antibodies against NeuN (feminizing locus on X-3; MAB377; EMD Millipore, Billerica, MA, USA), GFAP (180063; Invitrogen, Life Technologies, Carlsbad, CA, USA), Iba-1 (ab5076; Abcam Inc., Cambridge, MA, USA), OLIG2 (oligodendrocyte transcription factor; AB9610; EMD Millipore), and finally by a mixture of conjugated secondary antibodies for 2 h at room temperature. The images and analysis were performed using a fluorescence microscope (DM6000B; Leica Microsystems, Buffalo Grove, IL, USA) for **Fig. 1** and using confocal microscopy (SP8; Leica Microsystems) for Fig. 5.

Experimental protocols

IL-33 concentration/expression in the spinal cord (L4–L6) was determined by ELISA (d 3, 7, 14, and 21) and Western blot (d 7).

The cellular source of IL-33 in the spinal cord was determined using CCI IL-33$^{cit/+}$ mice by immunofluorescence 7 d after surgery. CCI was induced on ST2$^{+/+}$ and ST2$^{-/-}$ mice, and the intensity of mechanical hyperalgesia was measured 1–23 d by the electronic pressure-meter test. ST2$^{+/+}$ CCI mice received intrathecal treatment with sST2 (100, 300, and 1000 ng per mouse) on d 7 after CCI (the peak of mechanical hyperalgesia) followed by mechanical hyperalgesia measurement at indicated time points. IL-33 (10, 30, and 100 ng per mouse) was also injected intrathecally followed by mechanical hyperalgesia evaluation and determination of its hyperalgesic effect in naive and CCI ST2$^{+/+}$ and ST2$^{-/-}$ mice. In another set of experiments, spinal cord samples from sham-surgery and CCI ST2$^{+/+}$ and ST2$^{-/-}$ mice were collected 3, 7, 14, and 21 d after surgery for Western blot analysis to determine the effect of ST2 deficiency on the CCI-induced activation of PI3K, mTOR, ERK, JNK, p38, and NF-κB. Furthermore, naive ST2$^{+/+}$ and ST2$^{-/-}$ mice received intrathecal injection of TNF-α (1 ng) and IL-1β (1 ng) followed by mechanical hyperalgesia measurement at indicated time points and IL-33 level determination by ELISA after 2 h. To determine the role of TNF-α/TNFR1 and IL-1β in IL-33–induced hyperalgesia, IL-33 was injected intrathecally (100 ng) in WT, TNFR1$^{+/+}$, and TNFR1$^{-/-}$ mice, or IL-1ra–treated (30 mg/kg, i.p.) mice. Mechanical hyperalgesia was measured, and TNF-α and IL-1β concentrations (after 2 h) in the spinal cord (L4–L6) of ST2$^{+/+}$ and ST2$^{-/-}$ sham-surgery and CCI mice 7 d after surgery were analyzed. To determine the molecular signaling in nociception in the spinal cord triggered by IL-33 during neuropathy, mice received intrathecal coinjection of IL-33 and one of the following inhibitors at indicated doses: wortmannin (PI3K inhibitor, 1–10 μg); rapamycin (mTOR inhibitor, 1–10 μg); PD98059 (MEK1 inhibitor, prevents ERK activation, 1–10 μg); SP600125 (JNK inhibitor, 1–10 μg); SB202190 (p38 inhibitor, 1–10 μg); and PDTC (NF-κB inhibitor, 30 and 300 μg). The effect of IL-33 on glial cells was evaluated by intrathecal coinjection of IL-33 (100 ng) and minocycline (microglia inhibitor, 15, 50, and 150 μg), fluorocitrate (astrocyte inhibitor, 0.53, 1.6, and 4.8 μg), or α-aminoadipate (astrocyte inhibitor, 10, 30, 100, and 300 nmol) followed by evaluation of mechanical hyperalgesia 1–7 h later. Western blot and confocal microscope analysis were used to determine the activation of Iba-1 (microglia) and GFAP (astrocytes) by IL-33 intrathecal injection (24 h) in ST2$^{+/+}$ and ST2$^{-/-}$ mice. The effect of ST2 deficiency on CCI-induced activation of Iba-1 and GFAP was determined by Western blot. The effectiveness of intrathecal treatment with wortmannin, rapamycin, PD98059, SP600125, SB202190, PDTC, minocycline, fluorocitrate, and α-aminoadipate to reduce CCI-induced mechanical hyperalgesia was determined on d 7 after surgery.

Statistical analyses

The results are representative of 2 independent experiments and are presented as the means $\pm$ SEM ($n = 6$ per group in each experiment). One-way ANOVA followed by Tukey's t test was performed to evaluate the differences between responses. Statistical differences were considered to be significant at $P < 0.05$.

RESULTS

IL-33 production and cellular source of IL-33/cit in the spinal cord during CCI

CCI was performed on naive BALB/c mice, and IL-33 level in the spinal cord (L4–L6) lysates was determined by ELISA and Western blot. CCI induced a significant
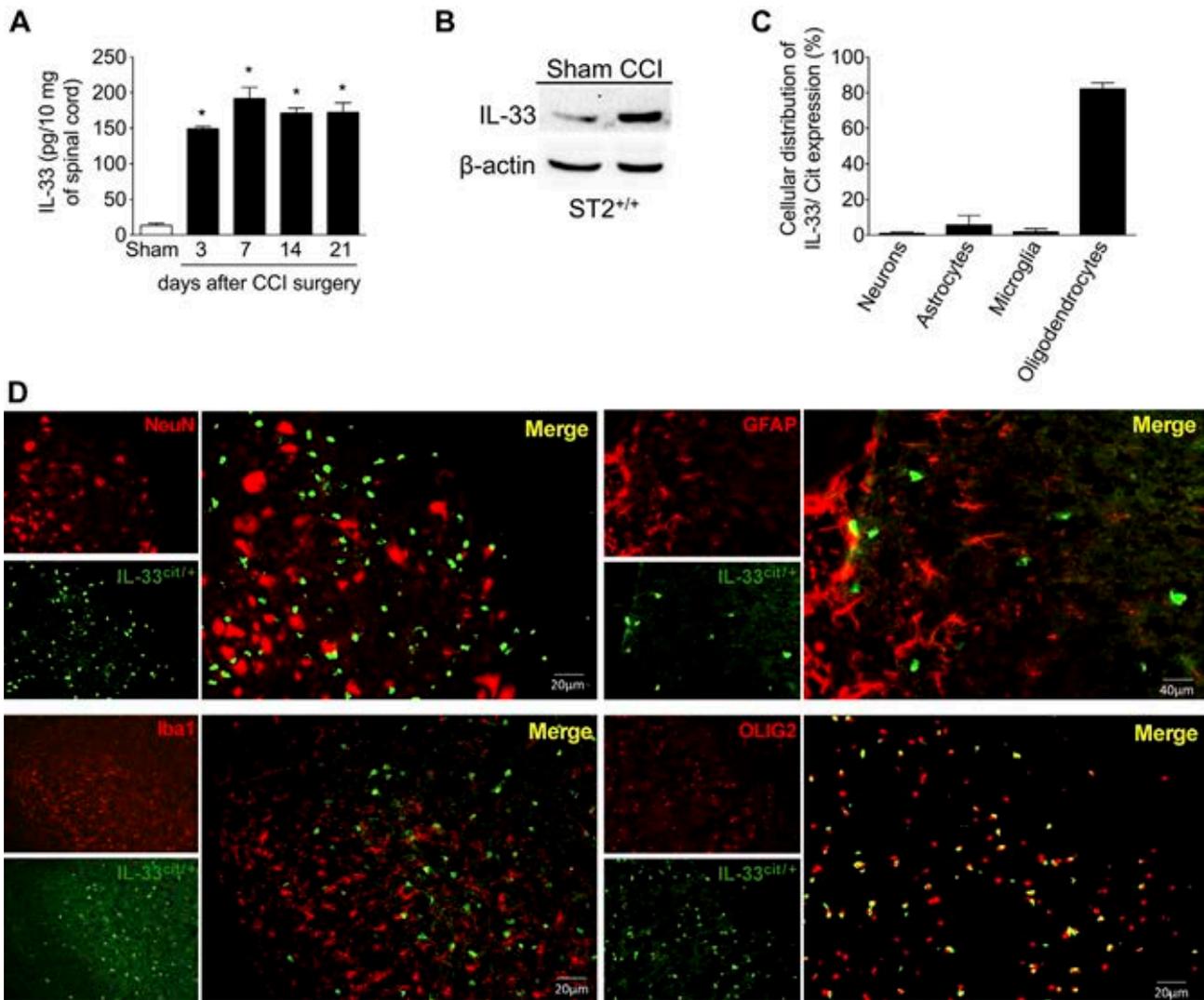
Figure 1. Detection of IL-33 and IL-33/cit in the spinal cord. IL-33 concentration/expression was determined 3–21 d after CCI surgery by ELISA (*A*) and 7 d after CCI surgery by Western blot (*B*) in spinal cord (L4–L6) samples. CCI was performed on C57BL/6 IL-33cit/+ mice (green), and spinal cord samples were stained with primary antibodies for neuron (NeuN, red), astrocyte (GFAP, red), microglial (Iba-1, red), and oligodendrocyte (OLIG2, red) detection (*D*). Double immunostaining is shown in yellow (*D*). The percentage of cellular types expressing IL-33/cit is presented (*C*), and the representative immunostainings are shown (*D*). *n* = 6, representative of 2 separate experiments (*A* and *B*); *n* = 1000 cells examined (*C*) from *n* = 2 mice (*D*). One-way ANOVA followed by Tukey's *t* test was performed. *$P < 0.05$ compared with sham-surgery group.

increase in the levels of IL-33 compared with sham-surgery mice 3–21 d after CCI as observed by ELISA (Fig. 1*A*). This result indicates a potential association of IL-33 and CCI over 21 d of disease. Western blot analysis corroborated that IL-33 levels were elevated in CCI mice at d 7 (Fig. 1*B*). In order to identify the potential cellular source of IL-33, we performed CCI in IL-33cit/+ mice and after 7 d determined the colocalization of IL-33/cit and spinal cord (L4–L6) cell markers, including neurons (NeuN), astrocytes (GFAP), microglia (Iba-1), and oligodendrocytes (OLIG2) by immunofluorescence. The main IL-33/cit-expressing cells in the spinal cord were OLIG2$^+$ cells. A minor expression of IL-33/cit was found in cells expressing NeuN, GFAP, or Iba-1. These results indicate that spinal cord IL-33 is mainly derived from oligodendrocytes during CCI-induced neuropathic pain (Fig. 1*C*, *D*).

Effect of ST2 deficiency and IL-33 injection in CCI-induced mechanical hyperalgesia

We then investigated the role of IL-33 and ST2 in CCI-induced mechanical hyperalgesia. CCI induced significant mechanical hyperalgesia between 3 and 23 d compared with the sham-surgery group (**Fig. 2***A*). After 23 d, the mechanical hyperalgesia began to decline but remained significant up to 35 d (data not shown). CCI-induced mechanical hyperalgesia was markedly reduced in ST2$^{-/-}$ mice compared with WT mice (Fig. 2*A*). Consistent with this observation, intrathecal injection of sST2 (a decoy receptor for IL-33) reduced the CCI-induced mechanical hyperalgesia in a dose-dependent manner (Fig. 2*B*). The intrathecal injection of sST2 did not affect basal mechanical thresholds *per se* (data not shown). We also observed that SNL-induced neuropathic pain was reduced in ST2$^{-/-}$
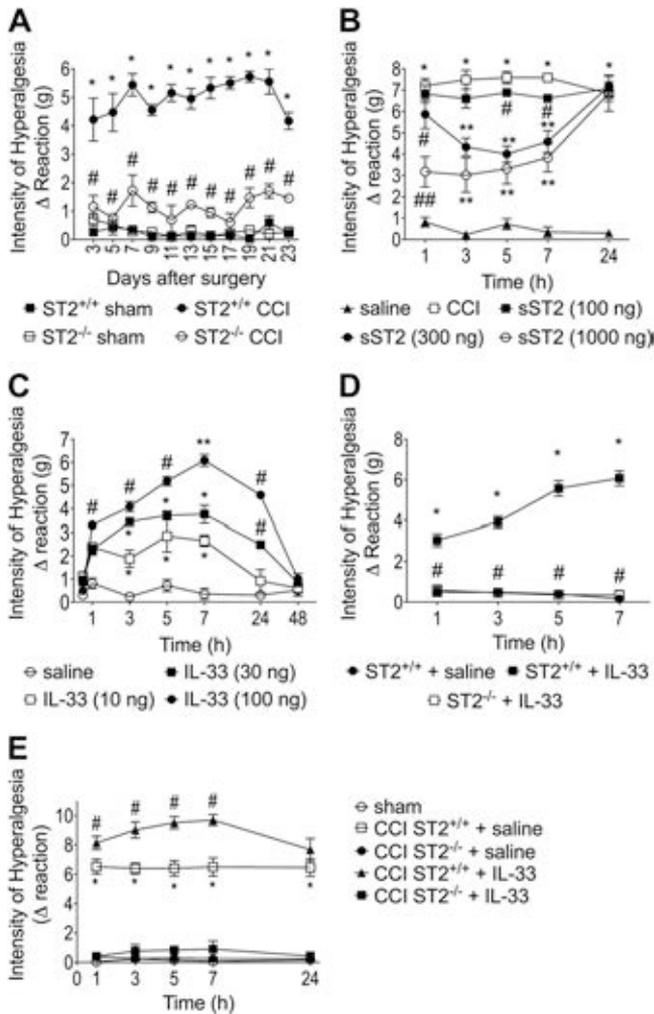
Figure 2. Effects of ST2 deficiency and IL-33 injection on CCI-induced mechanical hyperalgesia. $ST2^{+/+}$ and $ST2^{-/-}$ mice underwent CCI or sham surgery. *A*) Mechanical hyperalgesia was evaluated at indicated time points. *B*) $ST2^{+/+}$ and $ST2^{-/-}$ sham-surgery and CCI mice received intrathecal injection of sST2 on d 7 after surgery, and the mechanical hyperalgesia was evaluated at indicated time points after sST2 treatment. *C–E*) IL-33 was injected intrathecally (i.t.) into $ST2^{+/+}$ naive mice (*C*), IL-33 (100 ng, i.t.) was injected into naive $ST2^{+/+}$ and $ST2^{-/-}$ mice (*D*), and IL-33 was injected into $ST2^{+/+}$ and $ST2^{-/-}$ sham-surgery and CCI mice (*E*). Mechanical hyperalgesia was evaluated at indicated time points after IL-33 injection in naive mice and at 7 d after CCI surgery ($n = 6$, representative of 2 separate experiments). One-way ANOVA followed by Tukey's *t* test was performed. *$P < 0.05$ compared with saline or sham-surgery group; #$P < 0.05$ compared with CCI $ST2^{+/+}$ group or IL-33 10 ng, IL-33 in $ST2^{+/+}$ mice or vehicle $ST2^{+/+}$ CCI group; **$P < 0.05$ compared with the lower dose of sST2; ##$P < 0.05$ compared with 100 and 300 ng sST2.

compared with $ST2^{+/+}$ mice (Supplemental Fig. S1). These results, therefore, demonstrate that the IL-33/ST2 signaling pathway plays an important role in CCI- and SNL-induced mechanical hyperalgesia. We next determined a direct effect of IL-33 on CCI-induced hyperalgesia. Naive mice were injected intrathecally with IL-33 (10, 30, and 100 ng per mouse), and the mechanical hyperalgesia was determined. IL-33 induced significant mechanical hyperalgesia in a dose-dependent manner,

with the highest hyperalgesia induced by 100 ng IL-33 (Fig. 2*C*). The mechanical hyperalgesia induced by intrathecal injection of IL-33 was absent in $ST2^{-/-}$ mice (Fig. 2*D*). Moreover, IL-33 injected intrathecally on d 7 after CCI significantly enhanced CCI-induced mechanical hyperalgesia in $ST2^{+/+}$ mice, but not in $ST2^{-/-}$ mice (Fig. 2*E*). These results therefore further confirm that IL-33 plays an important role in CCI-induced hyperalgesia.

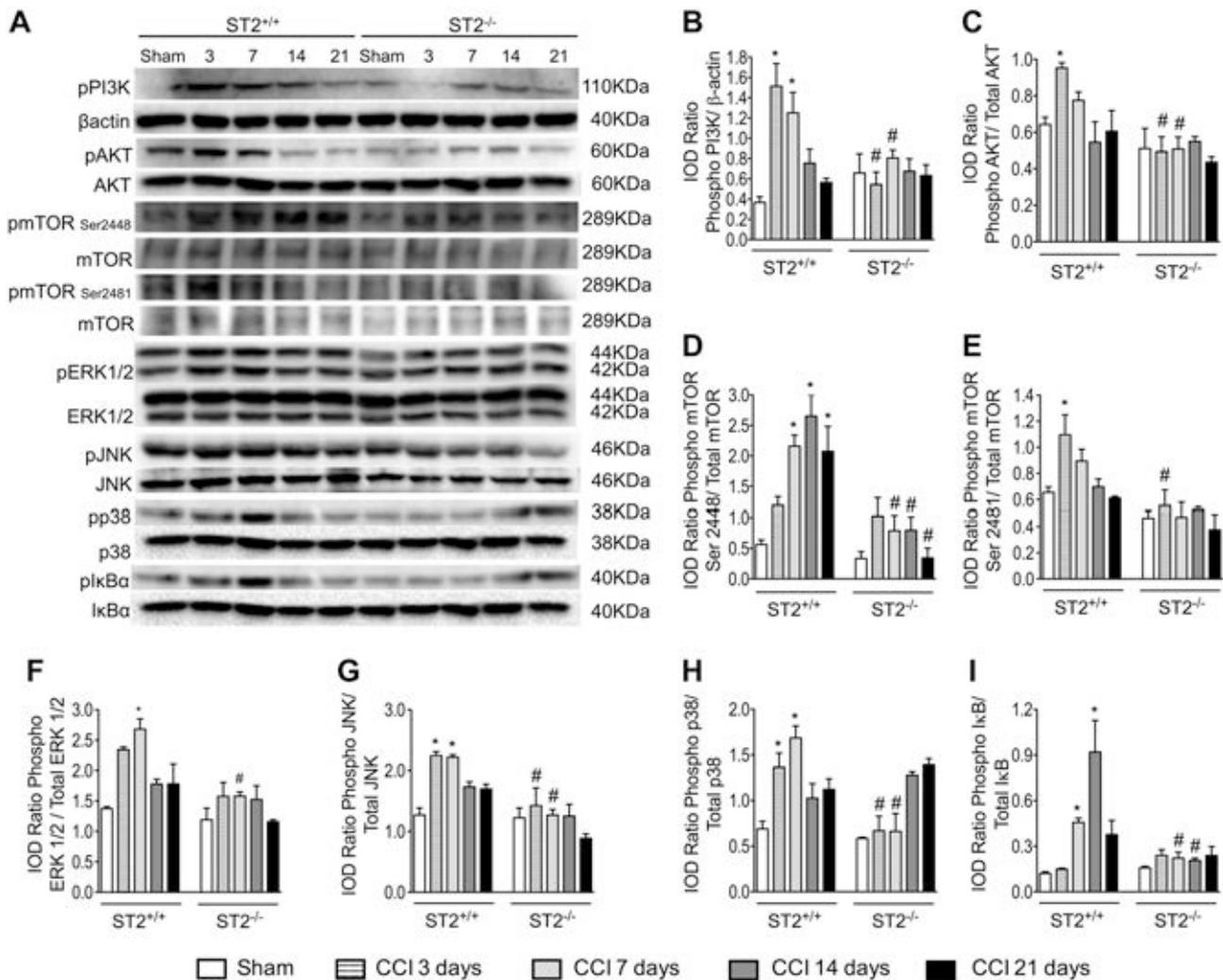### CCI-induced activation of PI3K, PKB, mTOR, MAPKs, and NF-κB depends on IL-33/ST2

We next investigated the role of the IL-33/signaling pathway in CCI-induced phosphorylation of PI3K, PKB, mTOR, MAPK, and NF-κB. CCI was performed on WT and $ST2^{-/-}$ mice, and spinal cords (L4–L6) were harvested 3–21 d after surgery. Western blot analysis of the spinal cord tissues shows that all the transcription factors investigated were activated, peaking between d 3 and 14 after surgery (**Fig. 3**). In contrast, these transcription factors were only modestly activated in the $ST2^{-/-}$ mice that underwent similar surgery, and the level of activation was significantly lower compared to that of the WT mice (Fig. 3). These results, therefore, indicate that CCI-induced activation of PI3K, PKB, mTOR, MAPK, and NF-κB is ST2 dependent.

IL-33–induced hyperalgesia is dependent on activation of PI3K, mTOR, MAPKs, and NF-κB

We further investigated the molecular signaling pathway of IL-33–induced hyperalgesia. Mice were injected intrathecally with IL-33 (100 ng) together with one of the following inhibitors: wortmannin (PI3K inhibitor), rapamycin (mTOR inhibitor), PD98059 (MEK1/2 inhibitor, prevents ERK1/2 activation), SP600125 (JNK inhibitor), or SB202190 (p38 inhibitor) at doses of 1, 3, and 10 μg per mouse, or PDTC (NF-κB inhibitor) at doses of 30 and 300 μg per mouse. Mechanical hyperalgesia was measured from 1 to 7 h after injection. All the inhibitors used reduced the IL-33–induced hyperalgesia in a dose-dependent manner (**Fig. 4**). These results indicate that IL-33–induced mechanical hyperalgesia is dependent on the activation of PI3K, mTOR, ERK, JNK, p38, and NF-κB. CCI-induced hyperalgesia also follows a similar pathway because it is also inhibited by intrathecal treatments with wortmannin, rapamycin, PD98059, SP600125, SB202190, and PDTC (Supplemental Fig. S2).

IL-33– and CCI-induced hyperalgesia is associated with microglial and astrocyte responses in an ST2-dependent manner

We then determined the cell types associated with IL-33– and CCI-induced mechanical hyperalgesia. WT mice were injected intrathecally with IL-33 (100 ng) together with graded doses of minocycline (microglia inhibitor), fluorocitrate (astrocyte inhibitor), or α-aminoadipate (astrocyte inhibitor), and mechanical hyperalgesia was measured up to 7 h postinjection. Minocycline, fluorocitrate, and α-aminoadipate inhibited IL-33–induced hyperalgesia in

Figure 3. IL-33/ST2 is required for CCI-induced activation of PI3K, PKB, mTOR, MAPKs, and NF-κB. CCI or sham surgery was performed on ST2$^{+/+}$ and ST2$^{-/-}$ mice, and samples of spinal cord were collected for Western blot analysis at d 3, 7, and 14, and 21 after surgery. *A*) Representative Western blots of PI3K, PKB, mTOR, MAPKs (ERK, JNK, and p38), and NF-κB. *B–I*) The IOD (intensity of optical density) ratios are presented as follows: PI3K:β-actin (*B*); phospho-PKB (Ser473):total PKB (*C*); phospho-mTOR (phosphorylated at Ser2448 *via* PI3K/PKB signaling pathway):total mTOR (*D*); phospho-mTOR (autophosphorylated at Ser2481):total mTOR (*E*); phospho-ERK 1/2:total ERK 1/2 (*F*); phospho-JNK:total JNK (*G*); phospho-p38:total p38 (*H*); and phospho-IκBα (Ser32):total IκBα (*I*). *n* = 4, representative of 2 separate experiments. One-way ANOVA followed by Tukey's *t* test was performed. *$P < 0.05$ comparing sham-surgery group and CCI group; #$P < 0.05$ comparing CCI ST2$^{+/+}$ and CCI ST2$^{-/-}$ groups at the same time point.

a dose-dependent manner (**Fig. 5*A*–*C***). Corroborating these behavioral/pharmacologic data, IL-33 intrathecal injection induced microglia (Iba-1, Fig. 5*D*, *F*) and astrocyte (GFAP, Fig. 5*E*, *G*) activation in the spinal cord as determined by Western blot and immunofluorescence, respectively. We next tested if minocycline, fluorocitrate, and α-aminoadipate have a similar effect on CCI-induced hyperalgesia. The inhibitors were injected intrathecally in WT mice on d 7 after CCI. Minocycline, fluorocitrate, and α-aminoadipate effectively inhibited hyperalgesia induced by CCI (**Fig. 6*A*–*C***). The spinal cords of the mice treated with the inhibitors as described in Fig. 6*A*–*C* above were harvested on d 7 (3 h after treatment), and the concentration of IL-33 was determined by ELISA. IL-33 concentration elevated by CCI was completely abrogated by the treatment with minocycline, fluorocitrate, and

α-aminoadipate (Fig. 6*D*). We next determined the role of IL-33/ST2 signaling in the CCI-induced activation of microglia and astrocytes (Fig. 6*E*–*G*). WT and ST2$^{-/-}$ mice were subjected to CCI, and spinal cords were harvested 3–21 d after surgery. Western blot analysis of the spinal cord tissue shows that microglia (Iba-1, Fig. 6*E*, *G*) and astrocytes (GFAP, Fig. 6*F*, *G*) were activated by CCI, peaking at 3–7 d and 7–14 d, respectively. In contrast, both microglial and astrocyte activations were modest in the ST2$^{-/-}$ mice that underwent similar CCI, and the level of activation was significantly lower than that in the WT mice (Fig. 6*E*–*G*). Together, these results demonstrate that IL-33– and CCI-induced hyperalgesia is associated with microglia and astrocytes and that the CCI-induced microglial and astrocyte activation is dependent on the IL-33/ST2 signaling pathway. Furthermore,

Figure 4. IL-33–induced hyperalgesia is dependent on activation of PI3K, mTOR, MAPKs, and NF-κB. Mice were coinjected intrathecally with IL-33 and 1 of the following compounds: wortmannin (PI3K inhibitor) (*A*); rapamycin (mTOR inhibitor) (*B*); PD98059 (MEK1 inhibitor, prevents ERK activation) (*C*); SP600125 (JNK inhibitor) (*D*); SB202190 (p38 inhibitor) (*E*); and PDTC (NF-κB inhibitor) (*F*). Mechanical hyperalgesia was evaluated at indicated time points after IL-33 injection ($n = 6$, representative of 2 separate experiments). One-way ANOVA followed by Tukey's *t* test was performed. *$P < 0.05$ compared with saline group; #$P < 0.05$ compared with vehicle + IL-33 group; **$P < 0.05$ compared with the lowest dose of inhibitors tested.
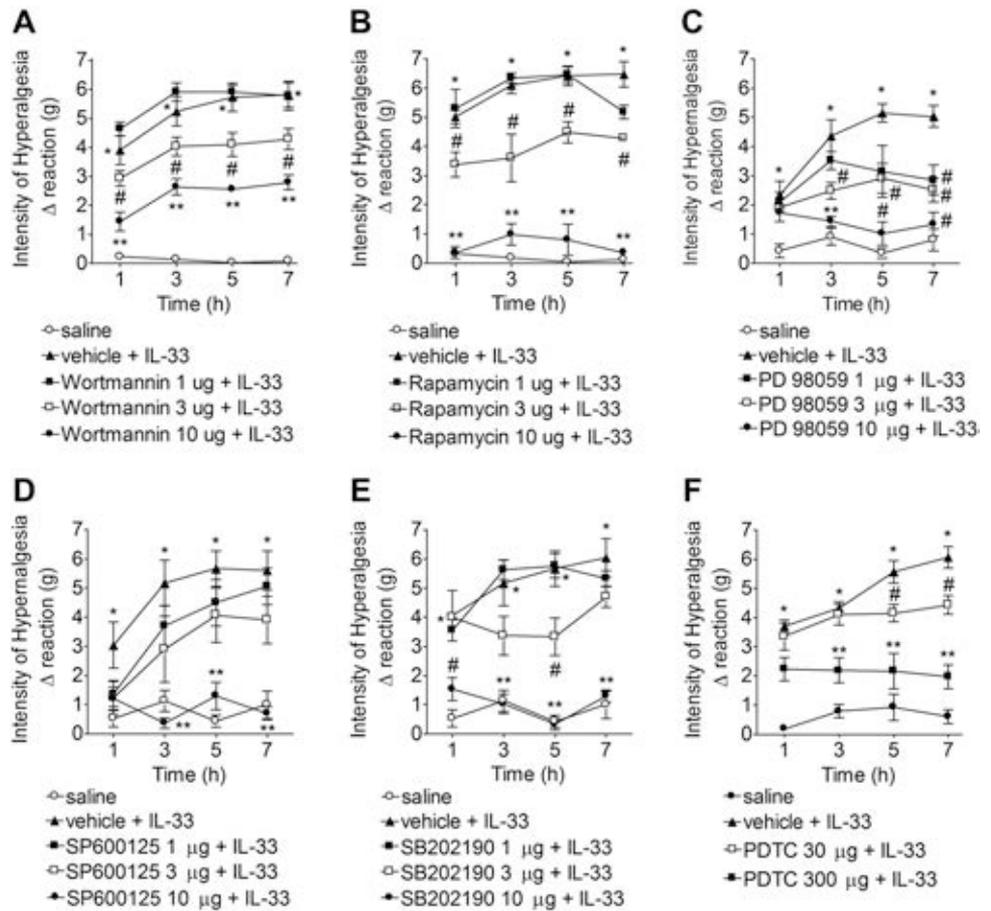
CCI-induced production of IL-33 is also microglial and astrocyte dependent.

### IL-33 induced TNF-α and IL-1β production in a PI3K-, mTOR-, MAPK-, and NF-κB–dependent manner

Finally, we investigated the role of TNF-α and IL-1β in IL-33–induced hyperalgesia. The mechanical hyperalgesia induced by the intrathecal injection of IL-33 was markedly reduced in TNFR1$^{-/-}$ mice compared with the TNFR1$^{+/+}$ mice (**Fig. 7A**), indicating that the IL-33–induced hyperalgesia is at least partly TNF-α dependent. The IL-33–induced hyperalgesia was also significantly reduced by treatment of mice with IL-1ra (30 mg/kg, i.p.) compared to saline-treated controls (Fig. 7*B*). Furthermore, IL-33 (intrathecal injection) induced significant production of TNF-α (Fig. 7*C*) and IL-1β (Fig. 7*D*) in the spinal cord. Corroborating these data in naive mice, CCI induced production of TNF-α (Fig. 7*E*) and IL-1β (Fig. 7*F*) in the spinal cord of ST2$^{+/+}$ mice, and this was absent in ST2$^{-/-}$ mice at 7 (Fig. 7*E, F*) and 14 (data not shown) d after surgery.

We therefore determined the role of these 2 cytokines in IL-33–induced hyperalgesia and the effect of the inhibitors of PI3K, mTOR, MAPK, NF-κB, microglia, and astrocytes. WT mice were injected intrathecally with IL-33 (100 ng) together with one of the inhibitors: PI3K (wortmannin, 10 μg); mTOR (rapamycin, 10 μg); ERK (PD98059, 10 μg);

JNK (SP600125, 10 μg); p38 (SB202190, 10 μg); NF-κB (PDTC, 300 mg/kg); microglia (minocycline, 50 μg); and astrocytes (fluorocitrate, 1.6 μg, and α-aminoadipate, 100 nmol). Spinal cords were harvested 2 h after injections, and the concentrations of TNF-α and IL-1β in the tissues were determined using ELISA. IL-33 markedly increased the concentrations of TNF-α and IL-1β, which were significantly inhibited by the presence of any one of the inhibitors (Fig. 7*G, H*). These results therefore demonstrate that IL-33 likely mediates mechanical hyperalgesia *via* the activation of PI3K, mTOR, MAPK, and NF-κB, leading to the induction of TNF-α and IL-1β in a microglial- and astrocyte-dependent manner. We then investigated the interdependency of IL-33 and TNF-α/IL-1β in hyperalgesia. The intrathecal injection of TNF-α (Supplemental Fig. S3*A*) and IL-1β (Supplemental Fig. S3*B*) induced significant mechanical hyperalgesia in ST2$^{+/+}$ mice, which was completely absent in ST2$^{-/-}$ mice. Moreover, 2 h after intrathecal injection of TNF-α (Supplemental Fig. S3*C*) and IL-1β (Supplemental Fig. S3*D*), there was a significant increase of IL-33 levels in the spinal cord, thus indicating a crosstalk between IL-33 and the 2 known hyperalgesic cytokines TNF-α and IL-1β.

DISCUSSION

The present study demonstrates that oligodendrocyte-derived IL-33 acting on ST2 contributes to CCI-induced
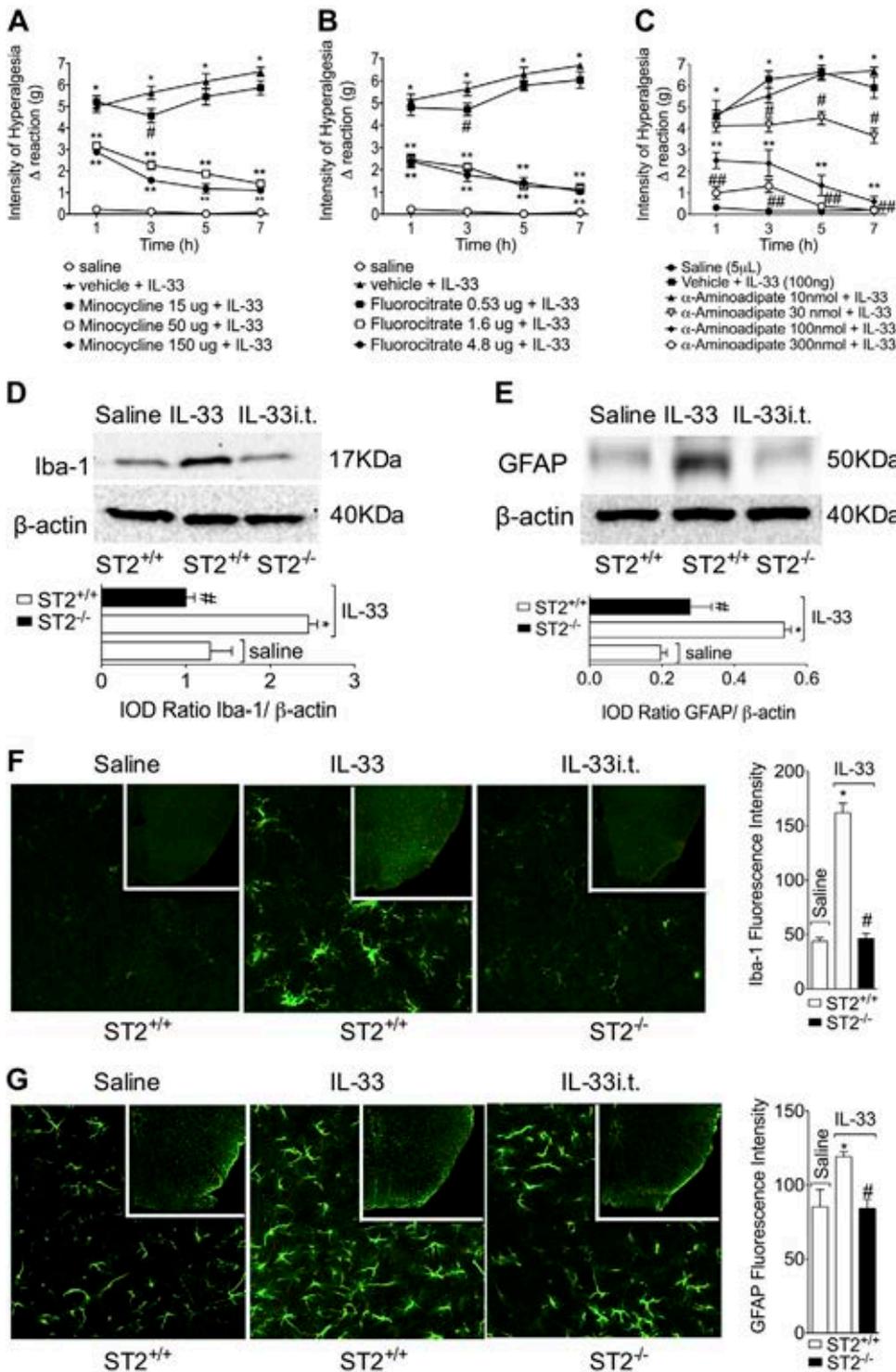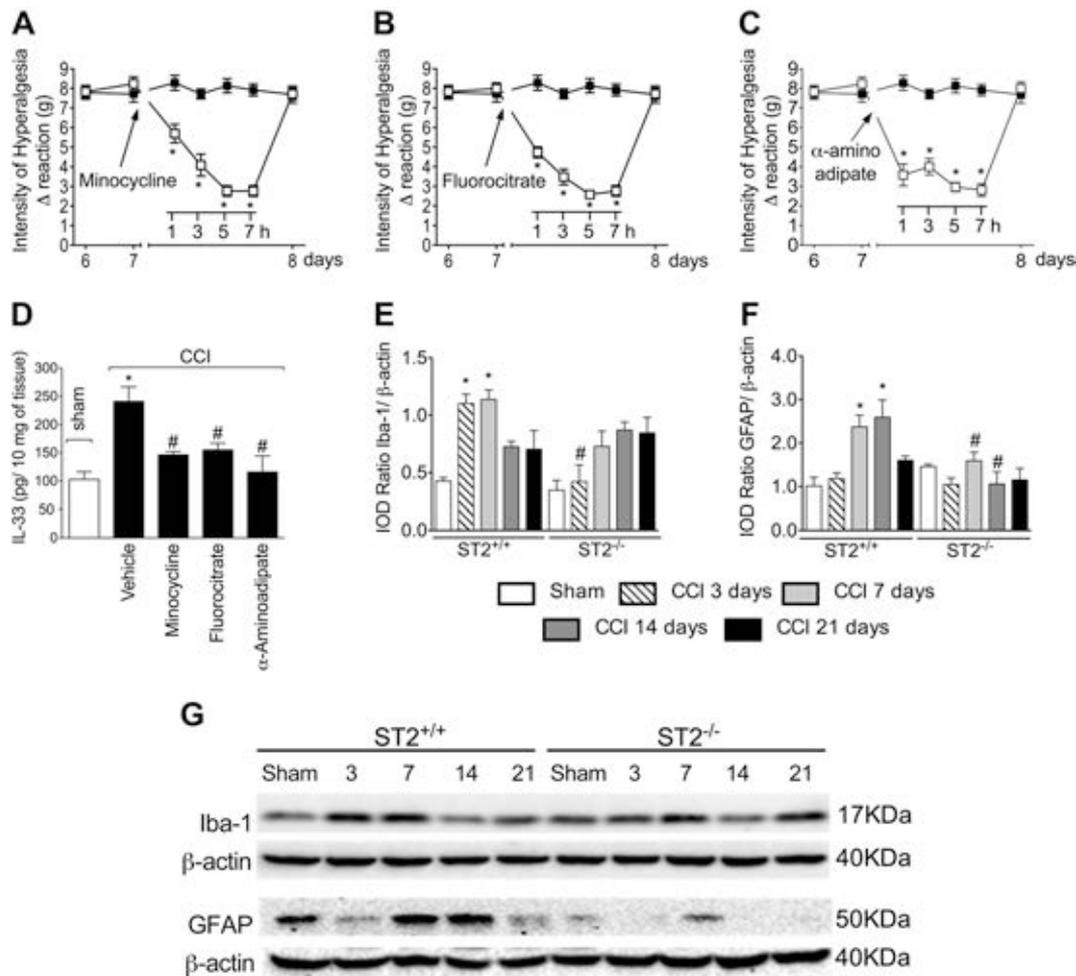
Figure 5. IL-33–induced hyperalgesia is associated with microglia and astrocytes in an ST2-dependent manner. Mice received coinjection (i.t.) of IL-33 and minocycline (microglia inhibitor) (*A*), fluorocitrate (astrocyte inhibitor) (*B*), or α-aminoadipate (selective astrocyte inhibitor) (*C*). Mechanical hyperalgesia was evaluated at indicated time points. Western blot assay (*D* and *E*) and immunofluorescence (*F* and *G*) were performed to evaluate the activation of microglia (*D* and *F*) and astrocytes (*E* and *G*) after IL-33 injection ($n = 6$ for hyperalgesia and $n = 4$ for Western blots and immunofluorescence, representative of 2 separate experiments). One-way ANOVA followed by Tukey's *t* test was performed. *$P < 0.05$ compared with saline group; #$P < 0.05$ compared with vehicle + IL-33 group; **$P < 0.05$ compared with the lowest dose of inhibitors tested; ##$P < 0.05$ compared with α-aminoadipate (100 nmol).

neuropathic pain by triggering TNF-α and IL-1β production and activating PI3K, mTOR, MAPKs ERK, JNK, and p38, NF-κB, and glial cells in the spinal cord. Although our group and others have demonstrated a prohyperalgesic role for IL-33/ST2 in inflammatory and cancer pain models by inducing hyperalgesic cytokine production (15–19), the pronociceptive role of IL-33 in neuropathic pain was not predicted because treatment with recombinant IL-33 alleviated secondary damage in the contused mouse spinal cord by significantly decreasing tissue loss,

demyelination, and astrogliosis, resulting in improved functional recovery (26).

Despite previous evidence of IL-33 expression by astrocytes under basal conditions (19, 27) and by spinal cord neurons and microglia in autoimmune encephalomyelitis (28), we observed minor IL-33/cit expression by spinal cord neurons, microglia, and astrocytes in CCI neuropathic mice. The major IL-33/cit expression was detected in spinal cord oligodendrocytes (~82%). To our knowledge, this is the first study to demonstrate that spinal cord

Figure 6. CCI-induced hyperalgesia is associated with microglia and astrocytes in an ST2-dependent manner. The mechanical hyperalgesia of CCI mice was evaluated on d 6–8 after surgery. On d 7, mice received intrathecal treatment with minocycline (50 µg) (*A*), fluorocitrate (1.6 µg) (*B*), or α-aminoadipate (100 nmol) (*C*). Spinal cord was collected for IL-33 analysis by ELISA (*D*). ST2$^{+/+}$ and ST2$^{-/-}$ mice underwent CCI or sham surgery, and samples of spinal cord were collected at 3, 7, 14, and 21 d after surgery. Intensity of optical density (IOD) ratios of Iba-1:β-actin (*E*) and GFAP:β-actin (*F*) and representative Western blots (*G*) are shown (*n* = 6 for hyperalgesia and ELISA analysis and *n* = 4 for Western blots, representative of 2 separate experiments). One-way ANOVA followed by Tukey's *t* test was performed. *$P < 0.05$ compared with sham-surgery group plus vehicle; #$P < 0.05$ compared with CCI plus vehicle ST2$^{+/+}$ group.

oligodendrocytes participate in neuropathic pain by producing IL-33. This result was not expected because the oligodendrocyte ablation triggers sensory changes resembling central neuropathic pain with cold allodynia and mechanical hyperalgesia not associated with autoreactive T- and B-cell infiltration in the spinal cord, microglial activation, or reactive astrogliosis (29). Oligodendrocytes have a central role as the main CNS source of IL-33 in the spinal contusion injury model (30), although IL-33 expression was also observed in astrocytes. IL-33 release by oligodendrocytes was attributed to cell damage, which was in accordance with the IL-33 release without new transcription. The IL-33 deficiency resulted in impaired CNS recovery after spinal cord injury due to lessened M2 response (30). We observed that the increase of IL-33 concentration/expression in the spinal cord by ELISA and Western blot as well as CCI induced a significant increase of IL-33 and ST2 mRNA expression compared to sham surgery at d 7 and 14 (data not shown), suggesting that in the

CCI model, there is up-regulation of the IL-33 system and not only the release of preformed IL-33. These differences in the IL-33 role in disease and IL-33 release with or without novel transcription are likely related to the great differences in the pathophysiologic mechanisms of each disease model. Spinal cord injury induces, in addition to neuronal loss, 50% death of astrocytes and oligodendrocytes adjacent to the site of injury (31). On the other hand, peripheral nerve injury induces little or no cell loss in the spinal cord and a striking proliferation of glial cells, mainly microglia, but also of integral membrane chondroitin sulfate proteoglycan-positive progenitors that mature to oligodendrocytes and limit astrocyte proliferation (32).

The Western blot analysis of the kinetics of CCI neuropathic pain shows that most of the molecular mechanisms addressed were activated at d 3 and 7 except for NF-κB activation (IκB degradation), which was initiated at d 7, peaking at d 14. mTOR activation was maintained from d 3 up to 21 d. The extended maintenance of mTOR
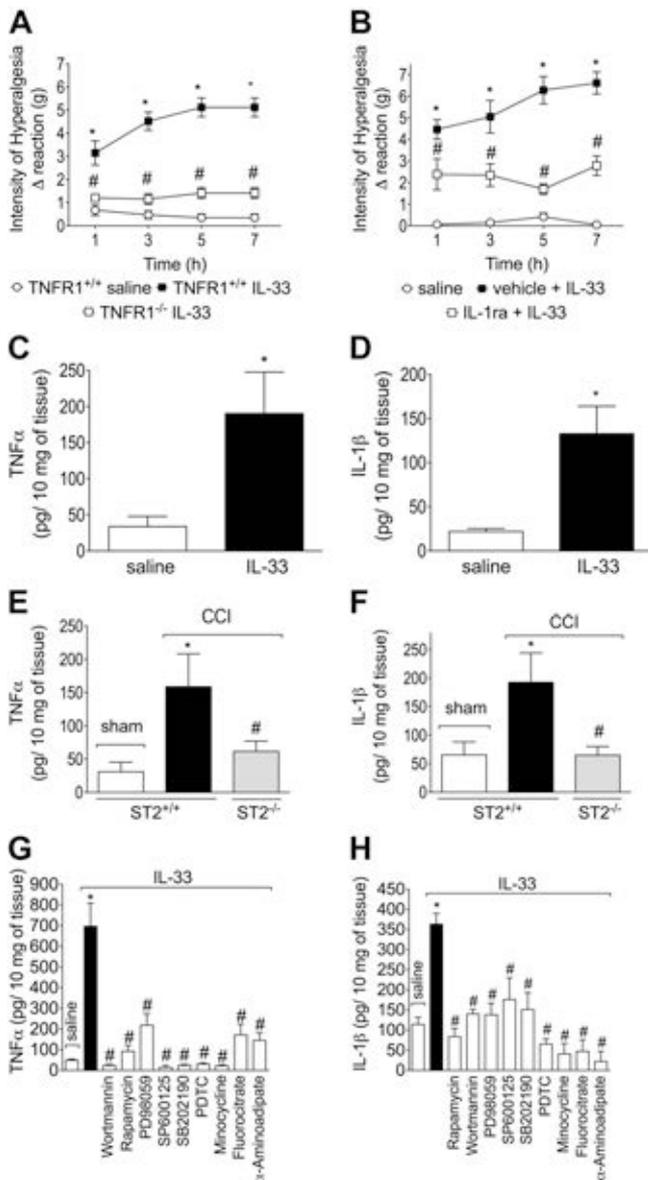
Figure 7. IL-33–induced mechanical hyperalgesia is TNF-α and IL-1β dependent, and TNF-α and IL-1β production depends on PI3K, mTOR, MAPK, NF-κB, and glial cell activation. IL-33 was injected (100 ng, i.t.) into naive TNFR1$^{-/-}$ (A) or IL-1ra–treated (30 mg/kg, i.p.) (B) mice, and the mechanical hyperalgesia was evaluated 1–7 h later. Spinal cord samples were collected 2 h after IL-33 injection for TNF-α (C) and IL-1β (D) measurement by ELISA. TNF-α (E) and IL-1β (F) concentrations were determined by ELISA in spinal cord samples of ST2$^{+/+}$ and ST2$^{-/-}$ mice 7 d after CCI surgery. Mice received intrathecal coinjection of IL-33 (100 ng) and one of the following compounds: wortmannin (10 μg per mouse); rapamycin (10 μg per mouse); PD98059 (10 μg per mouse); SP600125 (10 μg per mouse); SB202190 (10 μg per mouse); PDTC (300 μg per mouse); minocycline (50 μg per mouse); fluorocitrate (1.6 μg per mouse); or α-aminoadipate (100 nmol). The vehicle control result was obtained by pooling all vehicle controls because there was no difference among all vehicles used. Spinal cords were collected 2 h after IL-33 intrathecal injection for TNF-α (G) and IL-1β (H) measurement by ELISA (n = 6, representative of 2 separate experiments). One-way ANOVA followed by Tukey's t test was performed. *$P < 0.05$ compared with saline or sham-surgery group; #$P < 0.05$ compared with IL-33, IL-33 TNFR1$^{+/+}$, IL-33 vehicle of IL-1ra, or CCI ST2$^{+/+}$ group.

phosphorylation is consistent with the activation of lymphocytes by IL-33 (33), which are the key cell types involved in chronic neuropathic hyperalgesic conditions (34). Moreover, CCI induced IL-33 production in the spinal cord between 3 and 21 d, further corroborating IL-33 participation in the entire disease process.

In the spinal cord, ST2 and IL-1RAcP are expressed mainly on microglia and astrocytes. An earlier report shows that IL-33 induces the proliferation of microglia and enhances the production of proinflammatory cytokines TNF-α and IL-1β, and the anti-inflammatory cytokine IL-10 (27). Spinal cord neurons express ST2 in autoimmune encephalomyelitis, although the effect of activating ST2 expressed by neurons in pain has not been addressed (28). These observations are consistent with the present results demonstrating that the hyperalgesia induced by IL-33 depends on the activation of spinal cord microglia and astrocytes and that IL-33/ST2 is important for the activation of spinal cord microglia and astrocytes during CCI-induced neuropathy. However, the spinal cord cell types expressing ST2 in neuropathic pain remain to be further addressed.

The interrelationship of IL-33 and other cytokines could be an explanation for the induction of IL-33 production in CCI. TNF-α and IL-1β could induce IL-33 release by oligodendrocytes because these cells express TNFR1 and IL-1R1 (35). The interaction of IL-33 with TNF-α and IL-1β occurs in other diseases and disease models such as rheumatoid arthritis (15, 36, 37), vitiligo (38), psoriasis (39), allergic contact dermatitis (40), and bone cancer (19). These 3 cytokines act in synergy potentiating each other, inducing receptor expression and production, explaining the pronounced analgesia obtained by targeting any one of them. In agreement, TNF-α and IL-1β were individually sufficient and necessary for long-term potentiation induction *via* redundant pathways in rat lumbar spinal cord slices (41). Herein, IL-33–induced TNF-α and IL-1β production in the spinal cord was dependent on PI3K, mTOR, MAPKs, NF-κB, and glial cell activation. Importantly, CCI- and SNL-induced mechanical hyperalgesias were both reduced in ST2$^{-/-}$ mice compared with ST2$^{+/+}$ mice, indicating that the hyperalgesic role of IL-33/ST2 signaling is not restricted to one model of neuropathy.

The schematic **Fig. 8** summarizes the present findings and suggests further possible IL-33 mechanisms considering the literature. Spinal cord oligodendrocyte-derived IL-33 mediates CCI-induced neuropathic pain by acting on ST2 expressed by endothelial cells, microglia, astrocytes, and neurons (19, 27, 28, 43). Spinal cord microglial and astrocyte activation in CCI depends on IL-33/ST2. IL-33–induced ST2 activation triggers intracellular signaling pathways such as PI3K, mTOR, ERK, JNK, p38, and NF-κB, and microglia and astrocytes to induce TNF-α and IL-1β production. Both TNF-α and IL-1β contribute to enhancing synaptic transmission in the spinal cord and IL-33 production. For instance, TNF-α induces long-term potentiation in an NF-κB–dependent manner in nerve injury-induced neuropathic pain (44) and reduces the hyperpolarization-activated cation current inhibiting the spontaneous activity in GABAergic neurons in a p38-dependent manner, contributing to the development of neuropathic pain (45). TNF-α and IL-1β induce central sensitization and

Figure 8. Schematic representation of the IL-33/ST2 role in CCI-induced neuropathic pain. Regular arrows indicate possible cellular targets of IL-33 (cells expressing ST2 and IL-1RAcP) in the spinal cord (19, 27, 28, 42). Large arrow indicates that oligodendrocytes are the main source of IL-33 in the spinal cord in CCI. Dashed arrows indicate that microglia, astrocytes, and neurons play a minor role in IL-33 production. The activation of ST2/IL-1RAcP receptor complex by IL-33 in the spinal cord triggers intracellular molecular signaling consisting of PI3K/PKB, mTOR, MAPKs (ERK, JNK, and p38), and NF-κB, resulting in the production of TNF-α and IL-1β, which exacerbate the CCI-induced neuropathic pain together with IL-33.
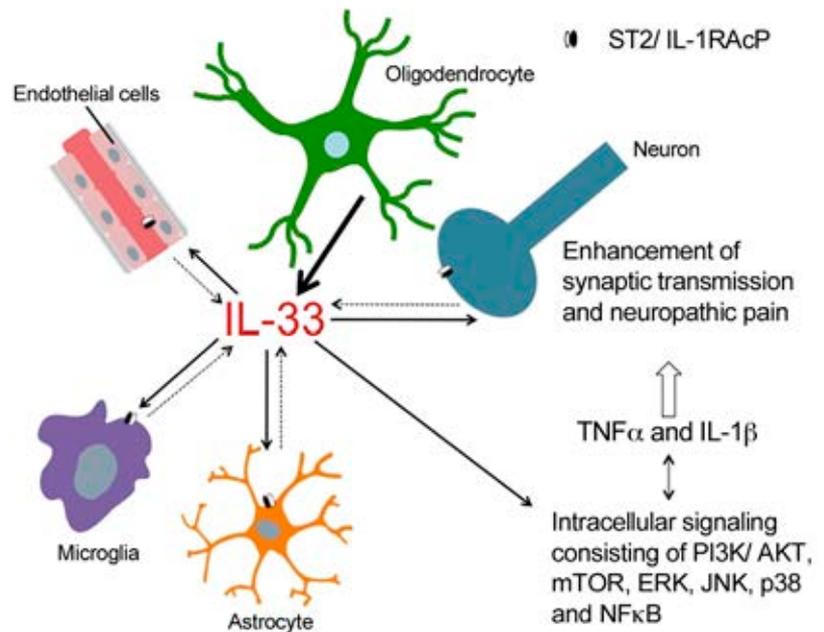
hyperalgesia by increasing excitatory synaptic transmission, decreasing inhibitory synaptic transmission, and inducing long-term synaptic plasticity through cAMP response element-binding protein-mediated gene transcription in superficial dorsal horn neurons (46). There is also evidence that synaptic transmission enhancement by TNF-α and IL-1β is not through neuronal cytokine receptors but, rather, indirectly by activation of glial cell cytokine receptors (41).

In conclusion, the prominent role of IL-33/ST2 signaling in neuropathic pain and other hyperalgesic conditions suggests it as a potential analgesic target, warranting further investigation. FJ

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