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RESEARCH PAPER

IL-33/ST2 signalling contributes to carrageenin-induced innate inflammation and inflammatory pain: role of cytokines, endothelin-1 and prostaglandin E_2

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BACKGROUND AND PURPOSE

IL-33 signals through ST2 receptors and induces adaptive and innate inflammation. IL-33/ST2 is involved in adaptive inflammation-induced pain. Here, we have investigated the contribution of IL-33/ST2-triggered mechanisms to carrageenin-induced innate inflammation.

EXPERIMENTAL APPROACH

Carrageenin- and IL–33-induced inflammatory responses were assessed in BALB/c- (WT) and ST2-deficient ($^{-/-}$) mice as follows: oedema (plethysmometer), myeloperoxidase activity (colorimetric assay), mechanical hyperalgesia (electronic version of von Frey filaments), cytokine levels (ELISA), PGE_2 (RIA), mRNA expression (quantitative PCR), drug treatments targeting leukocyte recruitment (fucoidin), TNF-α (infliximab), CXCL1 (antibody to CXCL1), IL-1 (IL-1ra), endothelin ET_A (clazosentan) and ET_B (BQ788) receptors and COX (indomethacin).

KEY RESULTS

Carrageenin injection increased ST2 and IL-33 mRNA expression and IL-33 production in paw skin samples. Carrageenin-induced paw oedema, hyperalgesia and myeloperoxidase activity were reduced in $ST2^{-/-}$ compared with WT mice, effects mimicked by IL-33 injection in the paw. Furthermore, IL–33-induced hyperalgesia was reduced by fucoidin suggesting a role for recruited leukocytes in its hyperalgesic effect. IL–33-induced hyperalgesia in naïve mice was reduced by treatments targeting TNF, CXCL1, IL-1, endothelin receptors and COX while carrageenin-induced ST2-dependent TNF-α, CXCL1, IL-1β, IL-10 and PGE_2 production and preproET-1 mRNA expression. Combining IL-33 and carrageenin at doses that were ineffective as single treatment induced significant hyperalgesia, oedema, myeloperoxidase activity and cytokine production in a ST2-dependent manner.

CONCLUSIONS AND IMPLICATIONS

IL-33/ST2 signalling triggers the production of inflammatory mediators contributing to carrageenin-induced inflammation. These data reinforces the importance of IL-33/ST2 signalling as a target in innate inflammation and inflammatory pain.

Abbreviations

ET-1, endothelin-1; i.pl, intraplantar; IL-1ra, IL-1 receptor antagonist

Introduction

IL-33 is a member of the IL-1 family of cytokines, which signals through the ST2/IL-1RAcP receptor complex (Schmitz *et al.*, 2005; Ali *et al.*, 2007). IL-33 seems to be an important therapeutic target or therapy in inflammatory conditions such as anaphylactic shock, septic shock, atherosclerosis, UV radiation, asthma, hepatitis and rheumatoid arthritis (Schmitz *et al.*, 2005; Verri *et al.*, 2008; 2010; Pushparaj *et al.*, 2009; Alves-Filho *et al.*, 2010; Arshad *et al.*, 2011; Byrne *et al.*, 2011). These roles of IL-33 are related to the activation of endothelial cells, mast cells, lymphocytes, macrophages, eosinophils and neutrophils (Schmitz *et al.*, 2005; Pecaric-Petkovic *et al.*, 2009; Alves-Filho *et al.*, 2010; Verri *et al.*, 2010).

IL-33 was initially recognized as a Th2 cytokine (Schmitz *et al.*, 2005). Further investigation of the biology of IL-33/ST2 signalling showed that it is an important component in Th1/Th17 and innate inflammation. Thus, IL-33 mediates antigen-induced arthritis dependent on TNF-α, IL-1β and IFN-γ (Verri *et al.*, 2008; 2010) and IL-17 production (Xu *et al.*, 2008). IL-33 also amplified Th1 and Th2 responses by acting on basophils, Th2 lymphocytes, iNKT and NK cells (Smithgall *et al.*, 2008). In support of a role of IL-33 in innate inflammation, glia secreted IL-33 in response to LPS (Hudson *et al.*, 2008) and IL-33 administration reduced the systemic inflammatory response induced by bacterial products in a model of sepsis (Alves-Filho *et al.*, 2010). IL-33 also increased expression of LPS receptor components resulting in enhanced cytokine production, suggesting a LPS self-regulatory feedback through IL-33 (Espinassous *et al.*, 2009). Furthermore, IL-33 deficiency resulted in reduced papain-induced lung innate inflammation and dextran-induced colitis accompanied by T–cell-independent epithelial damage (Oboki *et al.*, 2010). Moreover, IL-33 induced cytokine production by type 2 innate immune cells such as natural helper cells, nuocytes, and innate helper 2 cells (Kouzaki *et al.*, 2011; Kim *et al.*, 2012). However, ST2 deficiency increased innate and acquired Th1 and Th2 immunity in response to a murine mammary carcinoma (Jovanovic *et al.*, 2011). Therefore, the functions of IL-33 are not completely predictable.

Carrageenin is an extensively used and accepted model of inflammation. In general, it is injected in the paw to evaluate oedema and hyperalgesia, but it can also be injected in other cavities such as air pouch, peritoneal and pleural cavities to evaluate leukocyte recruitment, mainly of neutrophils, and production of chemotatic mediators (Cunha *et al.*, 2005; Valério *et al.*, 2007). The intraplantar injection of carrageenin induces mechanical hyperalgesia in mice by triggering a cytokine cascade initiated by TNF-α and CXCL1 production, which induce IL–1β-dependent PGE_2 production. In turn, PGE_2 sensitizes the nociceptor, which can be detected as mechanical hyperalgesia (Cunha *et al.*, 2005). Furthermore, endothelin-1 (ET-1) acting on ET_A and ET_B receptors mediates carrageenin-induced mechanical hyperalgesia (Baamonde *et al.*, 2004; receptor nomenclature follows Alexander *et al.*, 2011) and ET-1-induced hyperalgesia depends on PGE_2 production (Verri *et al.*, 2007). Recruited neutrophils also contribute to carrageenin-induced mechanical hyperalgesia by producing PGE_2 (Cunha *et al.*, 2008).

As IL-33 mediates mechanical hyperalgesia (Verri *et al.*, 2008) and neutrophil recruitment (Verri *et al.*, 2010) in adaptive inflammation, and is involved in innate inflammation (Hudson *et al.*, 2008; Espinassous *et al.*, 2009; Alves-Filho *et al.*, 2010; Oboki *et al.*, 2010; Zhang *et al.*, 2011), we have investigated whether IL-33/ST2 contributed to carrageenin-induced innate inflammation and inflammatory pain and the underlying mechanisms. Furthermore, because IL-33 potentiated antigen-induced production of inflammatory cytokines (Andrade *et al.*, 2011), we also assessed whether IL-33 could act in synergy with carrageenin to induce paw inflammation and hyperalgesia.

Methods

Animals

Sex matched BALB/c (WT, $ST2^{+/+}$) and BALB/c background ST2-deficient ($^{-/-}$) mice (Brint *et al.*, 2004), 20–25 g were bred in the Faculty of Medicine of Ribeirao Preto, University of Sao Paulo, Brazil. All animal care and experimental procedures complied with the International Association for the Study of Pain guidelines and approved by the Ethics Committee of the Faculty of Medicine of Ribeirao Preto, University of Sao Paulo, Brazil. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). A total of 908 animals were used in these experiments.

Electronic pressure meter test

The mechanical hyperalgesia test (Cunha *et al.*, 2004) consisted of evoking a hindpaw flexion reflex with a handheld force transducer (electronic anaesthesiometer; IITC Life Science, Woodland Hills, CA, USA) adapted with a 0.5 mm^2 polypropylene tip. The results are expressed as the differential (Δ) withdrawal threshold (in g) calculated by subtracting the mean measurements at indicated time points after stimulus from the zero-time mean measurements. Withdrawal threshold was 8.6 ± 0.5 g (mean ± SEM, $n = 40$) before injection of the hyperalgesic agents.

Paw oedema test

The volume of the paw was measured with a plethysmometer (Ugo Basil, Comerio, VA, Italy) before (V0) and at indicated time points after (VT) the intraplantar (i.pl.) stimulus with carrageenan (3 or 100 μg diluted in 25 μL of saline per paw) (Valério *et al.*, 2007). The amount of paw swelling was determined for each mouse and the difference between VT and V0 was taken as the oedema value (oedema mm^3 per paw).

Myeloperoxidase activity

The myeloperoxidase activity of paw homogenates was used to evaluate the migration of leukocytes to the hind paw skin of mice. It consists of a kinetic colorimetric assay (Bradley *et al.*, 1982) with modifications (Casagrande *et al.*, 2006). The results were presented as the myeloperoxidase activity (number of neutrophils 10^4 per paw).

Cytokine measurement

Animals were terminally anaesthetized (1.5% isoflurane; Abbott, (Abbott Park, IL, USA) and the plantar skin tissues

removed and homogenized in 500 µL of buffer containing protease inhibitors. Concentrations of TNF-α, IL-1β, CXCL1, IL-10 (BD Bioscience, San Jose, CA, USA) and IL-33 (eBioscience, San Diego, CA, USA) were determined by ELISA using paired antibodies.

Real-time PCR

Mice were killed 2 h after i.pl. injection of carrageenin and the plantar cutaneous hind paw tissues harvested. Samples were homogenized in Trizol reagent, and total RNA was extracted using the SV Total RNA Isolation System (Promega Biosciences, Fithburg, WI, USA). Quantitative PCR (qPCR) was performed in an ABI Prism 7500 Sequence Detection System using the SYBR-green fluorescence (Applied Biosystems, Grand Island, NY, USA). The primers were previously described (Verri *et al.*, 2008).

Determination of PGE_2 production

Two hours after i.pl. injection of carrageenin, paw skin tissue samples were collected in 0.5 mL of a mixture of acetone: 1 M HCl: water (10:1:5, v : v : v). After homogenizing with a turrax, the samples were centrifuged (20 min at 2000 *g* at 4°C) and the supernatant decanted before drying the pellet in a centrifugal evaporator at 37°C. The pellet was reconstituted in 500 µL of Tris/HCl buffer (10mM Tris, pH adjusted with HCl to 8.0). The concentrations of PGE_2 were determined by RIA (Amersham, Pittsburgh, PA, USA) (Verri *et al.*, 2007).

Experimental protocols

WT mice received intraplantar (i.pl., subcutaneous in the hind paw) injection of carrageenin (100 µg per paw) or saline. Samples of the paw skin were collected at 2 h for qPCR analysis for ST2, IL-33 and preproET-1 mRNA expression. IL-33 levels were determined 0.5, 1, 3 and 5 h after carrageenin by ELISA. Mechanical hyperalgesia, oedema and myeloperoxidase activity were evaluated at indicated time points after carrageenin (3 or 100 µg·per paw) or IL-33 (3, 30, 100 and 300 ng·per paw) injection in WT and $ST2^{-/-}$ mice. In other experiments, WT mice were treated with fucoidin (20 $mg \cdot kg^{-1}$, i.v, 15 min), infliximab (anti-TNF-α antibody, 10 $mg \cdot kg^{-1}$, i.p, 48 h and 60 min before stimuli), anti-CXCL1 antibody (αCXCL1; 700 $ng \cdot mice^{-1}$, i.p. injection, in saline, 30 min before stimulus), control IgG (same treatment protocol as for infliximab or αCXCL1) or IL-1 receptor antagonist (IL-1ra; 30 $mg \cdot kg^{-1}$, i.v., 15 min) before IL-33 administration (100 or 300 ng·per paw) followed by evaluation of mechanical hyperalgesia and myeloperoxidase at indicated time points. TNF-α, CXCL1, IL-1β, IL-10 and PGE_2 levels in paw skin samples were determined by ELISA or RIA at 2 h or indicated time points after carrageenin injection. In the last series of experiments, mice were treated with clazozentan (10 $mg \cdot kg^{-1}$, s.c., 15 min), BQ 788 (30 nmol per paw, 30 min) or indomethacin (5 $mg \cdot kg^{-1}$, i.p., 40 min) before i.pl. injection of IL-33 (100 ng· per paw) or saline. Mechanical hyperalgesia was evaluated 3 and 5 h after. To determine the synergism between IL-33 and carrageenin, mice received single treatments with carrageenin (3 µg per paw) or IL-33 (3 ng·per paw) or a combination of both followed by mechanical hyperalgesia, oedema and MPO activity determination at indicated time points. The same protocol was used to determine the production of TNF-α and IL-1β in WT ($ST2^{+/+}$) and $ST2^{-/-}$ mice. The doses of reagents used *in vivo* were chosen based on previous reports (Cunha *et al.*, 2005; 2008; Verri *et al.*, 2006; 2008; 2009; 2010; Pinto *et al.*, 2010). Drug treatments or genetic deficiency did not affect the baseline response of the mice to mechanical stimulation (data not shown).

Data analyses

Results are presented as means ± SEM; each experiment was performed at least twice. Two-way ANOVA was used to compare the groups and doses at all times when the parameters were measured at different times after the stimulus injection. The analysed factors were treatments, time and time versus treatment interaction. One-way ANOVA followed by Bonferroni's *t*-test was performed for each time. Comparison of two groups was performed using *t*-test. $P < 0.05$ was considered significant.

Materials

The following materials were obtained from the sources indicated: anti-CXCL1 antibody (Peprotech, Rocky Hill, NJ, USA), isotype control IgG (R&D Systems, Minneapolis, MN, USA), BQ 788 (Tocris Bioscience, Ellisville, MO, USA), carrageenin (FMC Corporation (Philadelphia, PA, USA), clazosentan (Actelion Pharmaceutical Ltd., Allschwil/Basel, Switzerland), fucoidin (Sigma Chemical, St Louis, MO, USA), IL-1ra (National Institute of Biological Standards and Control, Hertfordshire, UK), indomethacin (Prodome, Campinas, Brazil), infliximab (Remicade, Merck & Co., Whitehouse Station, NJ, USA). Recombinant human IL-33 was generated as previously described (Komai-Koma *et al.*, 2007).

Results

Carrageenin induces IL-33 and ST2 mRNA expression and IL-33 production in the paw skin

Carrageenin (100 µg·per paw) or saline (25 µL) was injected in WT mice and after 2 h paw skin samples were collected for qPCR analysis. Carrageenin induced a significant increase of ST2 (Figure 1A) and IL-33 (Figure 1B) mRNA expression. In agreement, carrageenin induced a significant increase of IL-33 production after 0.5–5 h (Figure 1C).

Reduction of carrageenin-induced mechanical hyperalgesia, oedema and neutrophil recruitment in $ST2^{-/-}$ mice

Carrageenin (100 µg per paw) or saline was injected in WT and $ST2^{-/-}$ mice, and mechanical hyperalgesia (Figure 2A) and oedema (Figure 2B) were evaluated 1–5 h after carrageenin. At 5 h, mice were killed and myeloperoxidase activity was determined in paw skin samples (Figure 2C). Carrageenin-induced mechanical hyperalgesia between 1 and 5 h, and oedema between 0.5 and 5 h in WT mice compared with vehicle group (Figure 2A and B, respectively). On the other hand, $ST2^{-/-}$ mice presented reduced hyperalgesia and oedema at the same time points (Figure 2A and B, respectively). The myeloperoxidase activity was increased by carrageenin injection in WT mice compared with the saline group and this increase was reduced in $ST2^{-/-}$ mice (Figure 2C).

IL-33 injection mimics carrageenin-induced inflammatory responses in a ST2-dependent manner in naïve mice

Mice received IL-33 (30–300 ng·per paw) and mechanical hyperalgesia (Figure 3A) and oedema (Figure 3B) were evaluated after 0.5–5 h. At 5 h samples of paw skin tissue were collected for myeloperoxidase activity determination (Figure 3C). The dose of 30 ng·per paw of IL–33-induced mechanical hyperalgesia between 1 and 5 h compared with vehicle group (Figure 3A), and the doses of 100 and 300 ng·per paw of IL-33 induced significant hyperalgesia compared with vehicle and the dose of 30 ng·per paw between 0.5 and 5 h (Figure 3A). IL-33 induced significant paw oedema between 0.5 and 3 h, only at 100 ng·per paw (Figure 3B). At 5 h, IL-33 induced a dose-dependent increase of myeloperoxidase activity, compared with levels after vehicle only (Figure 3C). In ST2$^{-/-}$ mice, IL-33 did not induce mechanical hyperalgesia, oedema or myeloperoxidase activity (Figure 3D–F). Thus, the injection of IL-33 mimicked carrageenin-induced inflammatory responses in WT mice, although the oedematogenic effect of IL-33, given i.pl., was clearly less than that of carrageenin. This effect was not further investigated, except for the synergism experiments presented in Figure 10.

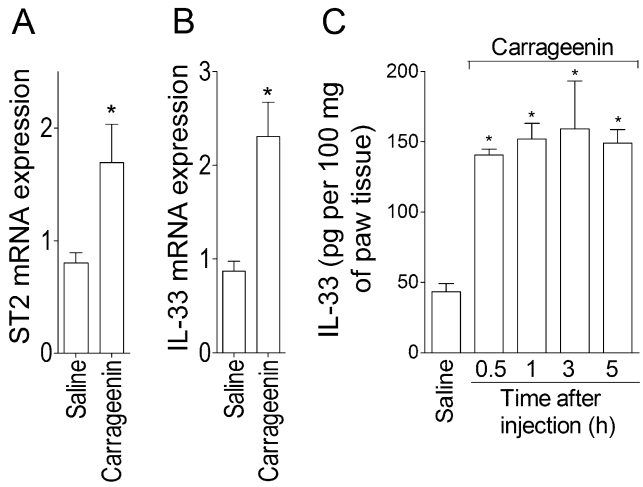

Figure 1

Carrageenin induces expression of mRNA for IL-33 and ST2 and IL-33 production in the paw skin. Mice received intraplantar (i.pl.) injection of carrageenin (100 µg per paw) or saline (25 µL), and after 2 h samples of paw skin were collected for quantitative (q) PCR analysis of ST2 (panel A) and IL-33 (panel B) mRNA expression or between 0.5 and 5 h for IL-33 determination by ELISA assay (panel C). $n = 6$ for qPCR and $n = 4$ for ELISA, representative of two separate experiments. *$P < 0.05$ compared with the saline group.

Effect of fucoidin in IL–33-induced mechanical hyperalgesia in naïve mice

WT mice were treated with fucoidin (which binds to L-selectin and inhibits leukocyte recruitment; 20 mg·kg^{-1}, i.v., 15 min) before IL-33 injection (100 or 300 ng·per paw), and mechanical hyperalgesia was evaluated at 3 and 5 h and myeloperoxidase activity at 5 h (Figure 4). Two doses of IL-33 were used because 100 and 300 ng achieved maximal mechanical hyperalgesia whereas 300 ng·was needed to produce maximal myeloperoxidase activity in the paw skin. Treatment with fucoidin inhibited the mechanical hyperalgesia at 3 and 5 h (Figure 4A and C) and myeloperoxidase activity at 5 h (Figure 4B and D) induced by 100 ng·(Figure 4A and B) and 300 ng·(Figure 4C and D) of IL-33.

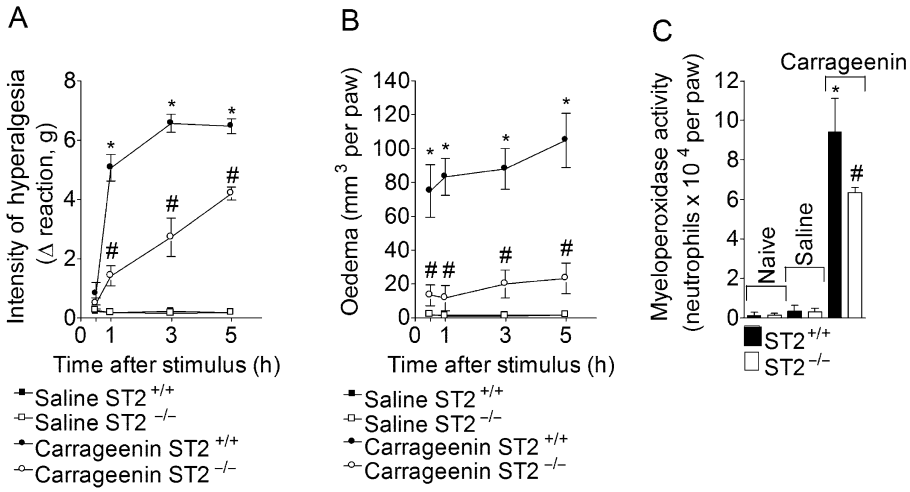

Figure 2

IL-33/ST2 mediates carrageenin-induced paw inflammation. WT (ST2$^{+/+}$) and ST2$^{-/-}$ mice received i.pl. injection of carrageenin (100 µg·per paw) or saline. Mechanical hyperalgesia (panel A) and oedema (panel B) were determined at indicated time points followed by myeloperoxidase assay at 5 h (panel C) in paw skin samples. $n = 5$, representative of two separate experiments. *$P < 0.05$ compared with the saline group and $^{\#}P < 0.05$ compared with the carrageenin group.

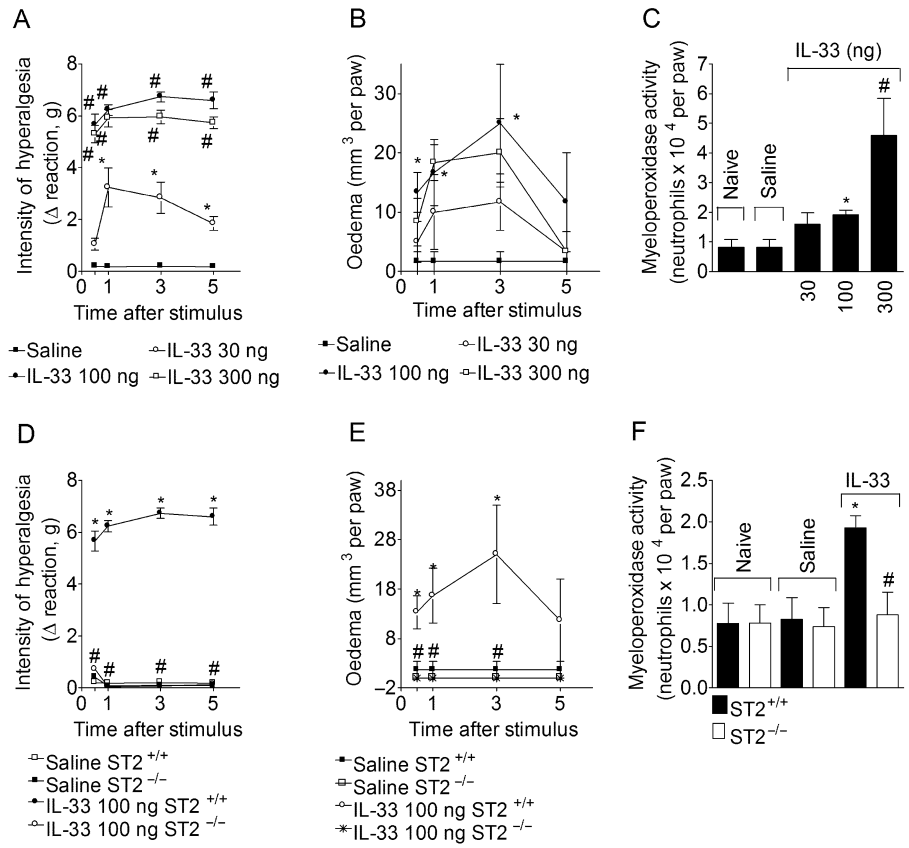

Figure 3

IL-33 induces hyperalgesia, oedema and neutrophil migration in a ST2-dependent manner in normal mice. IL-33 (30–300 ng) or saline (25 μL) was injected i.pl. in WT ($ST2^{+/+}$) mice. Mechanical hyperalgesia (panel A) and oedema (panel B) were determined at indicated time points followed by myeloperoxidase activity determination at 5 h (panel C) in paw skin samples. IL-33 (100 ng) or saline was injected i.pl. in WT and $ST2^{-/-}$ mice. Mechanical hyperalgesia (panel D) and oedema (panel E) were determined at indicated time points followed by myeloperoxidase activity determination at 5 h (panel F). $n = 6$, representative of two separate experiments. $^{*}P < 0.05$ compared with the saline group and $^{\#}P < 0.05$ compared with the lower dose of IL-33 or IL-33 injection in $ST2^{+/+}$ mice.

IL–33-induced mechanical hyperalgesia depends on TNF-α, CXCL1 and IL-1β in naïve mice

Mice were treated with infliximab (anti–TNF-α antibody, 10 mg·kg^{-1}, i.p. 48 h and 60 min before stimuli injection, Figure 5A and D), anti-CXCL1 antibody (αCXCL1, 700 ng·per paw, co-injection, Figure 5B and E) or IL-1ra (30 mg·kg^{-1}, i.v., 15 min, Figure 5A and D) before IL-33 injection (100 ng·per paw) and mechanical hyperalgesia was evaluated at 3 h (Figure 5A–C) and 5 h (Figure 5D–F). All treatments inhibited IL–33-induced mechanical hyperalgesia (Figure 5). Control IgG antibody (αcontrol) was used under the same treatment protocols as for infliximab (Figure 5A and D) or αCXCL1 (Figure 5B and E).

Carrageenin-induced production of TNF-α, CXCL1, IL-1β and IL-10 is decreased in ST2 deficient mice

Carrageenin (100 μg·per paw) or saline was injected in WT and $ST2^{-/-}$ mice and paw skin samples were collected after 2 h for assay of TNF-α, CXCL1 and IL-1. Carrageenin induced significant production of TNF-α (Figure 6A), CXCL1 (Figure 6B) and IL-1β (Figure 6C) in WT mice compared with the vehicle treatment, and these effects were inhibited in $ST2^{-/-}$ mice. Carrageenin also induced significant production of IL-10 at 0.5, 1, 3 and 5 h in WT mice and levels of this cytokine were also reduced in $ST2^{-/-}$ mice at 3 and 5 h after carrageenin injection (Figure 7).

ET-1 mediates IL–33-induced mechanical hyperalgesia and mRNA expression in naïve mice

Mice were treated with clazosentan (ET_A receptor antagonist, 10 mg·kg^{-1}, s.c., 30 min) or BQ788 (ET_B receptor antagonist, 30 nmol·per paw, 30 min) before IL-33 (100 ng per paw) injection (Figure 8A and B). Both clazosentan and BQ788 inhibited IL–33-induced mechanical hyperalgesia at 3 h (Figure 8A and C, respectively) and 5 h (Figure 8B and D). In agreement, carrageenin alone increased expression of mRNA for preproET-1 at 2 h after carrageenin injection in WT mice compared with the vehicle group, and this increase was lacking in $ST2^{-/-}$ mice (Figure 8E).

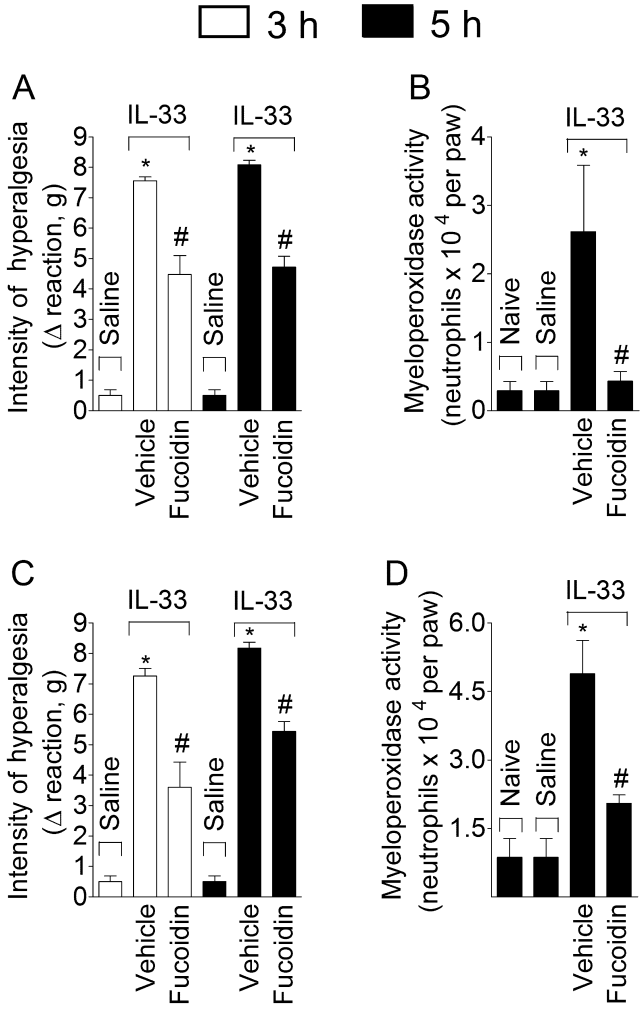

Figure 4

IL–33-induced hyperalgesia depends on neutrophil recruitment. IL-33 (100 ng – panels A and B or 300 ng – panels C and D) or saline was injected i.pl. and mechanical hyperalgesia (panel A and C) was determined at indicated time points followed by myeloperoxidase activity determination at 5 h (panel B and D) in paw skin samples. $n = 6$, representative of two separate experiments. $^{*}P < 0.05$ compared with the saline or naive group and $^{\#}P < 0.05$ compared with the IL-33 vehicle group.

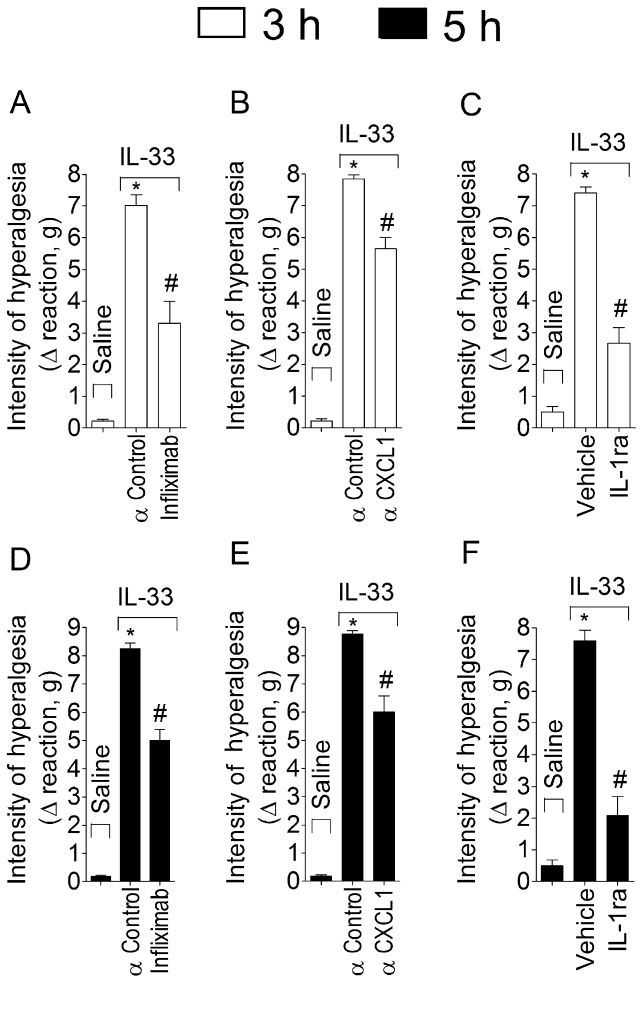

Figure 5

IL–33-induced mechanical hyperalgesia depends on TNF-α, CXCL1 and IL-1β. Mice were treated with infliximab (anti-TNF-α antibody, 10 mg·kg^{-1}, i.p. 48 h and 60 min before stimuli injection; panels A and D), anti-CXCL1 antibody (αCXCL1, 700 ng·per paw, co-injection; panels B and E), isotype IgG control antibody (αcontrol, same treatment protocol as for infliximab or αCXCL1) or IL-1 receptor antagonist (IL-1ra, 30 mg·kg^{-1}, i.v., 15 min) (panel C and F) before IL-33 i.pl. injection (100 ng·per paw). Mechanical hyperalgesia was measured 3h (panels A, B and C) and 5 h (panels D, E and F) after IL-33 injection. $n = 6$, representative of two separate experiments. $^{*}P < 0.05$ compared with saline group and $^{\#}P < 0.05$ compared with IL-33 group.

PGE_2 mediates IL–33-induced mechanical hyperalgesia in WT mice

Mice were treated with indomethacin (COX inhibitor, 5 mg·kg^{-1}, i.p.) 40 min before IL-33 injection (100 ng·per paw). As a result, IL–33-induced mechanical hyperalgesia was reduced at 3 h (Figure 9A) and 5 h (Figure 9B). Carrageenin also induced PGE_2 production in WT mice and this effect was absent in ST2$^{-/-}$ mice (Figure 9C).

IL-33 synergizes with carrageenin to induce paw inflammation and hyperalgesia

Mice received i.pl. injections of carrageenin (3 µg·per paw) alone, IL-33 (3 ng per paw) alone or a combination of IL-33 and carrageenan, in the same low doses. IL-33 and carrageenin given alone now did not induce significant mechanical hyperalgesia (Figure 10A) or oedema (Figure 10B) at 1, 3 and 5 h, or myeloperoxidase activity at 5 h. On the other hand, injection of IL-33 plus carrageenin induced significant mechanical hyperalgesia (Figure 10A), oedema (Figure 10B) and myeloperoxidase activity (Figure 10C). Furthermore, the combination of IL-33 and carrageenin also induced synergy in the production of TNF-α (Figure 10D) and IL-1β (Figure 10E), which was ST2-dependent (Figure 10D and E).

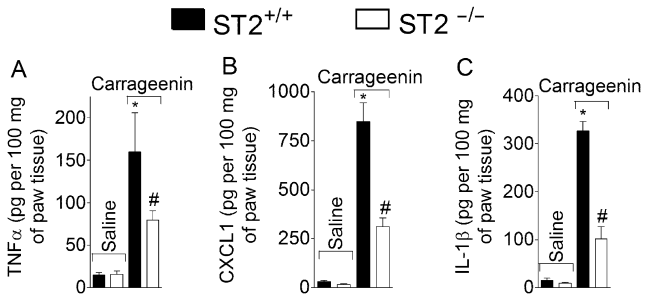

Figure 6

Carrageenin induced ST2-dependent production of TNF-α, CXCL1 and IL-1β in WT ($ST^{+/+}$) mice. Carrageenin (100 μg) was injected i.pl. and after 2 h samples of cutaneous plantar tissue were collected for determination of TNF-α (panel A), CXCL1 (panel B), and IL-1β (panel C) levels by ELISA. $n = 4$, representative of two separate experiments. $^{*}P < 0.05$ compared with saline group and $^{\#}P < 0.05$ compared with carrageenin $ST2^{+/+}$ group.

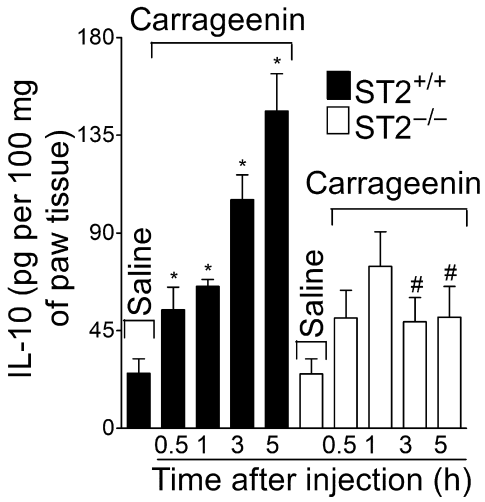

Figure 7

Carrageenin induced ST2-dependent production of IL-10 in mice. Carrageenin (100 μg) was injected i.pl. and after 0.5–5 h samples of cutaneous plantar tissue were collected for determination of IL-10 levels determination by ELISA. $n = 4$, representative of two separate experiments. $^{*}P < 0.05$ compared with saline group and $^{\#}P < 0.05$ compared with carrageenin $ST2^{+/+}$ group.

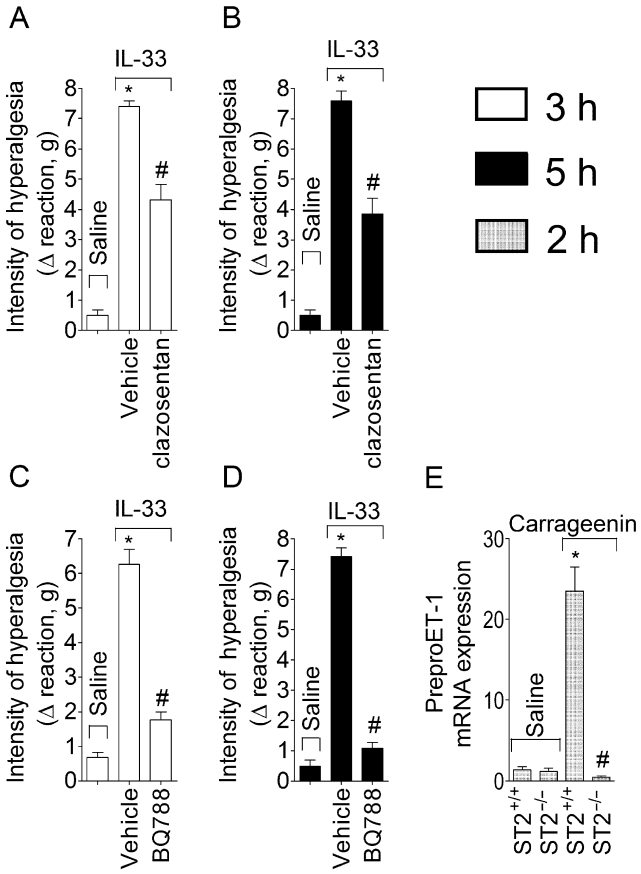

Figure 8

IL-33/ST2 mediates carrageenin-induced hyperalgesia via ET-1 acting on ET_A and ET_B receptors. Mice were treated with clazosentan (ET_A receptor antagonist, 10 $mg \cdot kg^{-1}$, 30 min, panels A and B) or BQ 788 (ET_B receptor antagonist, 30 nmol·per paw, 30 min, panels C and D) before IL-33 (100 ng·per paw) injection. Mechanical hyperalgesia was measured 3 and 5 h after IL-33 injection. Carrageenin (100 μg) or saline was injected i.pl. in $ST2^{+/+}$ and $ST2^{-/-}$ mice and samples of cutaneous tissue were collected after 2 h for qPCR analysis of preproET-1 mRNA expression (panel E). $n = 6$, representative of two separate experiments. $^{*}P < 0.05$ compared with saline group and $^{\#}P < 0.05$ compared with IL-33 or carrageenin $ST2^{+/+}$ group.

Discussion and conclusions

Despite the initial description of IL-33 as a Th2 cytokine, it has become evident that it is, rather, a pleiotropic cytokine mediating Th1, Th2, Th17 and innate inflammatory responses (Schmitz *et al.*, 2005; Komai-Koma *et al.*, 2007; Smithgall *et al.*, 2008; Verri *et al.*, 2008; Xu *et al.*, 2008; Alves-Filho *et al.*, 2010; Oboki *et al.*, 2010). We have previously demonstrated the hyperalgesic role of IL-33/ST2 in a model of antigen-induced arthritis in mice (Verri *et al.*, 2008). Therefore, we hypothesised that IL-33/ST2 signalling would also be important in innate inflammation-induced hyperalgesia. The carrageenin model of innate inflammation was chosen due to its wide applicability in the study of novel analgesic and anti-inflammatory drugs. The present data demonstrate that IL-33/ST2 contributes to carrageenin-induced inflammation consisting of oedema, myeloperoxidase activity and mechanical hyperalgesia by triggering the production of other cytokines (TNF-α, CXCL1 and IL-1β), along with ET-1 and PGE_2, and also reducing the production of the anti-hyperalgesic cytokine IL-10. Furthermore, we demonstrated that injection of both IL-33 and carrageenin at doses that did not cause responses alone, induced mechanical hyperalgesia, oedema, increase of myeloperoxidase activity and cytokine production, suggesting a synergism in inducing inflammation between carrageenin-and IL-33.

IL-33 has a role in the activation of mast cells, glia cells, macrophages, epithelial and endothelial cells (Schmitz *et al.*, 2005; Hudson *et al.*, 2008; Moussion *et al.*, 2008; Bourgeois

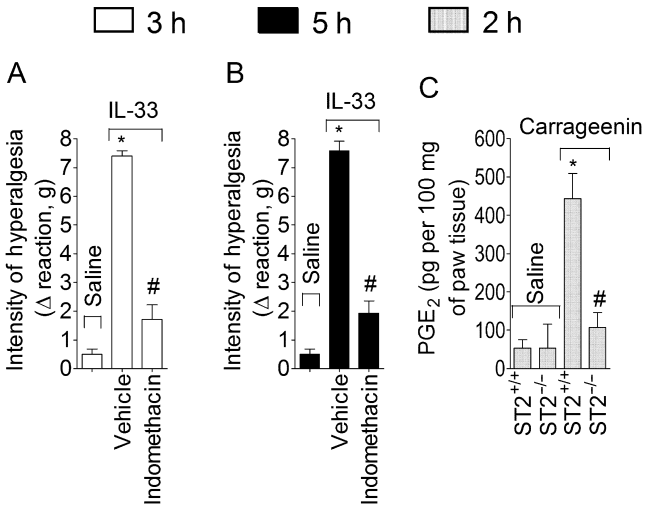

Figure 9

IL-33/ST2 mediates carrageenin-induced hyperalgesia via PGE_2. Mice were treated with indomethacin (COX inhibitor, 5 mg·kg^{-1}), 40 min, panels A and B) before IL-33 (100 ng·per paw) injection. Mechanical hyperalgesia was measured 3h (panel A) and 5 h (panel B) after IL-33 injection. Carrageenin (100 μg) or saline was injected i.pl. in $ST2^{+/+}$ and $ST2^{-/-}$ mice and samples of cutaneous tissue were collected after 2 h for PGE_2 levels determination by RIA (panel C). $n = 6$, representative of two separated experiments. $^{*}P < 0.05$ compared with the saline group and $^{\#}P < 0.05$ compared with the IL-33 or carrageenin $ST2^{+/+}$ group.

et al., 2009; Kurowska-Stolarska *et al.*, 2009; Nelson *et al.*, 2011; Zhang *et al.*, 2011), resulting in innate inflammation. The present results add to the earlier data demonstrating that IL-33/ST2 contributes to carrageenin-induced innate inflammation, which is itself dependent on the activation of resident tissue cells and recruitment of leukocytes.

It is interesting to note that there is rapid release of IL-33 upon stimulation of endothelial and epithelial cells (Moussion *et al.*, 2008). This finding, together with its actions as a cytokine and an intracellular nuclear factor show similarities with the alarmin HMGB-1 and raise the possibility that IL-33 could be a novel alarmin (Carriere *et al.*, 2007; Moussion *et al.*, 2008; Byrne *et al.*, 2011). Compatible with a fast and early release of IL-33 in innate inflammation, this cytokine was produced in significant amounts upon carrageenin stimulus within 30 min, and production of IL-33 continued at significant levels, at least, up to 5 h. There was also induction of IL-33 and ST2 mRNA expression, which indicated a continuous stimulation of IL-33 expression and not only its release and/or activation. Similar levels of IL-33 and/or ST2 mRNA (Savinko *et al.*, 2012; Schmieder *et al.*, 2012) and IL-33 production (Alves-Filho *et al.*, 2010) have been found in a range of conditions. This early production of IL-33 induced by carrageenin could account for the early participation of IL-33/ST2 in carrageenin-induced oedema, at 30 min. In agreement with IL-33 participation in oedema, this cytokine increased endothelial permeability in an *in vitro* model by increasing NO levels (Choi *et al.*, 2009). Injection of IL-33 alone in WT mice mimicked the paw inflammatory effects of carrageenin. Although the oedema induced by IL-33 was statistically significant, it did not attain the magnitude of oedema after carrageenin. Carrageenin-induced oedema is known to involve several other mediators, which potentiate the activity of each other (Ferreira and Vargaftig, 1974; Williams *et al.*, 1983; Katz *et al.*, 1984). Thus, it is to be expected that IL-33 alone would not fully mimic the carrageenin-induced oedema. Furthermore, the doses of IL-33 used were chosen from a dose–response curve and were similar to previous data on induction of pain (Verri *et al.*, 2008) and to the doses of other single cytokines necessary to induce inflammation (Joosten *et al.*, 2006). For instance, IL-32γ, TNF-α and IL-1β induce significant joint oedema at 100 ng·per joint, as determined by ^{99m}Tc-uptake (Joosten *et al.*, 2006).

The participation of IL-33/ST2 in carrageenin-induced hyperalgesia was evident starting at 1 h, and IL-33 injection achieved similar levels of mechanical hyperalgesia compared with carrageenin. The inhibition of leukocyte recruitment by fucoidin suggests the participation of recruited leukocytes in the IL–33-induced mechanical hyperalgesia. Nevertheless, the role of recruited leukocytes in the IL–33-induced hyperalgesic response was limited because abolishing the recruitment of leukocytes only partially reduced the mechanical hyperalgesia and correlations between IL–33-induced hyperalgesia and myeloperoxidase activity were not evident at any of the doses tested. The $ST2^{-/-}$ mice also presented partial reduction of myeloperoxidase activity upon carrageenin stimulus. Furthermore, it is possible that the participation of IL-33/ST2 in the recruitment of leukocytes such as neutrophils would become more evident at later stages of inflammation because cytokines such as TNF-α induce the expression of ST2 in neutrophils (Verri *et al.*, 2010) and because IL-33 is processed into mature form by neutrophil elastase and cathepsin G (Lefrançais *et al.*, 2012) indicating that, as the inflammatory process continued, the cellular responsiveness to IL-33 would increase and the recruited neutrophils would further contribute to activation of IL-33.

There is activation of a cascade of cytokines in the carrageenin-induced mechanical hyperalgesia model in which TNF-α and CXCL1 induce the production of IL-1β that, in turn, induces the production of PGE_2 (Ferreira *et al.*, 1988; Cunha *et al.*, 1992; 2005). Furthermore, TNF–α- and IL–1β-induced mechanical hyperalgesia were inhibited by ET receptor antagonists (Verri *et al.*, 2006), Both ET_A and ET_B receptor antagonists reduce carrageenin-induced mechanical hyperalgesia (Baamonde *et al.*, 2004) and ET-1 induces PGE_2 production in immunized mice (Verri *et al.*, 2007). Thus, cytokines seem responsible for ET-1 production in carrageenin inflammation. In the present study, IL–33-induced mechanical hyperalgesia was inhibited by targeting TNF-α, CXCL1, IL-1β, ET_A and ET_B receptors, and PGE_2. Further, the carrageenin-induced production of TNF-α, CXCL1, IL-1β and PGE_2, and preproET-1 mRNA expression were inhibited in $ST2^{-/-}$ mice. Therefore, IL-33/ST2 signalling seems to be an early event in carrageenin-induced inflammatory mechanical hyperalgesia by inducing the production of cytokines, chemokines, ET-1 and PGE_2. In addition to the contribution of TNF-α, CXCL1, IL-1β, ET-1 and PGE_2 to inflammatory hyperalgesia, these mediators also contribute to oedema and/or leukocyte recruitment (Williams, 1982; Faccioli *et al.*, 1990;

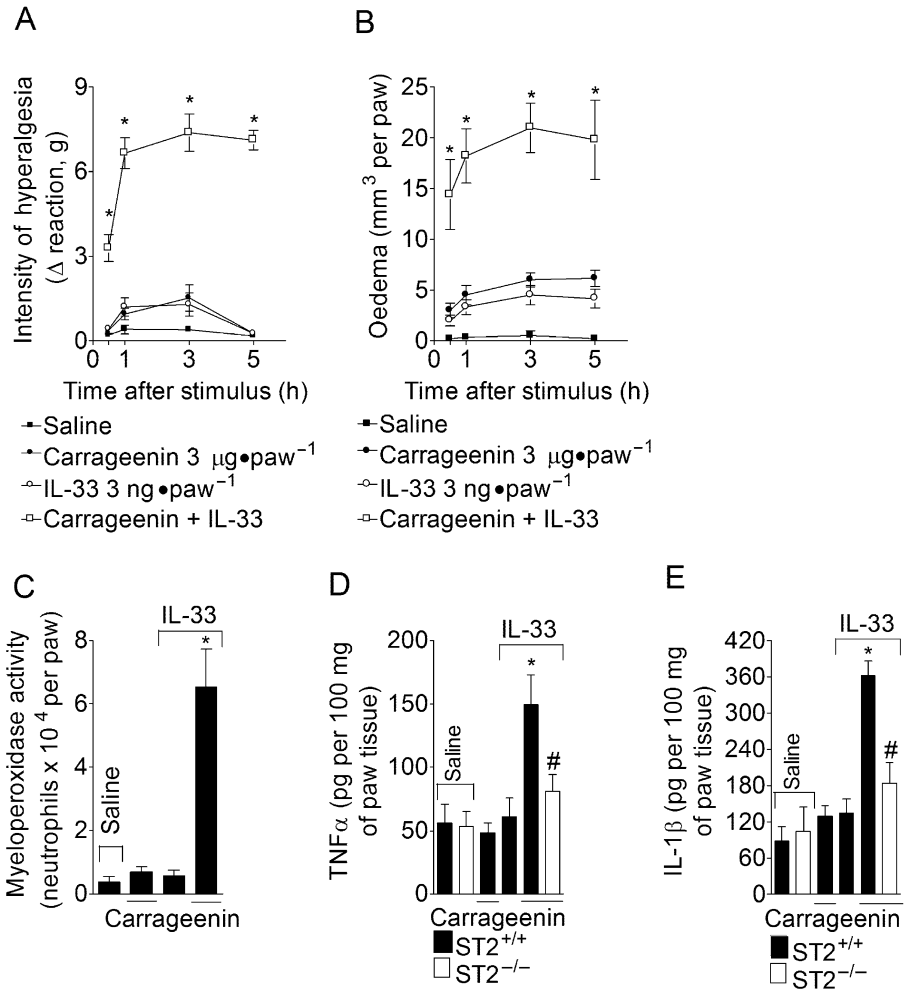

Figure 10

IL-33 synergizes with carrageenin to induce inflammation, hyperalgesia and cytokine production. Mice received i.pl. injection of IL-33 (3 ng·per paw), carrageenin (3 μg·per paw) or IL-33 plus carrageenin (same doses). Mechanical hyperalgesia (panel A) and oedema (panel B) were determined at indicated time points followed by myeloperoxidase activity determination at 5 h (panel C) in paw skin samples. TNF-α (panel D) and IL-1β (panel E) levels were determined by ELISA. $n = 6$ for hyperalgesia, oedema and myeloperoxidase activity, $n = 4$ for ELISA, representative of two separate experiments. $^{*}P < 0.05$ compared with the saline, carrageenin or IL-33 in $ST2^{+/+}$ group and $^{\#}P < 0.05$ compared with the carrageenin plus IL-33 in $ST2^{+/+}$ mice group.

McColl and Clark-Lewis, 1999; Verri *et al.*, 2007; Joosten *et al.*, 2006; Conte *et al.*, 2008; Zarpelon *et al.*, 2012).

Treatment with soluble ST2-Fc fusion protein inhibits intestinal ischaemia/reperfusion-induced lethality and inflammation by inducing IL-10 production (Fagundes *et al.*, 2007). IL-10 is an endogenous anti-hyperalgesic cytokine in the carrageenin model (Poole *et al.*, 1995). and carrageenin induced production of IL-10 in our experiments. However, in $ST2^{-/-}$ mice, the output of this cytokine was reduced at 3 and 5 h, demonstrating clearly that the loss of hyperalgesia in the $ST2^{-/-}$ mice could not be due to increased IL-10 levels.

It is interesting to point out that IL-33 potentiates antigen-induced production of cytokines in a mast cell line (Andrade *et al.*, 2011). We therefore proposed that IL-33 could also exhibit such activity during carrageenin inflammation. We observed a synergy between IL-33 and carrageenin at doses that were ineffective, as single treatments, in inducing hyperalgesia, oedema and myeloperoxidase activity. Moreover, this synergy was also observed in terms of TNF-α and IL-1β production, and the synergy was ST2-dependent. Therefore, it is likely that, although relatively high doses of IL-33 given alone did induce inflammation and pain in normal mice, during carrageenin paw inflammation much lower doses of IL-33 are required because this cytokine synergizes with carrageenin.

In conclusion, we have shown here that IL-33/ST2 was involved in carrageenin-induced inflammatory oedema, leukocyte recruitment and mechanical hyperalgesia. The mechanisms triggered by IL-33/ST2 involve the production of pro-inflammatory cytokines, ET-1 and PGE_2. This prominent role of IL-33/ST2 suggests that it is now important to determine whether drugs that inhibit carrageenin-induced inflammation also affect IL-33/ST2 signalling and supports further pre-clinical and clinical studies on IL-33/ST2 targeting thera-

pies in innate inflammation. Moreover, even if the primary use of therapies targeting IL-33/ST2 signalling is not intended to reduce pre-existing pain, they might contribute to reduce it. Pain is a likely side effect of therapies using recombinant IL-33 as it has been observed with other cytokines such as IL-12 and granulocyte-colony stimulating factor (Verri *et al.*, 2005; Carvalho *et al.*, 2011) although a non-steroidal anti-inflammatory drug would be sufficient to reduce such side effects, as suggested here.

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Conflict of interest

The authors declare no conflict of interest.

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