

Transient receptor potential ankyrin 1 (TRPA1) plays a critical role in a mouse model of cancer pain

Caren Tatiane De David Antoniazzi¹, Romina Nassini², Flávia Karine Rigo³, Alessandra Marcon Milioli³, Fernando Bellinaso¹, Camila Camponogara⁴, Cássia Regina Silva^{5,6}, Amanda Spring de Almeida¹, Mateus Fortes Rossato⁶, Francesco De Logu², Sara Marchesan Oliveira⁴, Thiago Mattar Cunha⁶, Pierangelo Geppetti², Juliano Ferreira⁷ and Gabriela Trevisan ^{1,3}

¹Graduate Program in Pharmacology, Federal University of Santa Maria (UFSM), Santa Maria, Rio Grande do Sul, Brazil

²Department of Health Sciences, Section of Clinical Pharmacology and Oncology, University of Florence, Florence, Italy

³Graduate Program in Health Science, University of the Extreme South of Santa Catarina, Unesc, Criciúma, Santa Catarina, Brazil

⁴Graduate Program in Biological Sciences: Toxicological Biochemistry, Federal University of Santa Maria (UFSM), Santa Maria, Rio Grande do Sul, Brazil

⁵Biochemistry and genetics Institute, Federal University of Uberlândia, Uberlândia, Minas Gerais, Brazil

⁶Department of Pharmacology, Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, São Paulo, Brazil

⁷Graduate Program in Pharmacology, Federal University of Santa Catarina (UFSC), Florianópolis, Santa Catarina, Brazil

There is a major, unmet need for the treatment of cancer pain, and new targets and medicines are required. The transient receptor potential ankyrin 1 (TRPA1), a cation channel expressed by nociceptors, is activated by oxidizing substances to mediate pain-like responses in models of inflammatory and neuropathic pain. As cancer is known to increase oxidative stress, the role of TRPA1 was evaluated in a mouse model of cancer pain. Fourteen days after injection of B16-F10 murine melanoma cells into the plantar region of the right hind paw, C57BL/6 mice exhibited mechanical and thermal allodynia and thigmotaxis behavior. While heat allodynia was partially reduced in TRPV1-deficient mice, thigmotaxis behavior and mechanical and cold allodynia were absent in TRPA1-deficient mice. Deletion of TRPA1 or TRPV1 did not affect cancer growth. Intrathecal TRPA1 antisense oligonucleotides and two different TRPA1 antagonists (HC-030031 or A967079) transiently attenuated thigmotaxis behavior and mechanical and cold allodynia. A TRPV1 antagonist (capsazepine) attenuated solely heat allodynia. *NADPH oxidase activity and hydrogen peroxide levels were increased in hind paw skin 14 days after cancer cell inoculation.* The antioxidant, α -lipoic acid, attenuated mechanical and cold allodynia and thigmotaxis behavior, but not heat allodynia. Whereas TRPV1, via an oxidative stress-independent pathway, contributes partially to heat hypersensitivity, oxidative stress-dependent activation of TRPA1 plays a key role in mediating thigmotaxis behavior and mechanical and cold allodynia in a cancer pain model. TRPA1 antagonists might be beneficial in the treatment of cancer pain.

Key words: hydrogen peroxide, HC-030031, NADPH oxidase, allodynia, chemotherapeutic drugs

*C.T.D.A. and R.N. contributed equally to this work

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Correspondence to: Gabriela Trevisan, Graduate Program in Pharmacology, Federal University of Santa Maria (UFSM), Roraima Avenue, 1000, building 21, room 5207, Zip code: 97105-900 Santa Maria, Rio Grande do Sul, Brazil, E-mail: gabrielatrevisansantos@gmail.com; Tel.: +55 55 32208976

Introduction

Despite the fact that prevention and novel treatments have reduced incidence and mortality, cancer remains a major public health problem worldwide. As a consequence of cancer, pain is one of the most feared and burdensome symptoms,¹ and unrelieved pain represents a major unmet medical concern in cancer patients.^{2,3} Although present at any time, pain usually increases with cancer progression, affecting from 75 to 90% of patients with metastatic or advanced-stage cancer.^{1,2} Advancements in the knowledge of pain and improvement in the use of analgesics have been limited. Thus, a considerable proportion of cancer patients complain of inadequate treatment,^{4,5} or report a number of severe adverse effects from currently available therapies.⁶ Poor understanding of the mechanism of cancer pain and lack of selective targets are major limitations for the development of novel, effective and safe drugs.

Activation of ion channels expressed in primary sensory neurons has been proposed to contribute to the initial processing of cancer pain.⁷ Acid-sensing ion channels (ASICs) and transient receptor potential vanilloid 1 (TRPV1), possibly

What's new?

While cancer is a frequent cause of pain, mechanisms underlying the association are poorly understood. Moreover, therapeutic options for cancer pain are limited, and affected patients are undertreated. Here, using a mouse model of cancer pain, the authors identify transient receptor potential ankyrin 1 (TRPA1), a cation channel expressed by pain receptors, as a primary transducer of cancer pain. In animals, TRPA1 deletion attenuated sensitivity to mechanical and cold pain stimuli. Similar effects were produced upon TRPA1 blockade *via* pharmacological inhibition and TRPA1-targeted antisense oligonucleotides. The findings warrant further investigation of TRPA1 antagonism as a means of treating cancer pain.

activated or sensitized by increased concentrations of protons or other mediators, appear to contribute to hypersensitivity in bone cancer pain models^{7,8} and in a soft tissue cancer model induced by injection of squamous cell carcinoma into the rat hind paw.⁹ Among the numerous regulatory proteins found in peptidergic nociceptors, the TRP ankyrin 1 (TRPA1) is a non-selective cation channel expressed by TRPV1-positive neurons, and a multisensor for a variety of noxious exogenous and endogenous stimuli, which plays a major role in different models of inflammatory and neuropathic pain.^{10–14} In addition to exogenous agonists, such as allyl isothiocyanate (AITC, in mustard oil and wasabi), cinnamaldehyde (in cinnamon) and allicin (in garlic), an unprecedented series of endogenous pro-inflammatory substances produced at sites of inflammation or tissue injury, including reactive oxygen, nitrogen and carbonyl species (ROS, RNS and RCS, respectively), directly gate TRPA1.^{10–12,15–17}

Some recent examples underline the unique ability of TRPA1 to sense the redox state of the milieu¹⁸ and, *via* this mechanism, to signal pain evoked by anticancer treatments. Chemotherapeutic drugs, including bortezomib, oxaliplatin and paclitaxel, elicit cold and mechanical allodynia in mice *via* oxidative stress-dependent TRPA1 activation.^{12–14} Furthermore, TRPA1 activation by two channel agonists, the aromatase inhibitors, letrozole¹⁹ and the aromatase substrate, androstenedione, was exaggerated by ROS.²⁰ Notably, the synergistic action of clinically relevant concentrations of letrozole, androstenedione and ROS reproduced inflammatory and neuropathic pain in mice, similar to the musculoskeletal symptoms reported by breast cancer patients treated with aromatase inhibitors.²⁰

Cancer remarkably disrupts tissue architecture and alters the biochemistry and metabolism of the microenvironment.²¹ Oxidative stress generation in cancer cells,²² as well as in the cells surrounding the tumor,^{8,23,24} is one of the most common and major changes associated with cancer growth. However, the role of TRPA1 and oxidative stress in pain originated by cancer growth is unknown. Here, in a model of cancer pain evoked by the inoculation of a mouse melanoma cell line in the hind paw in mice, we show that, while TRPV1 partially contributes to oxidative stress-independent heat hypersensitivity, thigmotaxis behavior and mechanical and cold allodynia associated with tumor growth are entirely due to oxidative stress-dependent activation of nociceptor TRPA1.

Materials and Methods**Animals**

Male adult C57BL/6 (male, 20–25 g, 5–6 weeks), littermate wild-type (*Trpa1*^{+/+}) and TRPA1-deficient (*Trpa1*^{-/-}; B6129P-*Trpa1*^{tm1Kykwl}) mice (25–30 g, 6–8 weeks) and TRPV1-deficient mice (*Trpv1*^{-/-}; B6129X1-*Trpv1*^{tm1Jul}) backcrossed with C57BL/6 mice (*Trpa1*^{-/-} or *Trpv1*^{+/+}) for at least 10 generations (25–30 g, 6–8 weeks) were used. The animals were housed 4 per cage, with wood shaving bedding and nesting material and standard animal food (Puro Lab 22 PB pelleted form, Puro Trato, Rio Grande do Sul, Brazil) and tap water *ad libitum*. The room temperature was controlled (22 ± 1 °C), and the illumination maintained on a 12-h light/dark cycle (lights on from 7:00 A.M. to 7:00 P.M.). Mice were acclimatized in the experimental room for at least 1 h before testing, and they were used throughout the experiments. The experiments reported in the current study were carried out in accordance with the guidelines for laboratory animal care and the ethical guidelines for pain investigations in conscious animals set by the International Association for the Study of Pain.²⁵ The study was approved by the Ethics Committee on the Use and Care of Laboratory Animals of the Federal University of Santa Maria (UFSM, protocol #7658240417), University of the Extreme South of Santa Catarina (Unesc; protocol #082–2014–01), São Paulo University (USP; process #208/2014) and University of Florence (protocol #579/2017-PR). The behavioral studies followed the animal research reporting *in vivo* experiments (ARRIVE) guidelines.²⁶ The number of animals for each experiment was determined by sample size estimation based on previous results obtained in our laboratory. The intensity of the noxious stimuli used were the minimum levels necessary to demonstrate the consistent effects of the drug treatments. Behavioral evaluations were performed between 8:00 A.M. and 5:00 P.M. All experiments were performed by an operator blind to drug administration and *in vitro* treatment.

Reagents and drugs

If not otherwise indicated, all reagents were from Sigma-Aldrich Chemical Co. (St. Louis, MO).

Cell culture and procedure for tumor inoculation

B16-F10 murine melanoma cells (CRL-6475; ATCC, Manassas, VA) were obtained from the Rio de Janeiro Cell Bank (BCRJ code 0046) and were cultured in DMEM containing

10% FBS and 1% penicillin–streptomycin (10,000 U/100 µg/mL) at 37 °C with 5% CO₂ in a humidified atmosphere and B16-F10 murine melanoma cells were used when received without further authentication. For tumor inoculation, 20 µL of melanoma cells (2 × 10⁵ cells) were suspended in PBS and injected (subcutaneous, s.c.) into the plantar region of the mice's right hind paws.^{27,28} The s.c. injection of PBS was used as vehicle.

Paw edema

The paw thickness after B16-F10 tumor cell inoculation was measured with a digital caliper.²⁹ Paw thickness was verified 2–14 days after B16-F10 melanoma cell inoculation and compared to vehicle injection. The results were expressed as percentage increase of the paw thickness over the basal value.

Behavioral tests

All behavioral tests were assessed before tumor inoculation (baseline), 2–14 days after B16-F10 tumor cell inoculation or vehicle injection, and then after treatments at different time intervals (1, 2 or 3 h).

Thigmotaxis behavior in the open field test. We used an open field apparatus to investigate the thigmotaxis behavior (the tendency to stay near the walls, e. g., in an observation chamber) that are related to nociception.³⁰ Thus, C57BL/6, *Trpa1*^{+/+} and *Trpa1*^{-/-} mice were introduced to individual activity chambers (50 × 50 × 25 cm), to which they had not previously been exposed. Each chamber had an inner zone (20 × 20 cm) delimiting the chamber's center. Thigmotaxis behavior was considered as the time spent in the inner zone, the number of rearing (vertical movements) and the number of crossing (horizontal movements), which were analyzed for 30 min.

Mechanical allodynia. The mechanical allodynia was assessed in C57BL/6, *Trpa1*^{+/+}, *Trpa1*^{-/-}, *Trpv1*^{+/+} and *Trpv1*^{-/-} mice by measuring the paw withdrawal threshold by using the up-down paradigm as previously described³¹ with minor modifications.¹² Briefly, the mice were firstly acclimatized (1 h) in individual clear plexiglass boxes (9 × 7 × 11 cm) on an elevated wire mesh platform, to allow for access to the plantar surfaces of the hind paws. Von Frey filaments of increasing stiffness (0.02–4 g) were applied to the hind paw plantar surfaces of the mice with enough pressure to bend the filament. The absence of a paw being lifted after 5 s led to the use of the next filament with an increased weight, whereas a lifting paw indicated a positive response, leading to the use of a subsequently weaker filament. 50% mechanical paw withdrawal threshold response was calculated from the resulting scores, as previously described.³² The paw withdrawal threshold responses were expressed in grams (g), and they were evaluated before (baseline), 2–14 days after melanoma cell or vehicle inoculation, and after drug treatments (1, 2 and 3 h).

The paw withdrawal threshold was measured in the periphery of the melanoma mass because this region usually shows more sensitivity.³³ A significant reduction in paw withdrawal threshold when compared to baseline characterized the development of painful hypersensitivity (mechanical allodynia), and an increase in the paw withdrawal threshold response after treatment indicated an anti-allodynic effect.

Cold allodynia. Cold allodynia was assessed in C57BL/6, *Trpa1*^{+/+}, *Trpa1*^{-/-}, *Trpv1*^{+/+} and *Trpv1*^{-/-} mice by measuring the acute nocifensive response to the acetone-evoked evaporative cooling as previously described.¹² Briefly, a droplet (50 µL) of acetone, formed on the flat-tip needle of a syringe, was gently touched to the plantar surface of the mouse hind paw, and the time spent in elevation and licking of the plantar region over a 60-s period was measured. Acetone was applied 3 times at a 10- to 15-min intervals, and the average elevation/licking time was calculated.

Heat hyperalgesia. Thermal hyperalgesia was measured in C57BL/6, *Trpa1*^{+/+}, *Trpa1*^{-/-}, *Trpv1*^{+/+} and *Trpv1*^{-/-} by exposing the mid-plantar surface of the hind paw to a beam of radiant heat through a transparent surface, using a plantar analgesimeter for paw stimulation (Ugo Basile, Comerio, Italy). Paw withdrawal latency was recorded as the time from onset of the thermal stimulus to the withdrawal response. In each paw, mean withdrawal latency of three measures was calculated. The interval between trials on the same paw was at least 5 min. The cut-off latency was set at 20 s to avoid tissue damage.

Eye wiping test. The number of eye wiping movements, after the instillation of eye drops of capsaicin (1 nmol/5 µL), AITC (10 nmol/5 µL) or vehicles (2% and 4% DMSO, respectively) to the conjunctiva, was recorded for a 10-min time period.³⁴

Treatment protocols

TRPA1 selective antagonists and antioxidant. Intragastric (i.g.) HC-030031 (300 mg/kg/10 ml), A-967079 and α-lipoic acid (both, 100 mg/kg/10 ml, i.g) or their vehicle (dimethyl sulfoxide, DMSO, 1% in NaCl 0.9%) and intraperitoneal (i.p.) capsazepine (4 mg/kg/10 ml) or its vehicle (DMSO 1% in NaCl 0.9%), were administered at day 14 after B16-F10 tumor cell inoculation or PBS injection. Mice were tested before (baseline), at day 14 (time 0) or 1, 2 and 3 h after treatments, in the assessment of mechanical and cold allodynia. Thigmotaxis behavior was assessed 1 h after HC-030031 or α-lipoic acid treatments.

TRPA1 oligonucleotide antisense. C57BL/6 mice received intrathecal (i.th. 5 µL) TRPA1 antisense oligonucleotide (TRPA1 AS ODN; 5' TCTATGCGGTTATGTTGG 3'; 30 µg/kg) or its mismatch (TRPA1 MM ODN; 5' ACTACTACACTAGACTAC 3'; 30 µg/kg), 3 times/day for 3 consecutive days (days 11, 12 and 13, after B16-F10 tumor cells inoculation or

PBS injection). Mice were tested before (baseline) and at day 14 (time 0) after B16-F10 tumor cell inoculation or PBS injection, in mechanical and cold assessment, and in the thigmotaxis behavior.

Biochemical analysis

Hydrogen peroxide (H_2O_2) production assay. The H_2O_2 levels in the hind paw skin after B16-F10 tumor cell inoculation or PBS injection were determined by the phenol red-horseradish peroxidase (HRP) method.³⁵ Briefly, at day 14, after cell inoculation or vehicle injection, mice were euthanized, and the hind paw skin was removed and homogenized in 50 mM phosphate buffer (pH 7.4) containing 5 mM sodium azide at 4 °C for 60 s. The homogenate was centrifuged (12,000xg for 20 min at 4 °C). The supernatant obtained was used to determine H_2O_2 levels.³⁵ The H_2O_2 levels were expressed as μmol of H_2O_2 based on a standard curve of HRP-mediated oxidation of phenol red by H_2O_2 , corrected by protein content (in milligrams) of paw skin samples analyzed.

Evaluation of NADPH oxidase activity. NADPH oxidase activity was assessed in the hind paw skin samples 14 days after B16-F10 tumor cell inoculation or PBS injection, using a commercially available assay kit (CY0100, cytochrome C reductase, NADPH assay kit). Briefly, paw skin samples were homogenized in 50 mM phosphate buffer (pH 7.4) and centrifuged at 3,000x g for 10 min at 4 °C. The supernatant obtained was centrifuged for 40 min at 10,000x g at 4 °C. The final supernatant was used for NADPH activity determination. The NADPH oxidase activity was expressed as U/mL/mg of protein.

Histology and immunofluorescence

Anesthetized [ketamine (90 mg/kg) and xylazine (3 mg/kg), i. p.] mice were transcardially perfused with PBS, followed by 4% paraformaldehyde at day 14 after B16-F10 tumor cell inoculation. The paw with injected B16-F10 tumor cells was removed, post-fixed for 24 h, and paraffin embedded or cryoprotected overnight at 4 °C in 30% sucrose until cryosectioning. Cryosections (10 μm) were stained with hematoxylin and eosin (H&E) for histological examination. Sections (5 μm) of formalin fixed paraffin-embedded tissue were incubated with the after primary antibody: TRPA1 (ab58844, rabbit polyclonal, 1:400, Abcam, Cambridge, UK), (1 h, RT) diluted in antibody diluent (Roche Diagnostics, Mannheim, Germany). Sections were then incubated with fluorescent secondary antibodies: polyclonal Alexa Fluor 488 (1:600, Invitrogen, Milan, Italy) (2 h, RT, protected from light). Sections were then coverslipped using a water-based mounting medium with 4',6'-diamidino-2-phenylindole (DAPI) (Abcam, Cambridge, UK). The analysis of negative control (nonimmune serum) was simultaneously performed to exclude the presence of nonspecific immunofluorescent staining, cross-immunostaining, or fluorescence bleed-through. Tissues were visualized, and digital images were captured using an Olympus BX51.

Real-time PCR

RNA was extracted from cultured B16-F10 cells and trigeminal ganglion neurons. The standard Trizol[®] extraction method was used. RNA concentration and purity were assessed spectrophotometrically by measuring the absorbance at 260 nm and 280 nm. The RNA (100 ng) was reverse-transcribed using the iScript[™] cDNA Synthesis kit (Bio-Rad, Hercules, USA) according to the manufacturer's protocol. For relative quantification of mRNA, real-time PCR was performed on Rotor Gene[®] Q (Qiagen, Hilden, GE). The sets of primers-probes were as follows: 18S-FW (forward): 5'-CGCGGTTCTATTTTGTGGT-3', 18S-RE (reverse): 5'-AGTCGGCATCGTTTATGGTC-3' (NCBI Ref Seq: NR_003278.3); TRPA1-FW: 5'-CAGGATGCTACGGTTTTTCATTACT-3', TRPA1-RE: 5'-GCATGTGTCAATGTTTGGTACTTCT-3' (NCBI Ref Seq: NM_177781.4). The chosen reference gene was the 18S. SsoAdvanced[™] Universal SYBR[®] Green Supermix (Bio-Rad, Hercules, USA) was used for amplification, and the cycling conditions were the after: samples were heated to 95 °C for 1 min followed by 40 cycles of 95 °C for 10 s, and 65 °C for 20 s. PCR reaction was carried out in triplicate. Relative expression of TRPA1 mRNA was calculated using the $2^{-\Delta(\Delta\text{CT})}$ comparative method, with each gene normalized against the internal endogenous reference 18S gene for the same sample.

Statistical analysis

All values were expressed as mean \pm S.E.M and analyzed by Student's *t*-test, One-way or Two-way analysis of variance (ANOVA) followed by Bonferroni's *post hoc* test when appropriate. All tests were carried out using GraphPad 5.0 Software (San Diego, CA). The $p < 0.05$ values denote significant difference among groups.

Results

Intraplantar inoculation of cancer cells evokes mechanical and thermal allodynia and thigmotaxis behavior in mice

Intraplantar (i.p.) injection of B16-F10 melanoma cells in the hind paw of mice induced a progressive increase of paw volume (12–16 days), indicating the development of tumor mass (Figs. 1a and 1b). Mice inoculated with B16-F10 melanoma Cells exhibited mechanical (Fig. 1c) and thermal (cold and heat) allodynia (Fig. 1d) (10–16 days). Allodynia peaked 14 days after B16-F10 melanoma cell inoculation, to decline in the after days, probably because disruption of tissue architecture associated with excessive increase in cancer mass prevented the reproducible assessment of mechanical and thermal hypersensitivity (Fig. 1c). Furthermore, measurements of thigmotaxis behavior were performed 14 days after melanoma cells inoculation. At this time point, we observed a significant reduction in the number of rearing events and in the time spent in the inner zone in the open field test during 30 min of observation compared to vehicle (PBS) injected mice (Fig. 1e), whereas no changes in the number of crossings (Fig. 1e) were reported. Mice injected with PBS did not show any change in mechanical and thermal (cold and heat)

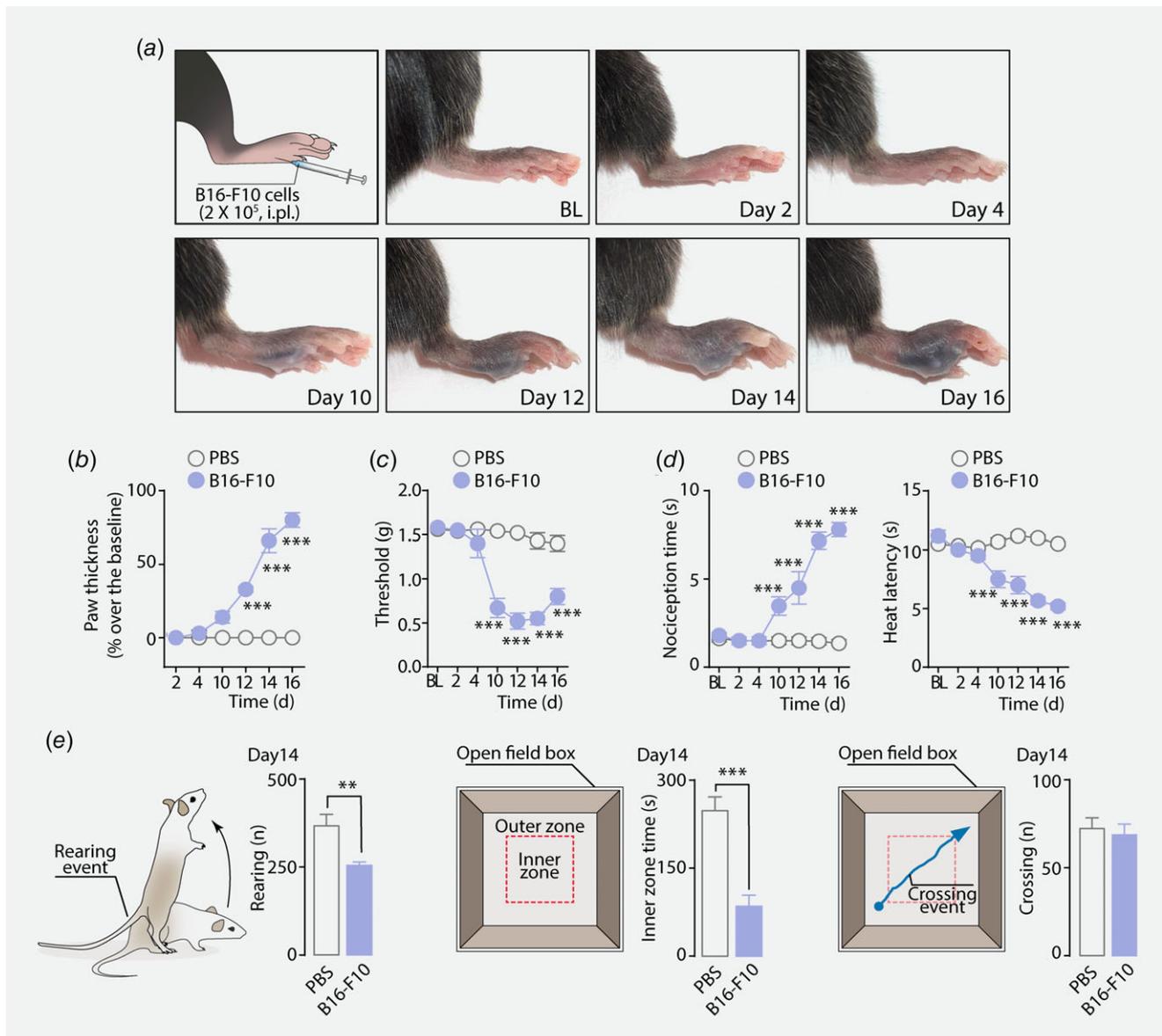


Figure 1. Intraplantar (i.pl.) inoculation of cancer cells evokes mechanical and cold allodynia, heat hyperalgesia and thigmotaxis behavior in mice. (a) representative microphotographs of tumor-bearing hind paw of C57BL/6 mice 2–16 days after inoculation (i.pl.) of B16-F10 melanoma cells (2×10^5 cells/mL, $20 \mu\text{L}$). The drawing in the upper right panel represents the site of inoculation. Time-dependent increase in paw thickness (b), mechanical (c), cold and heat (d) allodynia after B16-F10 melanoma cell inoculation. (e) Thigmotaxis behavior assessed by measuring number of rearing events, time spent in the inner zone, or crossing number in the open field test at day 14 after B16-F10 melanoma cell inoculation. Control mice were injected in the paw with PBS. BL, baseline measurement. Data are expressed as mean + S.E.M. ($n = 8$ mice per group). $**p < 0.01$, $***p < 0.001$, when compared to PBS-treated group. Two-way ANOVA, followed by Bonferroni's *post hoc* test or Student's *t*-test.

hypersensitivity over the 14 days of observation (Figs. 1b–1d), nor thigmotaxis behavior was observed at day 14 after the injection (Fig. 1e).

TRPA1, but not TRPV1, gene-deletion attenuates mechanical and cold allodynia and thigmotaxis behavior

Inoculation of B16-F10 melanoma cells in the hind paw of *Trpa1*^{+/+} mice caused a time-dependent increase in mechanical and cold allodynia (Fig. 2a), which were like those observed

in *C57BL/6* mice, and absent in *Trpa1*^{-/-} (Fig. 2a). Heat hyperalgesia was unaffected by *Trpa1* deletion (Fig. 2a). At day 14 after tumor cell inoculation, changes in thigmotaxis behavior (number of rearing events and time spent in the inner zone in the open field test) were observed in *Trpa1*^{+/+} mice, which were similar to those observed in *C57BL/6* mice (Fig. 2b), but these behaviors were absent in *Trpa1*^{-/-} mice. The crossings number was unaffected in *C57BL/6* mice and in both *Trpa1*^{+/+} and *Trpa1*^{-/-} mice (Fig. 2b). Thus, this

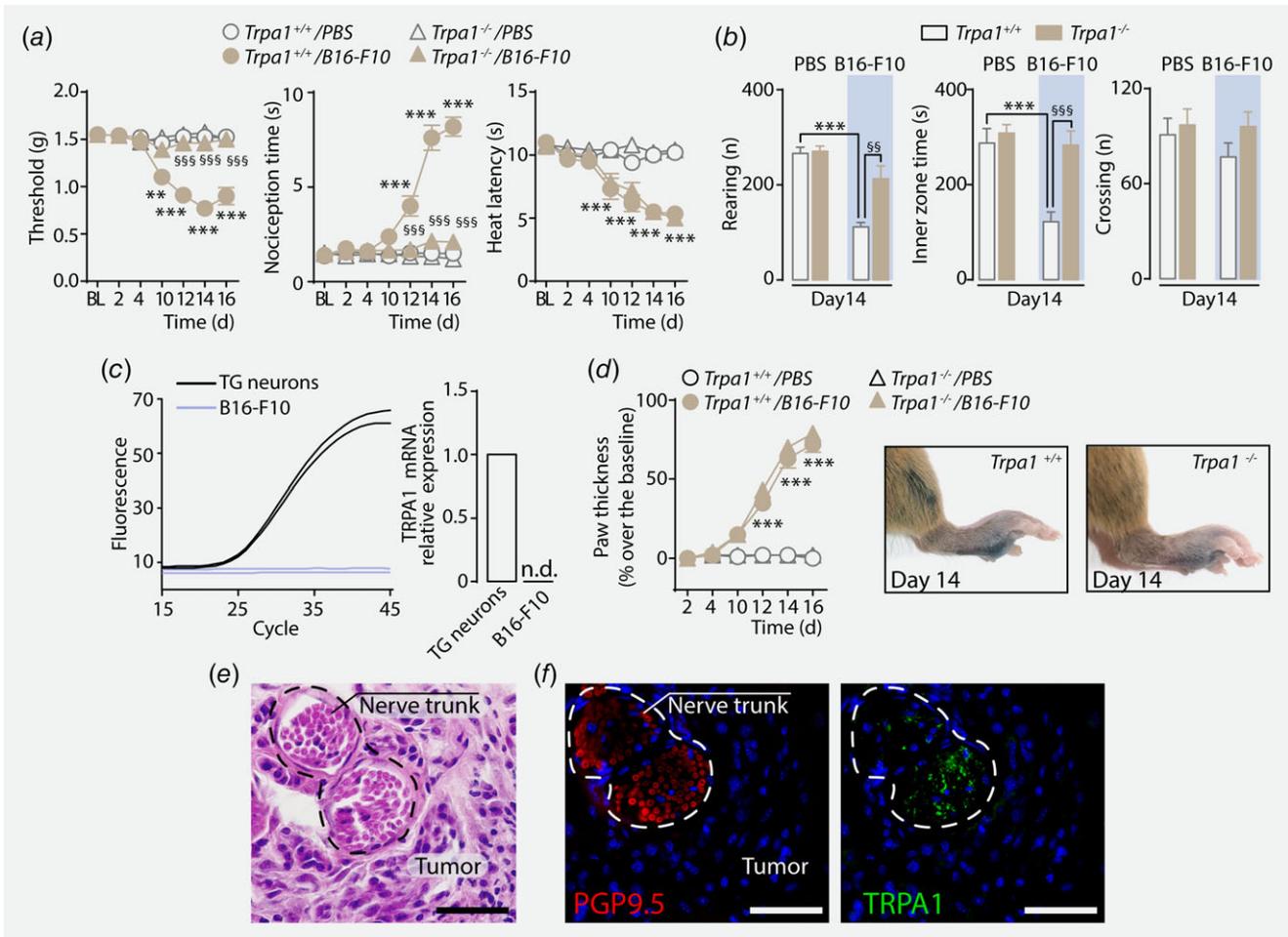


Figure 2. TRPA1 gene-deletion attenuates mechanical and cold allodynia, and pain-related behaviors in cancer pain model in mice. Time-dependent (2–16 days) changes in (a) mechanical and cold allodynia, and heat hypersensitivity in *Trpa1*^{+/+} and *Trpa1*^{-/-} mice. (b) Thigmotaxis behavior assessed by measuring number of rearing events, time spent in the inner zone, and crossing number in the open field test at day 14 after B16-F10 melanoma cell inoculation or PBS injection in *Trpa1*^{+/+} or *Trpa1*^{-/-} mice. (c) Representative real-time PCR plot and pooled data for TRPA1 mRNA relative expression in cultured trigeminal ganglion (TG) neurons and B16-F10 melanoma cells ($n = 3$ replicates from 2 independent experiments; n.d. not detectable). (d) Time-dependent changes in paw thickness and representative microphotographs of the tumor-bearing hind paw after B16-F10 melanoma cell inoculation in *Trpa1*^{+/+} or *Trpa1*^{-/-}. (e) Representative images of hematoxylin and eosin of hind paw 14 days after B16-F10 melanoma cells inoculation in C57BL/6 mice, showing nerve trunk surrounded by tumor cells. (f) Immunofluorescence staining of TRPA1 and PGP9.5 (a specific marker for nerve fibers) in the hind paw 14 days after B16-F10 melanoma cell inoculation in C57BL/6 mice (scale bar: 50 μ m). Control mice were injected in the paw with PBS. BL, baseline measurement. Data are expressed as mean + S.E.M. ($n = 8$ mice per group). ** $p < 0.01$, *** $p < 0.001$, when compared to *Trpa1*^{+/+}/PBS; §§§ $p < 0.001$, when compared to *Trpa1*^{+/+}/B16-F10. Two-way ANOVA or One-way ANOVA, followed by Bonferroni's *post hoc* test.

parameter was not further investigated, and the evaluation of thigmotaxis behavior refers only to number of rearing events and time spent in the inner zone.

TRPA1 mRNA (RT-qPCR) expression was elevated in cultured trigeminal ganglion neurons and was undetectable in B16-F10 melanoma cells in culture (Fig. 2c). This finding does not support the hypothesis that TRPA1 exerts a direct role in tumor growth. Furthermore, the absence of both mechanical and cold allodynia and thigmotaxis behavior in *Trpa1*^{-/-} mice cannot be associated with a reduced mass effect due to changes in tumor growth, since *Trpa1*^{+/+} and *Trpa1*^{-/-} mice showed a comparable time-dependent increase in paw thickness after

B16-F10 melanoma cells inoculation (Fig. 2d). Immunofluorescence analysis showed that, at day 14, PGP9.5⁺ nerve fibers were found in close proximity to the mass of proliferated B16-F10 melanoma cells (Fig. 2e). Some of the nerve fibers exhibited a remarkable positivity for the TRPA1 channel, suggesting a possible interaction between the tumor and TRPA1-expressing nerve bundle (Fig. 2f).

Deletion of TRPV1 did not affect tumor growth, nor mechanical and cold allodynia (Figs. 3a and 3b). However, a partial but significant reduction in the heat hyperalgesia was observed in *Trpv1*^{-/-} (Fig. 3c). Treatment with the TRPV1 selective antagonist, capsazepine (4 mg/kg, i.p.), attenuated

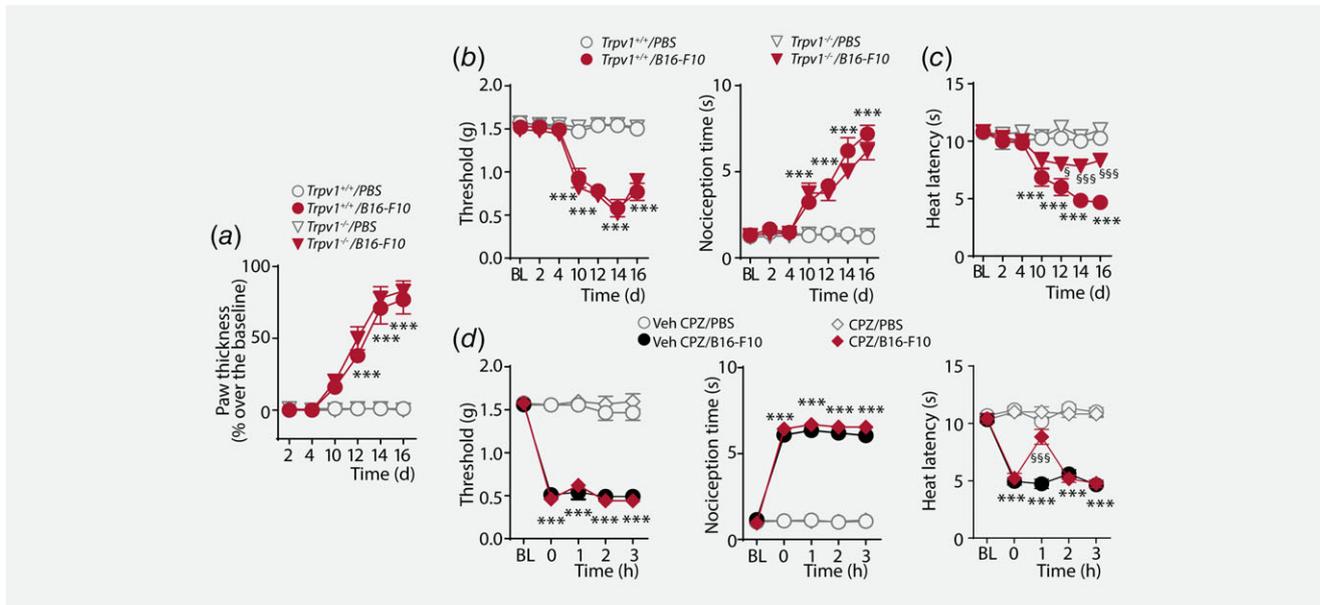


Figure 3. TRPV1 gene-deletion reduced the heat hyperalgesia in a cancer pain model in mice. Time course (2–16 days) of (a) paw thickness, (b) mechanical and cold allodynia and (c) heat allodynia in *Trpv1*^{+/+} and *Trpv1*^{-/-} mice. (d) Mechanical, cold and heat allodynia in capsaizepine (CPZ, 4 mg/kg, i.p.) treated mice at day 14 after B16-F10 melanoma cells (2×10^5 cells/mL, 20 μ L) intraplantar (i.pl.) inoculation. Control mice were injected in the paw with PBS. BL, baseline measurement. Data are expressed as mean + S.E.M. ($n = 8$ mice per group). *** $p < 0.001$, when compared to *Trpv1*^{+/+}/PBS or PBS-treated group; § $p < 0.05$, §§§ $p < 0.001$, when compared to *Trpv1*^{+/+}/B16-F10 or VehCPZ/B16-F10. Two-way ANOVA followed by Bonferroni's *post hoc* test.

heat hyperalgesia but did not affect mechanical and cold allodynia (Fig. 3d). This finding further strengthens a primary role of TRPA1 channel in the development of in B16-F10 melanoma-induced mechanical and cold allodynia.

TRPA1 antisense oligonucleotide and TRPA1 antagonists decreased mechanical and cold allodynia and thigmotaxis behavior

To confirm the contribution of nociceptor TRPA1 in mechanical and cold allodynia and thigmotaxis behavior evoked by B16-F10 melanoma cells inoculation in the hind paw, mice were treated with intrathecal TRPA1 AS or MM ODN. Administration of TRPA1 AS ODN, but not MM ODN, attenuated AITC-, but not capsaicin-evoked acute nociception, measured as eye wiping response (Fig. 4a), thus indicating selective disruption of TRPA1-mediated pain signaling. Administration of TRPA1 MM ODN did not affect baseline mechanical and cold threshold or thigmotaxis behavior in mice treated with vehicle or B16-F10 melanoma (Figs. 4b and 4c). However, TRPA1 AS ODN administration attenuated both mechanical and cold allodynia and restored the decrease in thigmotaxis behavior (Figs. 4b and 4c).

Systemic (i.g.) administration of two chemically unrelated TRPA1 receptor antagonists, HC-030031 (300 mg/kg) or A967079 (100 mg/kg), attenuated in a time-dependent manner both mechanical and cold allodynia evoked by inoculation of B16-F10 melanoma cells (Figs. 4d and 4e). Complete inhibition by both HC-030031 and A967079 was observed 1–2 h after

drug administration, whereas allodynia fully recovered 3 h after (Figs. 4d and 4e). HC-030031 administration reversed the thigmotaxis behavior compared to its vehicle (Fig. 4f).

Melanoma cell inoculation increased oxidative stress and antioxidant treatment attenuated mechanical and cold allodynia and thigmotaxis behavior

Increased levels of H_2O_2 were detected in the paw of mice 14 days after inoculation of B16-F10 melanoma cells, compared to mice injected with PBS (Fig. 5a). Similarly, NADPH oxidase activity was markedly increased in hind paw skin of mice inoculated with B16-F10 melanoma (Fig. 5b). Systemic (i.g.) administration of the antioxidant, α -lipoic acid (100 mg/kg), caused a time-dependent attenuation of both mechanical and cold allodynia but left heat allodynia unchanged, evoked by melanoma cells inoculation (Fig. 5c). Reversal of mechanical and cold hypersensitivity was obtained in 1 h, was maintained for 2 h thereafter, and terminated 3 h after α -lipoic acid administration. The vehicle of α -lipoic acid was ineffective (Fig. 5c). In addition, α -lipoic acid administration reversed the thigmotaxis behavior induced by tumor cells inoculation (Fig. 5d). The α -lipoic acid vehicle did not affect thigmotaxis behavior (Fig. 5d).

Discussion

Different animal models have recently been proposed for reproducing metastatic cancer-related pain, including tumor cells injection in the bone or the plantar pad.^{36–39} Previous studies showed that subcutaneous injection in the hind paw of B16-F10 melanoma cells

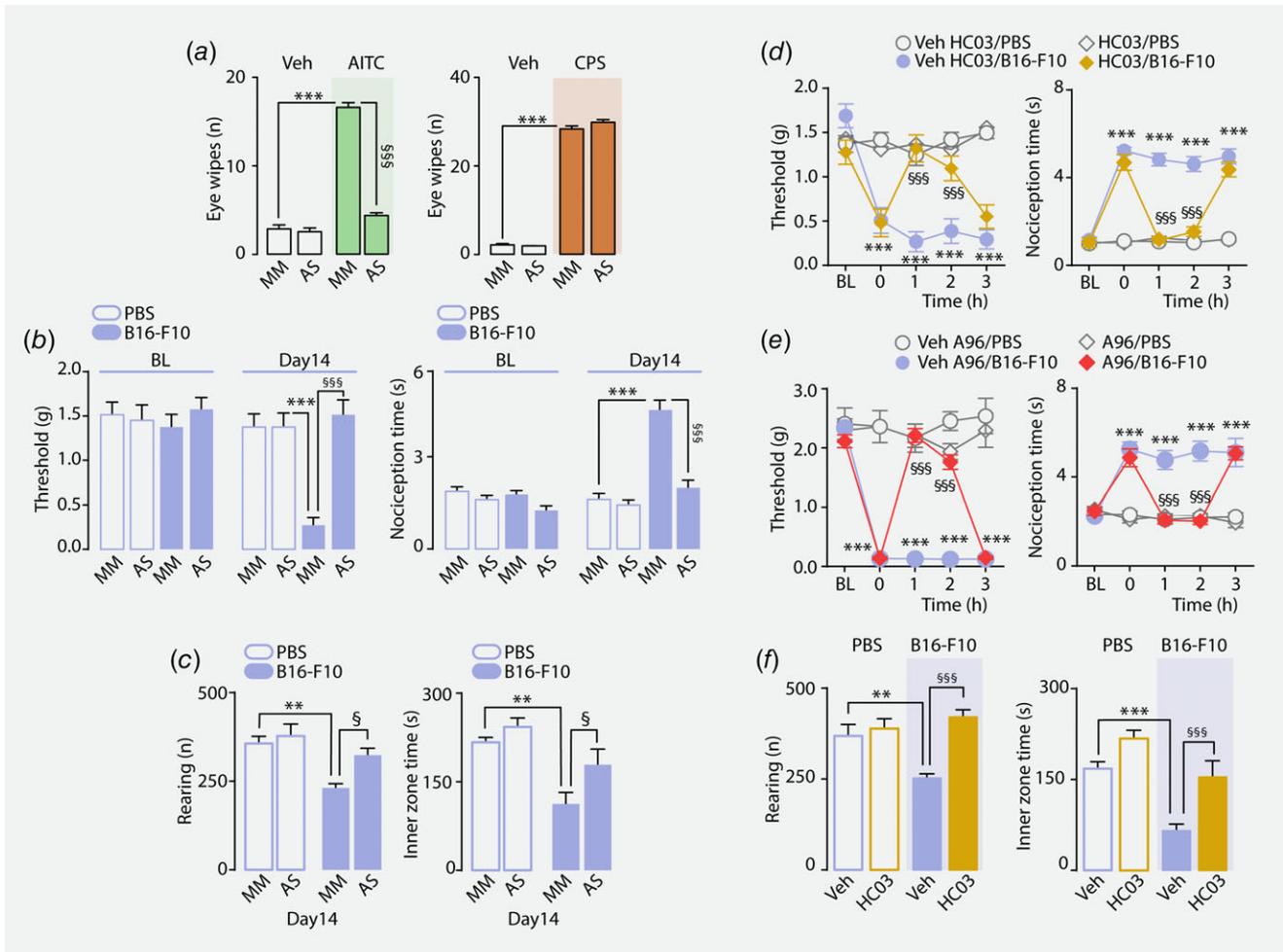


Figure 4. TRPA1 antisense oligonucleotide or TRPA1 antagonists administration decreased pain-related responses in a cancer pain model in mice. (a) Capsaicin- and AITC-evoked acute nociception (eye wiping response) after intrathecal (i.th.) antisense/mismatch (AS/MM) oligonucleotides (ODN). (b) Mechanical and cold allodynia after AS/MM ODN administration. (c) Thigmotaxis behavior assessed by measuring number of rearing events or the time spent in the inner zone in the open field test. (d) Mechanical and cold allodynia after intragastric (i.g.) HC-030031 (HC03, 300 mg/kg) administration; (e) Mechanical and cold allodynia after A967079 (A96, 100 mg/kg, i.g.) administration; (f) Thigmotaxis behavior assessed by measuring number of rearing events or the time spent in the inner zone in the open field test, measured 1 h after HC03 (300 mg/kg, i.g.) administration. All the tests were performed at day 14 after B16-F10 melanoma cells (2×10^5 cells/mL, 20 μ L) intraplantar (i.pl.) inoculation. Control mice were injected in the paw with PBS. BL, baseline measurement. Veh is the vehicle of different treatments. Data are expressed as mean + S.E.M. ($n = 8$ mice per group). $**p < 0.01$; $***p < 0.001$ when compared to PBS; $^{\$}p < 0.05$, $^{\$ \$ \$}p < 0.001$, when compared to B16-F10 or Veh/B16-F10. Two-way ANOVA followed by Bonferroni's *post hoc* test.

induces mechanical and cold allodynia that can be assessed 14 days after cells inoculation.^{27,28,40} Although mechanical and thermal allodynia is a hallmark of cancer pain, in the present study, we made the additional and original observation that mice exhibited thigmotaxis behavior 14 days after B16-F10 cell inoculation, including a decrease in number of rearing events and time spent in the inner zone in the open field test. As spontaneous pain is often present in cancer patients,⁵ spontaneous pain-related behaviors need to be included in the panel of responses evaluated in preclinical pain studies.^{30,41} Thus, the current mouse model seems to appropriately recapitulate the clinical condition by investigating both reflexive (using the von Frey hairs test and acetone test) and nonreflexive (thigmotaxis behavior) responses.

However, our major observation is that TRPA1 plays a critical role in mechanical and cold hypersensitivity, and in thigmotaxis behavior induced by B16-F10 melanoma tumor cells inoculation in the mouse hind paw. This conclusion is supported by two distinct genetic findings. First, TRPA1 homozygous gene-deletion prevented the development of either mechanical and cold allodynia and thigmotaxis behavior. The observation that *Trpa1*-deleted mice were completely protected indicated that the channel is necessary and sufficient for the pain-like responses and the thigmotaxis behavior evoked by cancer growth. Second, the knockdown of neuronal TRPA1 by intrathecally administered AS ODN, which we and others⁴² have reported to selectively abate TRPA1-mediated

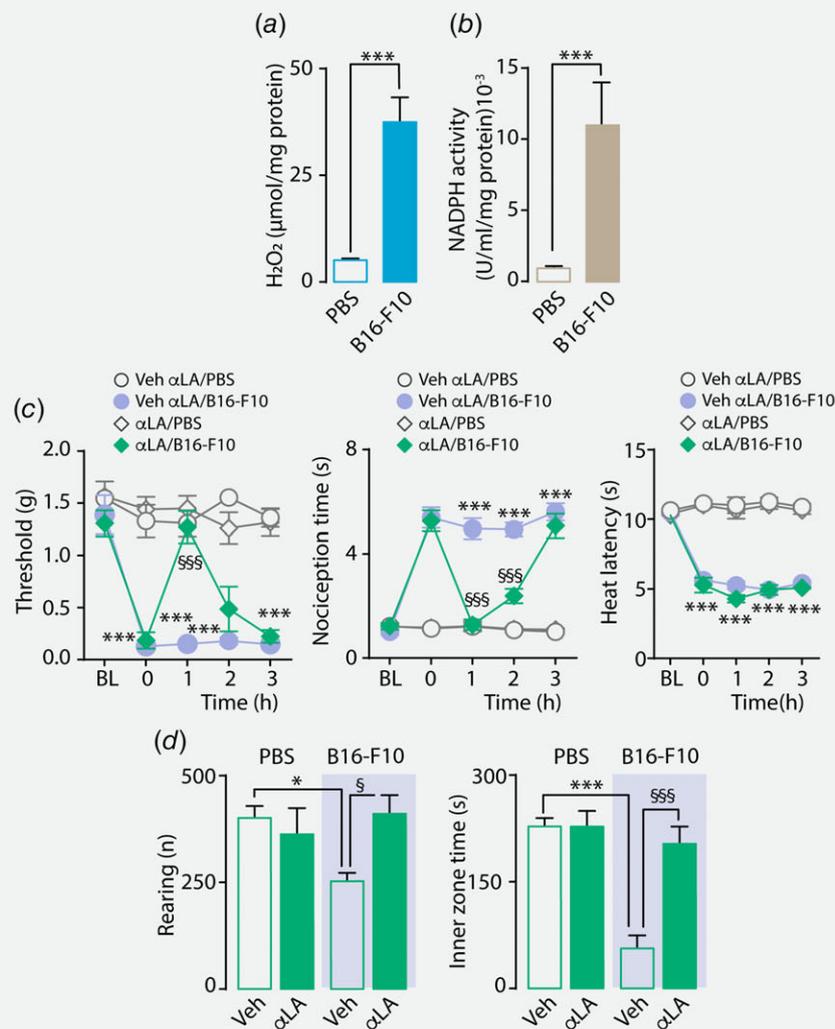


Figure 5. Melanoma cell inoculation increased the oxidative stress markers and α -lipoic acid attenuates pain-related responses. (a) H_2O_2 levels or (b) NADPH oxidase activity measured in the hind paw skin tissue after B16-F10 intraplantar (i.pl.) inoculation of melanoma cells (2×10^5 cells/mL, 20 μL). (c) Mechanical and cold allodynia and heat hypersensitivity after intragastric (i.g.) administration of the antioxidant α -lipoic acid (αLA , 100 mg/kg). (d) Thigmotaxis behavior assessed by measuring number of rearing events, or the time spent in the inner zone in the open field test, measured 1 h after αLA (100 mg/kg, i.g.) administration. All tests were performed at day 14 after B16-F10 melanoma cell (2×10^5 cells/mL, 20 μL) inoculation (i.pl.). Control mice were injected in the paw with PBS. BL, baseline measurement. Veh is the vehicle of αLA . Data are expressed as mean + S.E.M. ($n = 8$ mice per group). * $p < 0.05$, *** $p < 0.001$ when compared to PBS; § $p < 0.05$, §§§ $p < 0.001$, when compared to veh $\alpha\text{LA}/\text{B16-F10}$. Student's *t*-test, two-way ANOVA, or one-way ANOVA followed by Bonferroni's *post hoc* test.

nociceptive signals, transiently attenuated the pain responses and the thigmotaxis behavior. However, genetic TRPA1 deletion could not discriminate as to whether the channel mediates solely the initial activation of the proalgesic pathway or its targeting is continuously required to maintain the prolonged condition of hypersensitivity and nociception. The ability of two different channel antagonists, HC-030031 and A967079,^{12,13,43} to completely, but transiently, reverse cancer-related mechanical and cold allodynia and thigmotaxis behavior is possibly due to the according to the short half-lives of the two drugs.^{44,45} However, the temporary effect suggests that one or more, hitherto unknown, endogenous agents produced

during cancer progression are required to continuously target TRPA1 in order to sustain pain-like responses.

The tumor microenvironment is enriched by several inflammatory and immune mediators, and some of them might directly or indirectly target TRPA1 to signal pain.⁴⁶ In particular, excessive cancer cell turnover and metabolism or recruited immune and inflammatory cells present in the tumor milieu may increase oxidative stress.^{24,35} Since ROS and their byproducts, including the RNS, peroxynitrite and the RCS, 4-hydroxynonenal, are among the most active endogenous activators of TRPA1,^{16,47} we sought to find out whether, 14 days after cell inoculation when mechanical and cold allodynia, and

the thigmotaxis behavior were maximal, markers of oxidative stress were increased in the affected paw. The observation that H₂O₂ levels were markedly enhanced and that such increase was associated with enhanced NADPH oxidase activity confirmed in our present model that tumor growth is associated with a local increase in oxidative stress. ROS involvement in cancer pain has recently been proposed,⁸ and increased levels of NADPH oxidase activity have been found in a murine breast tumor model.⁴⁸ However, the underlying mechanism by which oxidative stress generates pain signals in cancer pain is poorly understood. The present results, showing that the antioxidant, α -lipoic acid, reversed mechanical and cold hypersensitivity, and the changes in the thigmotaxis behavior, underscore the association between oxidative stress generation and TRPA1 signaling in cancer-evoked pain-like responses. As both TRPA1 antagonism and ROS inhibition caused complete attenuation of pain-like responses, the most parsimonious hypothesis proposes that ROS and their byproducts generated within the tumor microenvironment continuously target TRPA1 on nociceptor nerve terminals surrounding cancer tissue to signal pain. The presence of TRPA1-expressing fibers in nerve bundles closely associated with tumor cells may provide some support to the present hypothesis. A pain-signaling pathway initiated by oxidative stress and mediated by TRPA1 has been proposed in models of chemotherapeutic-induced neuropathic pain, which typically occurs in patients treated with anticancer drugs.^{12–14,19} However, to the best of our knowledge, this is the first study showing the involvement of TRPA1 in a model of cancer pain. In addition, whereas a role for oxidative stress in models of cancer pain has been previously proposed,^{35,39,48} this is the first paper showing that increased oxidative stress sustains cancer pain *via* its ability to target TRPA1 in nociceptors.

TRPA1 overexpression has been proposed as an unfavorable prognostic marker in nasopharyngeal carcinoma,⁴⁹ and to

contribute to lung cancer progression.⁴⁶ B16-F10 melanoma cells inoculation caused a marked increase in paw thickness that may be considered a good approximation of cancer growth. Thus, the observation that, after B16-F10 melanoma cells inoculation, the time-dependent increase in paw size was similar in both *Trpa1*^{+/+} and *Trpa1*^{-/-} mice suggests that TRPA1 deletion is irrelevant for cancer growth. The absence of TRPA1 expression in the B16-F10 melanoma cells is in line with this observation. TRPA1 activation is associated with the release from nerve peripheral terminals of the proinflammatory neuropeptides substance P and calcitonin gene-related peptide, which promote neurogenic inflammatory responses, namely plasma protein extravasation and vasodilatation, respectively.¹⁰ Present findings that paw thickness was unchanged in *Trpa1*^{-/-} mice suggest that neither neurogenic inflammation nor the TRPA1-mediated component of neurogenic inflammation significantly contribute to increase the paw size during cancer growth.

Cancer pain is usually difficult to manage in the clinical setting. New mechanisms involved in this type of neuronal hypersensitivity must be explored, considering that unalleviated cancer pain markedly decreases a patient's quality of life.⁵ Despite increasing efforts to ameliorate the treatment of cancer pain, opioids remain the leading therapeutic option, with, however, marked limitations to their use.^{6,50} Thus, novel targets that contribute to the processing of cancer pain are urgently needed to identify effective and safe medicines. Heat allodynia associated with cancer growth was entirely independent from TRPA1 activation and oxidative stress and was partly due to TRPV1 activated by some hitherto unknown mediator. Notwithstanding, due to the marked contribution of TRPA1 to thigmotaxis behavior and mechanical allodynia, which are the most troublesome features of cancer pain,^{1,2} the development of TRPA1 antagonists appears to be a suitable therapeutic strategy for the alleviation of pain in cancer patients.

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