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ORIGINAL ARTICLE

IMT504 Provides Analgesia by Modulating Cell Infiltrate and Inflammatory Milieu in a Chronic Pain Model

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Abstract

IMT504 is a non-CPG, non-coding synthetic oligodeoxinucleotide (ODN) with immunomodulatory properties and a novel inhibitory role in pain transmission, exerting long-lasting analgesic effects upon multiple systemic administrations. However, its mechanisms of anti-nociceptive action are still poorly understood. In the present study in male adult rats undergoing complete Freund's adjuvant-induced hindpaw inflammation, we focused in the analysis of the immunomodulatory role of IMT504 over the cellular infiltrate, the impact on the inflammatory milieu, and the correlation with its anti-allodynic role. By means of behavioral analysis, we determined that a single subcutaneous administration of 6 mg/kg of IMT504 is sufficient to exert a 6-week-long full reversal of mechanical and cold allodynia, compromising neither acute pain perception nor locomotor activity. Importantly, we found that the anti-nociceptive effects of systemic IMT504, plus quick reductions in hindpaw edema, were associated with a modulatory action upon cellular infiltrate of B-cells, macrophages and $CD8^+$ T-cells populations. Accordingly, we observed a profound downregulation of several inflammatory leukocyte adhesion proteins, chemokines and cytokines, as well as of β-endorphin and an increase in the anti-inflammatory cytokine, interleukin-10. Altogether, we demonstrate that at least part of the anti-nociceptive actions of IMT504 relate to the modulation of the peripheral immune system at the site of injury, favoring a switch from pro- to anti-inflammatory conditions, and provide further support to its use against chronic inflammatory pain.

Keywords Complete Freund's adjuvant (CFA) · Cytokines · IMT504 · Lymphocytes · Oligonucleotide · Pain

Introduction

Pain due to peripheral inflammation naturally depends on nociceptors activation. Nociceptors respond to a variety of stimuli, including pathogen-associated molecular patterns detected after barrier interface breaching, damage-associated molecular patterns resulting from tissue injury-dependent cell destruction (e.g. ATP and protons), or cytokines, chemokines and growth factors (see Saloman et al. 2020). The activation of nociceptors, or the lowering of their activation thresholds by these different mediators, ultimately leads to sensitization and pain, mediated by the induction of changes in the activity of ion channels, including TRP and voltage-gated sodium channels (see Cook et al. 2018). Moreover, activated peripheral nociceptors also engage in neurogenic inflammation, a process where antidromic signals induce the release of a number of neurotransmitters and neuropeptides from the same sensitized nociceptors. As a result, edema and the generation of an inflammatory milieu rich in chemokines and cytokines that further attract inflammatory cells ensues, creating a vicious circle of nerve ending sensitization (see Xanthos and Sandkühler 2014). These different events highlight an intimate interaction between peripheral nociceptors and the immune system, influencing both inflammatory and neuropathic pain mechanisms (see Cook et al. 2018).

Improved treatment options for patients with ongoing inflammatory pain are urgently needed. Neither opioids nor

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non-steroidal anti-inflammatory drugs appear to provide sustained and effective pain-relief in chronic non-cancer pain conditions such as inflammatory pain (Woolf 2020). Interestingly, drugs targeting the cyclooxygenase 2 or the nerve growth factor (NGF), both involved in neuroimmune interactions, are proving to be clinically effective (Woolf 2020). Experimental targeting of the nuclear factor kappa B (NF-κB) signaling cascade, closely associated with cytokine signaling (Gonçalves dos Santos et al. 2020), has also become of interest. Thus, a decoy double-stranded oligodeoxynucleotide (ds-ODNs) targeting NF-κB was shown to reduce pro-inflammatory mediators in the serum of mice with hindpaw inflammation, plus improving the clinical arthritis and joint rehabilitation indexes (Wang et al. 2020).

Immunomodulatory oligodeoxynucleotides (ODNs) are synthetic molecules that stimulate cells of the vertebrate immune system, causing activation, proliferation and immunoglobulin secretion. Two classes of immunomodulatory ODNs have been identified according to their CpG content: CpG ODNs (Krieg et al. 1995) and non-CpG ODNs (Elias et al. 2003). Among the latter, we reported a new family of ODNs named PyNTTTTGT after its active motif (where Py: Pyrimidine; N: Adenine, Cytosine, Thymidine or Guanine; T: Thymidine; G: Guanine). CpG ODNs and PyNTTTTGT ODNs have a number of characteristics in common, but also remarkable differences. ODNs of both classes target B-cells and/or plasmacytoid dendritic cells (PDCs). The hallmark of CpG ODNs is their capability to stimulate secretion of IFN-α by PDCs interacting with the Toll-like receptor 9 (TLR9) (Krug et al. 2001; Bauer et al. 2001) which engages the MyD88 adaptor molecule and activates NF-kB and IRF7 signaling pathways (Kawai and Akira 2011). On the other hand, the hallmark of the PyNTTTTGT class is the induction of granulocyte macrophage colony stimulating factor (GM-CSF) by NK and natural killer T (NKT) cells in collaboration with IL-2 (Rodriguez et al. 2015) and stimulation of mesenchymal stem cells (MSCs) (Hernando-Insúa et al. 2007), characteristics that are absent or poorly expressed in CpG ODNs. Interestingly, IFN-α inhibits the PyNTTTTGT ODNs-dependent secretion of GM-CSF, and conversely, PyNTTTTGT ODNs inhibit the TLR9-dependent secretion of IFN-α by CpG-stimulated PDCs (Rodriguez et al. 2015). The mutual interference between these major classes of ODNs suggests that they may stimulate different and incompatible immune response pathways (Zhao et al. 2009).

Importantly, we have shown that the prototype of PyNTTTTGT-ODNs, IMT504, exerts powerful anti-nociceptive effects after multiple systemic administrations in rats with sciatic nerve crush (Coronel et al. 2008) or unilateral hindpaw inflammation (Leiguarda et al. 2018). Moreover, the effect is present under early (at the time of injury) or late (several days after injury) treatment protocols, resulting in quick and long-lasting reductions in mechanical allodynia (Coronel et al. 2008; Leiguarda et al. 2018). The anti-nociceptive mechanisms of IMT504, however, remain unknown.

In the present study, our aim was to expose if local modulation of the immune system and the inflammatory milieu are potential mechanisms responsible for the anti-allodynic effects of IMT504 in rats with hindpaw inflammation.

Materials and Methods

Animals

A total of 193, six-week old, male Sprague Sprague–Dawley rats (200–300 g, BioFucal, Buenos Aires, Argentina) were used in this study. Animals were kept in a 12 h light-cycle (lights on, 7:00 h; lights off, 19:00 h), with water and food ad libitum. Experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the Instituto de Investigaciones en Medicina Traslacional (IIMT; #17–02) and were carried out according to the Guide for the Care and Use of Laboratory Animals (NIH Publication 86–23).

Complete Freund's Adjuvant Model

Under aseptic conditions, rats ($n = 121$) were anaesthetized with Isoflurane (5% induction, 2.5% maintenance, 0.8 L/min O_2 flow rate; Piramal Healthcare, Morpeth, UK), and received an intraplantar injection of 100 μl of CFA (1:1, dissolved in normal saline; Sigma-Aldrich, MO, USA), using a 1 ml syringe with a 25G needle attached. Rats recovered from anesthesia in a warm and quiet environment before relocating into their home cages. Behavior of animals was evaluated at various time-points.

Experimental Drug

In all experiments, the ODN IMT504, with sequence 5′-TCATCATTTTGTCATTTTGTCATT-3′, was used. The HPLC-grade phosphorothioate (PS) ODN (Biosynthesis Inc., Lewisville, Texas, USA) was suspended in sterile saline (0.9% NaCl; 20 mg/ml; storage concentration) and assayed for LPS contamination. A control ODN consisting of 24-mer poly C ODN was used in the preliminary studies (not shown). Sequence specificity of ODN IMT504 was extensively described by Elias et al. (2003) and Rodriguez et al. (2006).

Experimental Design

Rats were randomly assigned to a number of experimental groups, including: 1) CFA-injured rats (CFA; $n = 3$); 2) CFA-injured rats receiving IMT504 (CFA-IMT504; $n = 76$) or vehicle (CFA-VEH; $n = 54$); 3) uninjured rats receiving

IMT504 (IMT504; $n = 26$) or vehicle (VEH; $n = 15$); and 4) uninjured and untreated rats (naïve (N); $n = 19$).

All treatment protocols (2, 6 or 10 mg/kg IMT504, single s.c. dose) were evaluated 7 d after CFA-induced injury. First, mechanical allodynia testing was exercised using all treatment protocols (n = 5–9/group) in order to determine the optimal dose. Subsequent pain-like behavior ($n = 4$–9/group), histological (n = 3/group), plasma extravasation (n = 7/group), cellular (n = 4/group) and molecular (n = 3–5/group) tests practiced at various survival times, were performed after administration of 6 mg/kg IMT504, identified as the optimal dose. Impact of ODN administration on locomotion was assessed 24 and 72 h after administration of 1 or 6 mg/kg IMT504 in naïve rats ($n = 11$/group).

Behavioral Testing

Behavioral assessment was performed during daytime (between 9:00 h and 18:00 h) in all animals before any intervention (basal responses) and at different time-points after injury and/or IMT504 or vehicle administration. Behavioral measurements were carried out by an investigator blinded to drug administration.

Mechanical Allodynia

After 15 min habituation inside transparent plastic domes on a metal mesh floor, a series of von Frey filaments (1.4, 2, 4, 6, 8, 10, 15 y 26 g; Stoelting, Inc., Wooddale, IL, USA) were applied to the medial aspect of the plantar surface of the ipsi- and contralateral hindpaws of naïve, CFA-VEH, CFA-IMT504 rats, starting with the 6 g filament. Mechanical withdrawal thresholds were calculated following the modified up-down method of Dixon, to establish the 50% withdrawal threshold (Chaplan et al. 1994).

Cold Allodynia

After acclimatization in individual cubicles (as above) for 15 min, a drop of acetone was gently brought in contact with the plantar surface of the ipsi- and contralateral hindpaws of CFA-VEH and CFA-IMT504 rats, according to standard methods (Choi et al. 1994). Applications were made 5 times every 5 min. Foot withdrawal was scored as positive (1); lack of withdrawal as negative (0). The cold withdrawal frequency was obtained from the average of the 5 stimulations applied at each time-point.

Acute Thermal Nociception

Acute thermal nociception was addressed 21 d after s.c. administration of VEH or IMT504 (6 mg/kg) in rats undergoing a seven-day-long CFA hindpaw inflammation, by means of the previously described tail immersion test (Zhou et al. 2014). The latency of tail-flick reflex to swift immersion of the last 3 cm of the tip of the tail of each rat on a hot bath (52 °C) was measured using a stopwatch (resolution of 0.01 s). This was repeated 3 times, with 10 s intervals, and an average response obtained.

Formalin Test

Following three-day, consecutive, 1 h acclimation periods inside transparent plastic domes on a glass floor (plus administration of s.c. VEH or IMT504 (6 mg/kg) on the first day of acclimation), rats received an intraplantar injection of a formalin solution (50 μl, 5%; Sigma) in the right hindpaw. Rats and individual timers (resolution 0.01 s) were recorded for 1 h, at the same time, using a webcam attached to a computer. The total number of hindpaw flinches/5 min bins during 1 h was quantified off-line (Bannon and Malmberg 2007).

Open-Field Test

The open-field test (OFT) consisted of custom—made acrylic open field boxes (High×Width×Long: 30 × 40 × 80 cm) Naïve rats were evaluated twice, 24 h and 72 h after a single dose of s.c IMT504 (1 or 6 mg/kg) or vehicle administration. Locomotor activity was recorded and analyzed using an automated system (Ethovision XT 11.0, Noldus, The Netherlands). Total distance traveled (in cm) was quantified in 5 min time bins for a total of 30 min. Animals were not habituated to the open-field previously to the first locomotion test assay.

Hindpaw Dorsoventral Thickness

Hindpaw dorsoventral thickness was measured in awake N, CFA-VEH, CFA-IMT504 rats at various time-points, and as previously described (Nwidu et al. 2016). Briefly, once the animals were calm and allowed the positioning of a caliper touching both the ventral and dorsal surfaces of the hindpaw, the dorsoventral thickness was measured. Care was taken not to compress the hindpaw, avoiding skin deformation during measurement.

Histological Analysis

CFA-injured rats receiving vehicle (CFA-VEH) or IMT504 (6 mg/kg, CFA-IMT504) were sacrificed with CO_2 and decapitated at different experimental points: 7, 10, and 49 days (3 and 42 days after treatment), and naïve rats were also included. Contra- and ipsilateral hindpaws were dissected out, kept in 10% paraformaldehyde (Sigma) for 3 d, embedded in paraffin wax (Sigma) and transversally sectioned (5 μm) using a microtome (Shandon AS325 Retraction, Marshall Scientific,

Hampton, NH, USA). Tissue sections were mounted onto glass slides and stained with standard hematoxylin-eosin for light microscopy analysis using a Nikon Eclipse E-800 photomicroscope (Nikon, Tokyo, Japan). The public domain open source image processing package based on the NIH program ImageJ, FIJI (Schindelin et al. 2012), was used to measure dermal thickness by selecting the width of the dermal layer in 10x microphotographs. Contralateral sides remained unaltered, comparable to naïve hindpaws (data not shown).

Evans Blue Extravasation

Plasma extravasation was evaluated in ipsi- and contralateral hindpaws of CFA-VEH and CFA-IMT504 (6 mg/kg) rats, 21 d after treatment. Under Isoflurane anesthesia (as above), rats were injected through the tail vein with a 50 mg/ml solution of Evans Blue at a dose of 1 μl/g. After 30 min, animals were deeply sedated (as above), quickly decapitated, and their hindpaws removed by cutting at the ankle joint. Tissue was incubated in formamide (Sigma) for 48 h and the extravasated dye was filtered and measured at 620 nm (NanoDrop One, ThermoFisher, Madison, WI, USA). The degree of plasma extravasation was expressed as difference of μg of dye/ml between contra- and ipsilateral hindpaws, and controlled against paw wet/dry weight ratio (Ercan et al. 2013). Contralateral sides remained unaltered (data not shown).

Flow Cytometry

Analysis of immune cell infiltration/recruitment in the right hindpaw of naïve rats, and the ipsilateral hindpaw of CFA-VEH and CFA-IMT504-treated (6 mg/kg) animals was carried out as previously described (Chiu et al. 2013). Rats were deeply sedated with Isoflurane (as above), quickly decapitated (1 or 21 d after treatment) and paw tissue dissected, minced and digested in a mixture of 1 mg/ml collagenase A and 2.4 U/ml Dispase II (Roche Applied Sciences, Madison, WI, USA) in Hepes-buffered saline solution (Sigma) for 90 min. After digestion, tissue were triturated by pipette, washed with Hank's balanced salt solution (HBSS; Corning, Life Sciences, Corning, NY, USA) 0.5% BSA (Sigma), and filtered through a 70-μm mesh. Cells were fixated in paraformaldehyde (PFA, Sigma) 4%, twice washed with 1 ml of FACS (1% BSA and 0.05% Sodium Azide (NaN3) and stored in 1x PBS (phosphate-buffered saline) for future incubation with antibodies. Cells were divided into 3 panels and stained with monoclonal antibodies (mAb) for 45 min in FACS buffer (all from BioLegend, San Diego, CA, USA, unless noted): anti–CD45RA-PE (0.25 μg/100 μl), anti–CD8a-PE (0.25 μg/100 μl), anti–CD3-AF647 (0.06 μg/100 μl), anti–CD45-AF488 (0.25 μg/100 μl), anti–CD172a-PE (0.25 μg/100 μl), anti-CD4-PE/Cy7 (0.25 μg/100 μl), anti-CD11b/c-PE/Cy7 (0.25 μg/100 μl), anti-HIS36-PE (0.5 μg/100 μl; BD Biosciences, Franklin Lakes, NJ, USA). Panels were designed as follow: Monocytes and macrophages (anti-CD45, anti-CD11b/c, anti-CD172a, anti-HIS36, anti-CD3 and anti-CD4), T-cells (anti-CD3 and anti-CD4 and anti-CD3 and anti-CD4CD8a) and B-cells (anti-CD45 and anti-CD45RA). After incubation, samples were fixated in PFA 4%, washed and stored in FACS. Flow cytometry was conducted on a FACSAria II flow cytometer (Becton Dickinson Biosciences, Franklin Lakes, NJ, USA) and analyzed by FlowJo software v10 (LLC, Ashland, OR, USA). Lymphoid and myeloid population analyses were presented as percentage of absolute values (total cells); subpopulation analyses are represented by absolute values (total cells). All samples were first gated on a forward scatter (FS)/side scatter (SS) plot and selected accordingly to exclude non-viable cells and debris.

Cytokines and Chemokines Analysis by Proteome Profiler Rat Cytokine Array

One and twenty-one days after treatment, CFA-VEH and CFA-IMT504 (6 mg/kg) rats were anesthetized (as above), quickly decapitated, and their ipsilateral hindpaw tissue freshly excised and homogenized in a solution of PBS1X with protease inhibitors (Sigma). After homogenization, samples were centrifuged (14,000 g/5 min), and the supernatant was used to evaluate the expression of proinflammatory secreted proteins. Duplicate levels of each secreted protein were determined using the Proteome profiler Rat Cytokine Array according to manufacturer's instructions (ARY008, R&D Systems, Minneapolis, MN, USA). The average signal (pixel density) of the pair of duplicate spots (mean) representing each cytokine was relativized to the positive control defined by the array for each cytokine, using the negative controls to subtract the averaged background. The quantification was performed using FIJI software (version 2.0.0).

ELISA Analysis of ß-Endorphin Expression

One and twenty-one days after treatment, CFA-VEH and CFA-IMT504 (6 mg/kg) rats were anesthetized (as above) and quickly decapitated. Naïve rats were also included. Briefly, ipsilateral hindpaw samples obtained as previously described (Fehrenbacher et al. 2012) were separately homogenized in 1 ml Lysis Buffer (ThermoFisher, Waltham, MA, USA), complemented with 10 μl protease cocktail inhibitor (Santa Cruz, Dallas, TX, USA), later sonicated (3 pulses of 10 s during 1 min) and centrifuged (14,000 rpm/30 min/4 °C) for further protein quantification. ß-endorphin levels were quantified following the manufacturer's instructions (EK-022-33, Phoenix Pharmaceuticals, Burlingame, CA, USA). After detection of ß-endorphin levels at O.D 450 nm, these were referred to the protein content in each sample. Therefore,

β-endorphin levels in this study are expressed as ng of peptide per mg of total protein.

Statistical Analysis

All data was expressed as mean ± S.E.M and evaluated using GraphPad Prism 7.0a (GraphPad Company, San Diego, CA, USA). Whenever appropriate, area under the curve (AUC) analysis was performed, including the entire data-set. Previous to statistical analysis, all data underwent standard normality test. Behavioral experiments were analyzed by Repeated-Measures ANOVA followed by corresponding post-hoc tests (Tukey's for mechanical allodynia and locomotion, Sidak's for cold allodynia and formalin test) or T-test (acute heat responses). Histological and plasma extravasation tests were analyzed by Multiple t-test and t-test, respectively. Flow cytometry and ELISA tests were analyzed by One-way ANOVA, followed by Tukey's post-hoc test. Cytokine array panel assay was analyzed using t-test. Hedges' g test was used for size-effect calculations and all AUC analyses were performed including the entire experimental data set. Alpha-value was set at $P \leq 0.05$. *P*-values were presented as follows: $^{*}P < 0.05$; $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

Results

A Single s.c. IMT504 Administration Suppresses Pain-like Behavior in a Dose-Dependent Manner

We began our analysis by determining the optimal single s.c. dose at which IMT504 exerted robust anti-allodynic effects, that could later be chosen for subsequent behavioral and prospective mechanistic effects of the ODN in this study. We observed that all CFA-injured rats quickly developed mechanical allodynia, with onset 12 h after injury, and lasting for several weeks in those treated with VEH (Fig. 1a). In contrast, CFA-injured rats receiving IMT504, 7 d after injury, exhibited dose-dependent effects on pain-like behavior (Fig. 1a). Administration of 6 or 10 mg/kg s.c. IMT504 resulted in a fast return to basal mechanical withdrawal thresholds, starting 24 h after administration and exhibiting full recovery from 3 d after treatment initiation and onwards. On the other hand, CFA rats receiving 2 mg/kg IMT504 reached stable basal withdrawal thresholds 14 d after administration of the ODN. Comparison between groups revealed significant statistical differences ($P < 0.0001$, $F(60,544) = 15.73$) (Fig. 1a), as represented in area under the curve (AUC) analysis (Fig. 1b; $P < 0.0001$, $F(3,28) = 22.40$). No changes were registered in contralateral basal mechanical withdrawal thresholds (data not shown). The lack of major differences between 6 and 10 mg/kg doses supported the use of the 6 mg/kg s.c. dose of IMT504 as the optimal dose, used throughout the rest of this study.

Response analysis to cold stimuli showed that, while all injured rats developed cold allodynia during the first 7 d after injury, treatment with IMT504 quickly reduced and blocked this behavior (Fig. 1c), starting 24 h after administration ($P < 0.0001$, $F(9,90) = 7.076$), as represented in the AUC analysis ($P = 0.0185$, $t = 2.80$, $df = 10$; Fig. 1d). CFA-VEH rats remained allodynic for a few more days than CFA-IMT504 rats, only reaching basal withdrawal levels 14 d after injury and onwards. No changes were registered in contralateral basal responses to cold stimuli (data not shown).

Finally, we also followed a separate group of rats during 63 d after treatment, to determine the time-point at which the anti-allodynic effects of IMT504 begin to fade in injured rats (6 mg/kg). We found that, after the initial 42 d of sustained blockade of mechanical allodynia, CFA-IMT504 rats show a progressive return to responses comparable to CFA-VEH rats ($P < 0.0001$, $F(16,160) = 10.94$; Supplementary Fig. 1).

Altogether, these results allowed determination of 6 mg/kg as the optimal dose of IMT504, and demonstrate that a single systemic administration of the ODN is able to modulate both mechanical and cold allodynia in rats with inflammatory pain, and that the effects are long-lasting.

IMT504 Does Not Alter Acute Nociception or Locomotor Activity

To address if the anti-allodynic effects of IMT504 relate to the modulation of regular nocifensive responses, we performed both the tail immersion and the formalin tests. On the one hand, 21 d after administration of IMT504, CFA-injured rats showed no significant alteration in tail withdrawal latency (Fig. 1e). On the other hand, performing the formalin test in naïve rats pre-treated with IMT504, 3 d before the test, showed unaltered responses in the number of ipsilateral hindpaw flinches, as compared to rats receiving VEH (Fig. 1f).

In order to evaluate if IMT504 alters locomotion, we analyzed locomotor activity in naïve rats receiving a single dose of VEH, 1- (sub-therapeutic dose) or 6 mg/kg IMT504. No significant differences in locomotion were detected 24 or 72 h after administration among the different treatment groups (Fig. 1g) ($P = 0.9212$, $F(2,20) = 0.082$). In contrast, all treatment groups showed a significant reduction ($P < 0.0001$, $F(1,10) = 45.20$) in total distance travelled at 72 h post-IMT504 injection (compared to the locomotor values observed at 24 h post injection), suggestive of some degree of habituation to the open-field as consequence of repetitive exposure. This is an expected outcome, since habituation of locomotor activity across repeated exposures (i.e. decreased exploration when exposed to the same stimuli) is a known phenomenon that has been well characterized in rodents (Leussis and Bolivar 2006).

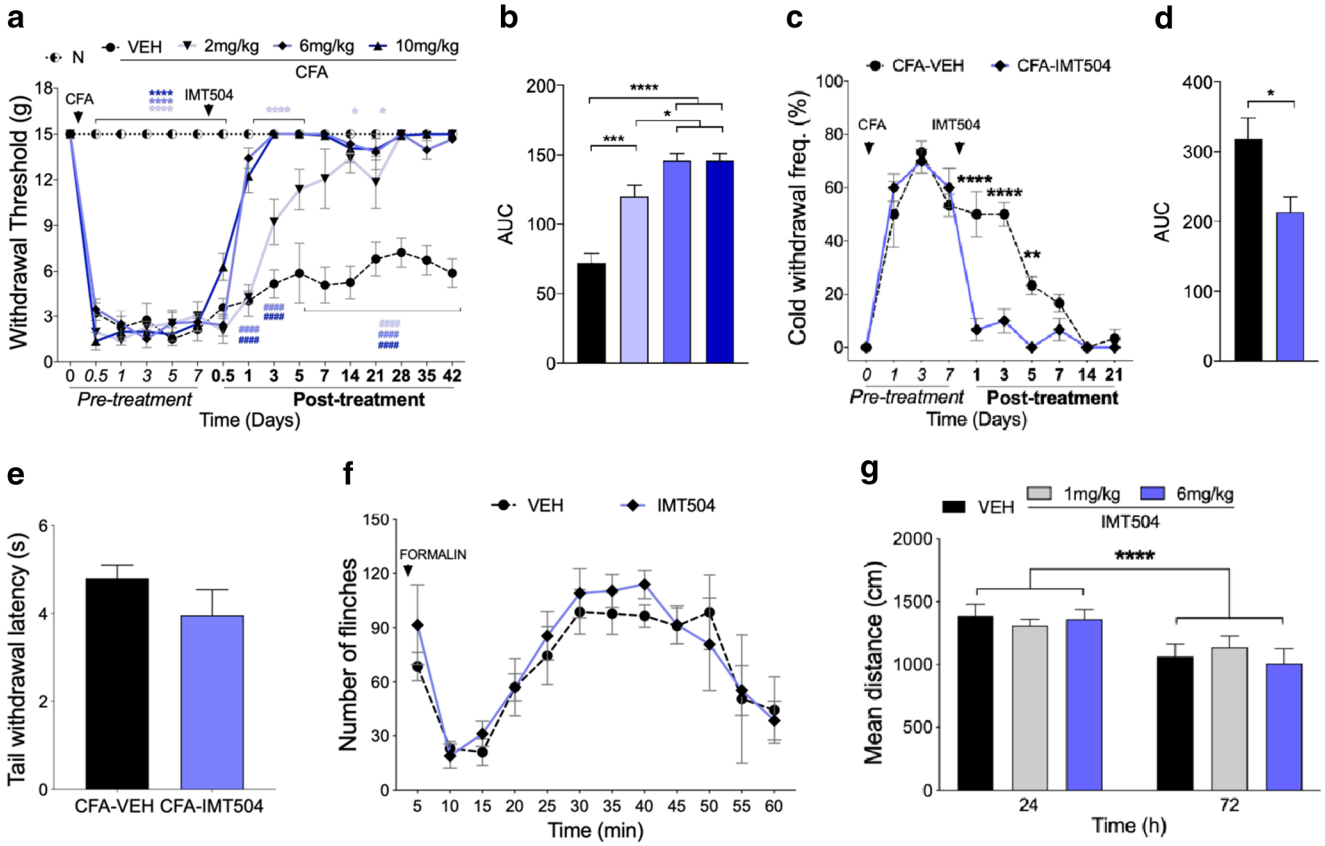


Fig. 1 Single s.c. IMT504 administration results in profound pain-like behavior modulation, while maintaining basal responses to acute pain and locomotor activity. **a** Mechanical allodynia was reverted in all rats receiving IMT504. Significant differences are shown between naïve ($n = 5$) and CFA-IMT504 rats (*; $n = 9$), and between CFA-VEH (n = 5) and CFA-IMT504 treated rats (#). **b** AUC analysis of results in (**a**) (Hedges'g: CFA-VEH vs. CFA-IMT504 (6 or 10 mg/kg) = 4.69, CFA-VEH vs. CFA-IMT504 (2 mg/kg) = 2.22; CFA-IMT504 (2 mg/kg) vs. CFA-IMT504 (6 or 10 mg/kg) = 1.299). **c** Cold allodynia was strongly reduced from 24 h and onwards in CFA-IMT504 rats (6 mg/kg), as compared to CFA-VEH rats ($n = 6$). **d** AUC of results in (**c**) (Hedges'g: CFA-VEH vs. CFA-IMT504 (6 mg/kg) = 1.62). **e** CFA-IMT504 rats did not show changes in responses to acute heat, as compared to CFA-VEH rats, 21 d after treatment ($n = 5$). **f** No significant differences were observed in the responses to intraplantar formalin between naïve rats receiving IMT504 or VEH ($n = 4$). **g** No significant differences were detected in locomotor activity when comparing VEH- or IMT504-treated (1 or 6 mg/kg) rats, either 24 or 72 h after IMT504 administration ($n = 11$). However, a significant reduction in locomotor activity was detected between time-points in all groups, suggestive of typical habituation to the open-field arenas

These results confirm that a single systemic administration of IMT504 does not compromise regular nocifensive responses to acute heat or chemical challenges, neither basal patterns of locomotor activity.

IMT504 Reduces CFA-Induced Hindpaw Inflammation and Edema

In order to determine if the anti-allodynic effects of IMT504 relate to local modulation of hindpaw inflammatory responses, we first analyzed hindpaw dorsoventral thickness, plasma extravasation and potential alterations in the histology of the hindpaw. All CFA-injured rats exhibited a considerable increase in ipsilateral hindpaw dorsoventral thickness, showing significant differences with naïve rats, already 12 h after injury (Fig. 2a). However, while CFA-VEH rats maintained increased dorsoventral thickness throughout the 49 tested days, rats receiving IMT504 (6 mg/kg) showed a reduction that became apparent 3 d after treatment ($P < 0.0001$, F(30,210) = 38.54 (Fig. 2a); this effect was also reflected by the AUC analysis ($P < 0.0001$, F(2,16) = 308.7) (Fig. 2b). No changes were registered in basal contralateral hindpaw thickness (data not shown).

Histological analysis in CFA-VEH rats revealed an increased dermal thickness and an abundant cellular infiltrate in the dermis and hypodermis, as observed 3 and 42 d after administration of VEH (Fig. 2c, d). In contrast, while CFA-IMT504 rats also showed increased dermal thickness 3 d after treatment, this was followed by a noticeable decrease, as exposed 42 days after treatment ($P = 0.0043$, t = 5.84, df = 4); this event was paralleled by a virtually complete disappearance of cellular infiltrate in the dermis and hypodermis (Fig.

2c, d). Evaluation of the contralateral hindpaw in rats receiving any treatment revealed no significant differences when compared to naïve rats (data not shown).

Analysis of vascular permeability by Evans Blue dye extravasation in the ipsilateral hindpaw, 21 d after treatment, revealed that IMT504 treatment results in significant reductions in plasma extravasation ($P = 0.0054$, $t = 3.38$, $df = 12$) (Fig. 2e), with no significant differences in paw wet/dry weight ratio ($P = 0.2173$; CFA-VEH: 4.36 ± 0.20, CFA-IMT504: 3.67 ± 0.43).

Altogether, these results support an anti-inflammatory role for a single systemic administration of IMT504, which includes progressive reductions in hindpaw edema, dermal thickness and cell infiltrate.

IMT504 Modulates the Tissue Immune Environment by Promoting T Cell Infiltration while Diminishing B Cells and Macrophages Frequencies

The demonstration of noticeable effects on hindpaw inflammation and cell infiltration prompted a more detailed analysis of the type of cells reacting to the administration of IMT504 in injured rats. Immune cellular infiltrate was characterized by flow cytometry in CFA-IMT504 rats (6 mg/kg), on days 1 and 21 d after treatment (8 and 28 d after injury) and compared with naïve and CFA-VEH rats.

Analysis 1 d after treatment revealed marked and similar increases in immune cells infiltration in both CFA-VEH and CFA-IMT504 rats, as compared to naïve rats, with predominance of myeloid cells ($CD45^+CD11b/c^+$; *1d*: $P = 0.0007$, $F(2,9) = 18.42$) over lymphoid cells ($CD45^+CD11b/c^-$; *1d*: $P < 0.0001$, $F(2,9) = 56.60$) (Fig. 3a, b). Twenty-one days after treatment, myeloid cells remained only slightly increased in both experimental groups, as compared to naïve rats (*21 d*, $P = 0.04$, $F(2,9) = 4.77$), whereas lymphoid cells frequency appeared to return to naïve-like values (*21 d*, n.s.) in both experimental groups (Fig. 3a, b). Subpopulations of myeloid and lymphoid lineages were subsequently analyzed using cell-specific markers (Gating strategies for all populations and subpopulations are shown in Supplementary Fig. 2).

When analysing the absolute number of monocytes ($CD45^+CD11b/c^+CD172a^+$) in CFA-VEH- and CFA-IMT504 rats, no differences were observed at neither time-point, although both experimental groups exhibited significantly higher values than naïve animals (*1 d*, $P < 0.0001$, $F(2,9) = 55.23$; *21 d*, $P = 0.045$, $F(2,5) = 6.12$) (Fig. 3a1). Analysis of macrophages ($CD4^+CD3\text{-}His36^+$) revealed significantly higher counts in both experimental groups and at both time-points evaluated, as compared to naïve rats (*1 d*, $P < 0.0001$, $F(2,9) = 48.85$; *21 d*, $P = 0.0008$, $F(2,7) = 23.77$); moreover, this population was the only one to exhibit a remarkable increase in absolute numbers when comparing between time-points (Fig. 3a2). However, CFA- IMT504 rats exhibited clearly lesser macrophage counts than CFA-VEH rats, as observed 21 d after treatment (Fig. 3a2).

As shown for macrophages, analysis of B cells ($CD45^+/CD45RA^+$) revealed a similar downregulating effect of IMT504. Thus, while B cells were found upregulated in both CFA-VEH and CFA-IMT504 rats, the latter group always exhibited lower cell counts (1d: $P = 0.0001$, $F(2,6) = 55.90$; 21d: $P = 0.003$, $F(2,7) = 14.86$) (Fig. 3b1). Accordingly, B cell counts in IMT504-treated rats were comparable to N rats 21 d after treatment (Fig. 3b1).

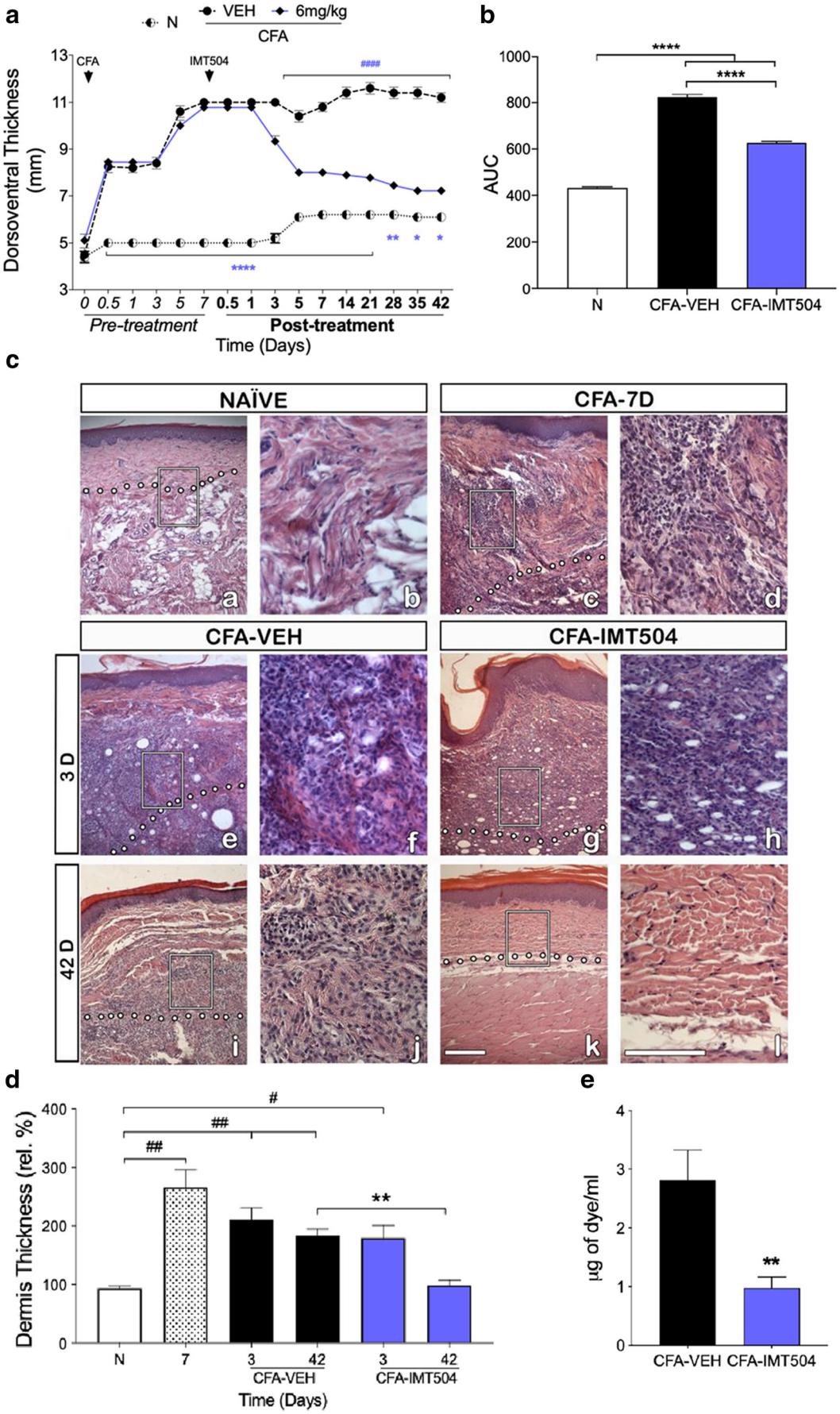
Finally, analysis of T cells revealed an upregulating effect of IMT504. Thus, while the counts of $CD4^+$ ($CD45^+/CD3^+/CD4^+$) and $CD8^+$ ($CD45^+/CD3^+/CD8^+$) T cells were strongly induced by injury in both experimental groups and at both time-points ($CD4^+$ (*1 d*, $P = 0.0003$, $F(2,9) = 21.99$; *21 d*, $P = 0.0047$, $F(2,8) = 11.30$); $CD8^+$ (*1 d*, $P < 0.0001$, $F(2,8) = 55.53$; *21 d*, $P = 0.0001$, $F(2,5) = 88.04$)) (Fig. 3b2, b3), IMT504-treated rats showed a significantly larger increase in $CD8^+$ T cell than the one observed in VEH-treated rats (Fig. 3b3). $CD4^+$ T cells also exhibited a tendency towards larger counts in CFA-IMT504 rats, although values did not reach statistical significance and the effect appeared more evident 1 day after treatment (Fig. 3b2).

Altogether, these results emphasize the possibility that a single administration of IMT504 is capable of modulating inflammatory pain by acting upon macrophage, B- and $CD8^+$ T cell populations. Moreover, the identification of effects over macrophages and $CD8^+$ T cells suggests newly found potential cell targets of IMT504.

IMT504 Modulates a Large Number of Cytokines and Chemokines, Favoring the Decrease of pro-Inflammatory Mediators, and Limiting the Upregulation of Endogenous β-Endorphin

Because the effects of IMT504 on cell infiltrate could also be associated with changes in the inflammatory milieu at the site of injury, we evaluated the expression of a number of cytokines 1 and 21 d after treatment, comparing CFA-VEH and CFA-IMT504 rats. One day after treatment (8 d after injury), significantly increased levels of soluble intercellular adhesion molecule 1 (sICAM), epithelial-derived neutrophil activating peptide (CXCL5), platelet-derived growth factor (CXCL7), L-selectin (CD62L), RANTES (CCL5), tissue inhibitor of metalloproteinase (TIMP-1) were observed in VEH-treated rats, as compared to IMT504-treated rats (sICAM ($P < 0.0001$); CXCL5 ($P < 0.0001$); CXCL7 ($P < 0.0001$); CD62L ($P = 0.034$); CCL5 ($P = 0.0006$); TIMP ($P < 0.0001$)). Interleukin-1 receptor antagonist (IL-1RA) levels appeared increased in both experimental groups at this time-point (Fig. 4a).

Analysis 21 d after treatment revealed a similar downregulating effect of IMT504, as compared with CFA-VEH rats,


a
N
VEH
6mg/kg
CFA
CFA
IMT504
####
Dorsoventral Thickness (mm)
13
11
9
7
5
3
**
*
*
****
0 0.5 1 3 5 7 0.5 1 3 5 7 14 21 28 35 42
Pre-treatment
Post-treatment
Time (Days)
b
****
****
AUC
1000
800
600
400
200
0
N
CFA-VEH
CFA-IMT504
c
NAÏVE
CFA-7D
CFA-VEH
CFA-IMT504
3 D
42 D
a
b
c
d
e
f
g
h
i
j
k
l
d
#
##
##
**
Dermis Thickness (rel. %)
400
300
200
100
0
N
7
3
42
3
42
CFA-VEH
CFA-IMT504
Time (Days)
e
µg of dye/ml
4
3
2
1
0
**
CFA-VEH
CFA-IMT504

◀ **Fig. 2** Single s.c. IMT504 administration reduces hindpaw edema and cellular infiltration. **a** Hindpaw dorsoventral thickness is considerably reduced in CFA- IMT504 rats (6 mg/kg, n = 9), as compared to CFA-VEH (n = 5) and naïve (N) groups (n = 5). **b** AUC analysis of results in (**a**) (Hedges'g: CFA-VEH vs $N = 20.31$, CFA-VEH vs CFA-IMT504 = 7.11; N vs CFA-IMT504 = 7.70). **c** Histological analysis of the hindpaw skin, 3 d after administration of the ODN, reveals that CFA-IMT504 rats (**c**g, h) exhibit the same degree of dermal thickness and cell infiltration as observed in CFA-VEH rats (**c**e, f) or CFA rats with a 7-d long inflammation (**c**c, d). Forty-two days after administration, CFA-IMT504 rats (**c**k, l) show disappearance of cellular infiltrate, and normal dermal thickness, both still compromised in CFA-VEH rats (**c**i, j; dotted lines show the approximate limit between dermis and hypodermis). Scale bars: 100 μm (**c**k = **c**a, c, e, g, i, k; 50 μm, **c**l = **c**b, d, f, h, j). **d** Dermal thickness analysis showed near basal values 42 d after IMT504 treatment ($n = 3$) (Hedges' g: CFA-VEH vs. CFA-IMT504 at 42 d after treatment = 4.76). **e** Evans blue dye extravasation was reduced in CFA-IMT504 rats (6 mg/kg), as compared with CFA-VEH rats ($n = 7$; Hedges'g:1.80)

both on the same cytokines already evaluated 1 d after injury, as well as on macrophage inflammatory protein-1 α (CCL3), macrophage inflammatory protein-3 (CCL20), IL1-RA, interleukin-1 α and β (IL-1α, IL-1β) and ciliary neurotrophic factor (CNTF) (sICAM ($P < 0.0001$); CXCL5 ($P < 0.0001$); CXCL7 ($P < 0.0001$); CD62L ($P < 0.0001$); CCL3 ($P < 0.0001$); CCL20 ($P = 0.023$); CCL5 ($P < 0.0001$); IL1-RA ($P = 0.025$); IL-1α ($P < 0.0001$); IL-1β ($P = 0.005$); TIMP ($P < 0.0001$); CNTF ($P < 0.0001$)) (Fig. 4b). In contrast, while VEH-treated rats lacked detectable levels of IL-10, IMT504-treated rats exhibited upregulated expression for this cytokine ($P = 0.046$) (Fig. 4b).

Finally, we also evaluated presence of endogenous β-endorphin in the hindpaw skin of CFA-VEH and CFA-IMT504, as it is known that its expression is locally upregulated in rats with hindpaw inflammation (See Discussion below). Analysis 1 d after treatment revealed a tendency towards increase; however, no significant differences were detected between groups ($P = 0.42$, $F(2,6) = 1.02$) (Fig. 5). In contrast, 21 d after treatment, CFA-IMT504 rats exhibited a significant reduction in the hindpaw expression levels of β-endorphin, as compared to CFA-VEH and reaching values comparable with naïve rats ($P = 0.004$, $F(2,6) = 15.97$) (Fig. 5).

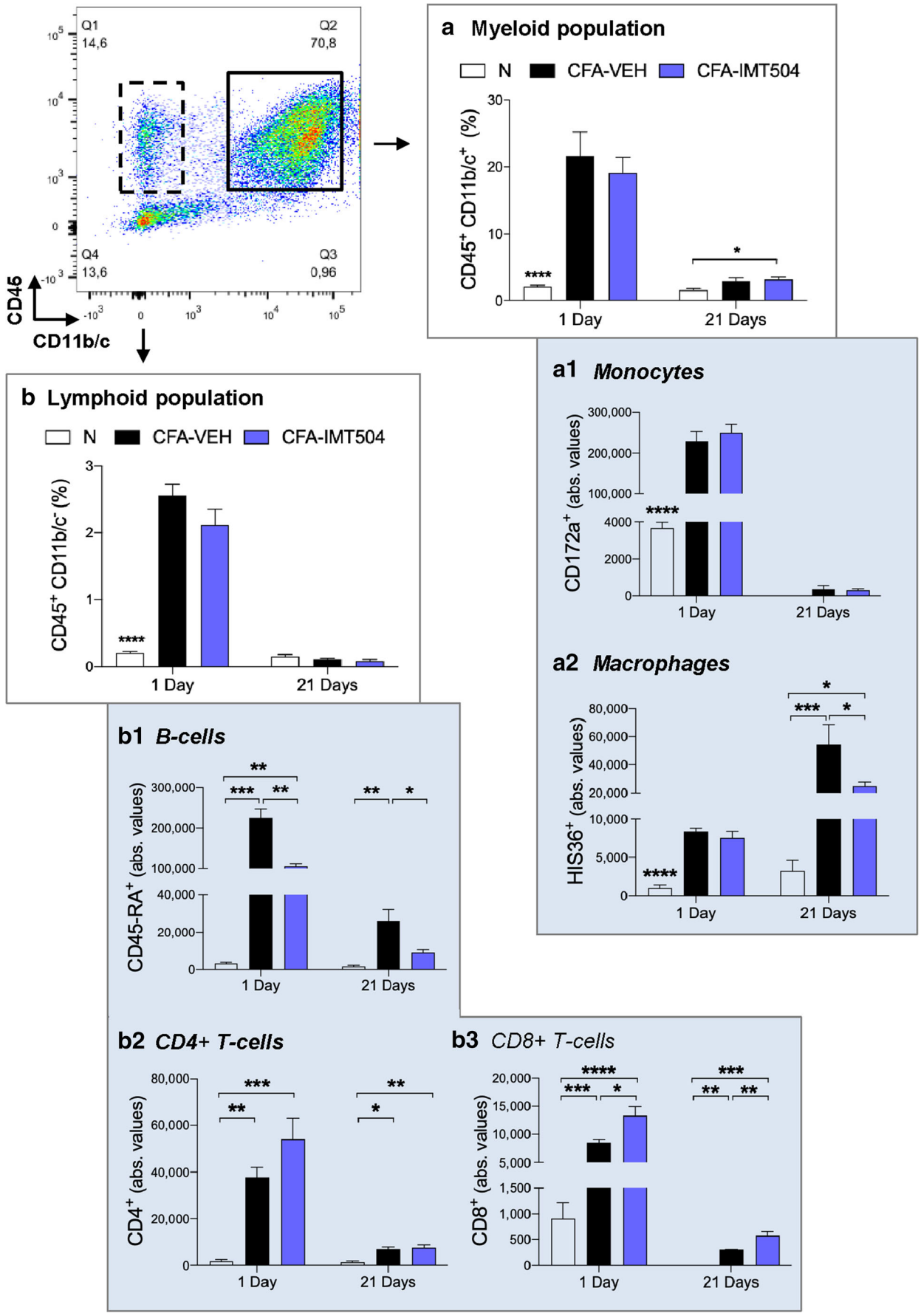
These results suggest that the anti-nociceptive and anti-inflammatory effects of systemic IMT504 relate to modulation of the inflammatory milieu, including downregulation in pro-inflammatory cytokines and chemokines. The decreases in endogenous peripheral β-endorphin levels observed in IMT504-treated rats may be related to the overall downregulating effects of the ODN in local cell infiltration (see below).

Discussion

The physiopathology of inflammatory pain is heavily influenced by the simultaneous action of several immune-derived ligands upon nociceptors. This explains the limited efficacy of currently available mono-target therapies, and stresses the need for a multi-target therapeutic approach to inflammatory pain (Jain et al. 2020). Here we show that even a single dose of IMT504, a non-coding, non-CPG ODN, fully reverts mechanical and cold allodynia for a prolonged period (up to 6 weeks after treatment) after CFA-induced hindpaw inflammation, in association with long-term local resolution of cellular infiltrate and edema. Interestingly, we reveal for the first time that these effects depend on the modulation of infiltrating myeloid and lymphoid cells, and of the expression levels of leukocyte adhesion molecules, pro- and anti-inflammatory cytokines, chemokines, and β-endorphin. Importantly, the efficacious blockade of pathological pain seems not to alter acute pain perception, as shown by normal responses to heat and formalin. In addition, our results showed no evidence of sedative effects (i.e. decreased locomotion/exploratory activity) after an acute IMT504 administration, as tested 24 and 72 hs post-injection.

The long-lasting and robust inflammatory reaction induced by intraplantar CFA (Wiedemann et al. 1991) primarily relies on the activation of the innate immune system and, to a lesser extent, also to the adaptive immune system (Rittner et al. 2001; Bas et al. 2016). In fact, in rat (Rittner et al. 2001) and mouse (Ghasemlou et al. 2015), circulating myeloid populations quickly infiltrate CFA-inflamed hindpaws, followed by more discrete and later (7 or more days) contributions of infiltrating lymphoid populations. Our results in CFA-VEH rats confirm these observations and also show that the global proportions of myeloid and lymphoid cells return to near-basal levels, 28 days after injury, despite continued CFA-insult, hindpaw edema and mechanical hypersensitivity. In contrast, while IMT504 administration did not appear to modify the upregulated global counts of myeloid and lymphoid cell populations 3 and 21 days after treatment, analysis of specific cell subpopulations revealed a more selective modulation of local immune infiltration by the ODN. In particular, B cells and macrophages exhibited lower frequencies, while $CD8^+$ T cells showed a moderate, yet significant, increase in IMT504-treated groups. Interestingly, full disappearance of infiltrating cells was evident 42 days after treatment only in IMT504-treated rats, as revealed by histological analysis. These results suggest that IMT504 can modulate cells from both the innate and adaptive immune response, ultimately interrupting cell infiltration.

The modulating role of IMT504 over the innate and adaptive immune response was also associated with considerable reductions in the protein levels of leukocyte adhesion molecules and several chemokines. Interestingly, these different mediators are increasingly addressed as relevant players in the mechanisms of chronic pain (Jiang et al. 2020). Thus, IMT504 induced profound downregulation in the expression of sICAM1, typically present in the cell surface of endothelial


Q1
14,6
Q2
70,8
Q4
13,6
Q3
0,96
CD45
CD11b/c
a Myeloid population
N
CFA-VEH
CFA-IMT504
CD45+ CD11b/c+ (%)
1 Day
21 Days
b Lymphoid population
CD45+ CD11b/c- (%)
a1 Monocytes
CD172a+ (abs. values)
a2 Macrophages
HIS36+ (abs. values)
b1 B-cells
CD45-RA+ (abs. values)
b2 CD4+ T-cells
CD4+ (abs. values)
b3 CD8+ T-cells
CD8+ (abs. values)

◀ **Fig. 3** Single s.c. IMT504 administration modulates macrophage, B and T cells infiltration in the hindpaw of rats with CFA-induced inflammation (n = 4). **a**, **b** Frequencies of myeloid ($CD45^+CD11b/c^+$; A) and lymphoid ($CD45^+CD11b/c^-$; B) cells at 1 and 21 d after treatment. Both CFA-VEH and CFA-IMT504 rats show increased percentage of myeloid and lymphoid cells 1 d after treatment, with values returning to near basal levels 21 d after injury. **(a1-b3)** Total number of monocytes (**a1**), macrophages (**a2**), B cells (**b1**), $CD4^+$ T cells (**b2**) and $CD8^+$ T cells (**b3**) of naïve (N), CFA-VEH and CFA-IMT504 rats, 1 and 21 d after treatment. CFA-IMT504 rats exhibited lesser increases in macrophages (21 d; Hedges'g: CFA-IMT504 vs CFA-VEH = 2.01, CFA-IMT504 vs N: 4.79) and B cell counts (Hedges'g CFA-IMT504: *1d:* vs CFA-VEH = 4.27, vs *N* = 11.65; *21d*: vs CFA-VEH = 2.2, vs *N* = 3.41), together with a potentiation in the number of $CD8^+$ T-cells at both time-points tested (Hedges'g CFA-VEH vs IMT504: *1d* = 2.23; *21d* = 3.35)

cells, macrophages and lymphocytes, and involved in the recruitment of a variety of immune cells at the site of injury (Bui et al. 2020), as well as of CD62L, a surface receptor relevant for leucocyte/endothelial cell interactions, and commonly found in the surface of T-cells (Ivetic 2018). Both sICAM1 and CD62L, are upregulated in the hindpaw of CFA-injured rats (Mousa et al. 2000; Machelska et al. 2002). On the other hand, IMT504 also downregulated the expression of chemokines such as CXCL5, CXCL7, CCL3, CCL5 and CCL20, involved in the chemo-attraction of immune cells in the inflamed paw, but also having direct pro-nociceptive actions (Jiang et al. 2020). Thus, in uninjured rats, intraplantar injection of CXCL5 induces neutrophil and macrophage infiltration, as well as mechanical (but not thermal) hypersensitivity (Dawes et al. 2011). CCL3, a macrophage chemo-attractant, is released by activated Schwann cells and macrophages themselves (Taskinen and Röyttä 2000; Kiguchi et al. 2010), and has been mostly characterized in neuropathic pain models (see (Jiang et al. 2020) and references therein), where it enhances the response of the transient receptor potential cation channel subfamily V member 1 (Zhang et al. 2005). Also, CCL5, which is involved in the recruitment of T-cells, eosinophils and basophiles, induces increases in Ca^{2+} mobilization and excitation of cultured DRG neurons, as well as mechanical allodynia upon intraplantar injection in uninjured rats (Oh et al. 2001); and CCL20, considered as a strong chemo-attractant for lymphocytes, is found upregulated in patients with temporomandibular joint osteoarthritis (Monasterio et al. 2018). Altogether, our present results suggest the hypothesis that the long-term anti-nociceptive actions of IMT504 may relate, at least in part, to progressive reductions in the expression of leukocyte adhesion molecules and chemoattractants, ultimately limiting cell infiltration, and also preventing chemokine-dependent pro-nociceptive signaling.

Pro-inflammatory cytokines such as IL6, TNFα, IL-1α and -β, and anti-inflammatory cytokines such as IL-10, and IL1-RA are relevant players in a balance that determines the induction or repression of pain signals (Cook et al. 2018; Gonçalves dos Santos et al. 2020). In the CFA model, increases in the cutaneous levels of macrophage-dependent TNFα and IL-1β drive the expression of the nerve growth factor (NFG) (Safieh Garabedian et al. 1995; Woolf et al. 1997) and prostaglandins (Cunha et al. 2005), ultimately mediating inflammatory hypersensitivity. In the present study, we show that both IL1-α and –β are upregulated in CFA-injured rats 28 days after injury, and that the administration of IMT504 strongly reduces their increase. IL-10, released by macrophages, dendritic cells, B-cells, mast cells and regulatory T-cells (see Cook et al. 2018; Gonçalves dos Santos et al. 2020), acts not only by down-modulating several pro-inflammatory cytokines, but also by inducing the expression of the IL-1RA (see Poole et al. 1995; Vanderwall and Milligan 2019), which inhibits mechanical hyperalgesia in rats with carrageenan-induced hindpaw inflammation (Cunha et al. 2000). IMT504, particularly 21 days after treatment, resulted in a modest upregulation in the levels of IL-10, in association with downregulated IL-1RA. This latter result was unexpected, although it could potentially reflect a positive reaction to downregulated expression of excitatory interleukins (Tisoncik et al. 2012). Altogether, it can be concluded that IMT504 favors the switch from a pro- to an anti-inflammatory environment at late stages of CFA-injury.

Two other relevant cytokines were found modulated by IMT504. On the one hand, CNTF, highly expressed by Schwann cells in peripheral nerves, is transiently upregulated in nerves undergoing spared nerve injury in mice and has been shown to participate in the modulation of neuroinflammatory responses (Hu et al. 2020). Here we found that CNTF is also upregulated in the hindpaw of CFA-injured rats, and that IMT504 prevents such an increase. On the other hand, TIMP-1, a matrix metalloproteinases inhibitor involved in tissue remodeling and inflammatory signaling, which has been shown to attenuate the development of inflammatory pain, as analyzed in TIMP-1-KO mice (Knight et al. 2019). Here we show a downregulated expression of TIMP-1 in the inflamed paw of IMT504-treated rats. While the exact meaning of the downregulating effects of IMT504 over CNTF and TIMP-1 in CFA-injured rats remains to be better established, it could be speculated that they responded to ODN-dependent reductions in pro-inflammatory conditions and tissue destruction, and thus a lesser need for tissue remodeling factors such as CNTF and TIMP-1.

One last finding in our study was that IMT504 treatment results in decreased hindpaw expression of endogenous β-endorphin. Peripheral opioid mechanisms of pain control are increasingly recognized as a relevant endogenous reaction against pain and inflammation (Stein and Machelska 2011) and previous studies have reported increased levels of endogenous opioids in the CFA chronic pain model (Przewlocki et al. 1992; Mousa et al. 2004; Labuz et al. 2006). Granulocytes, monocytes and macrophages (Rittner et al. 2001), B-cells (Mousa et al. 2004; Maddila et al. 2017) and

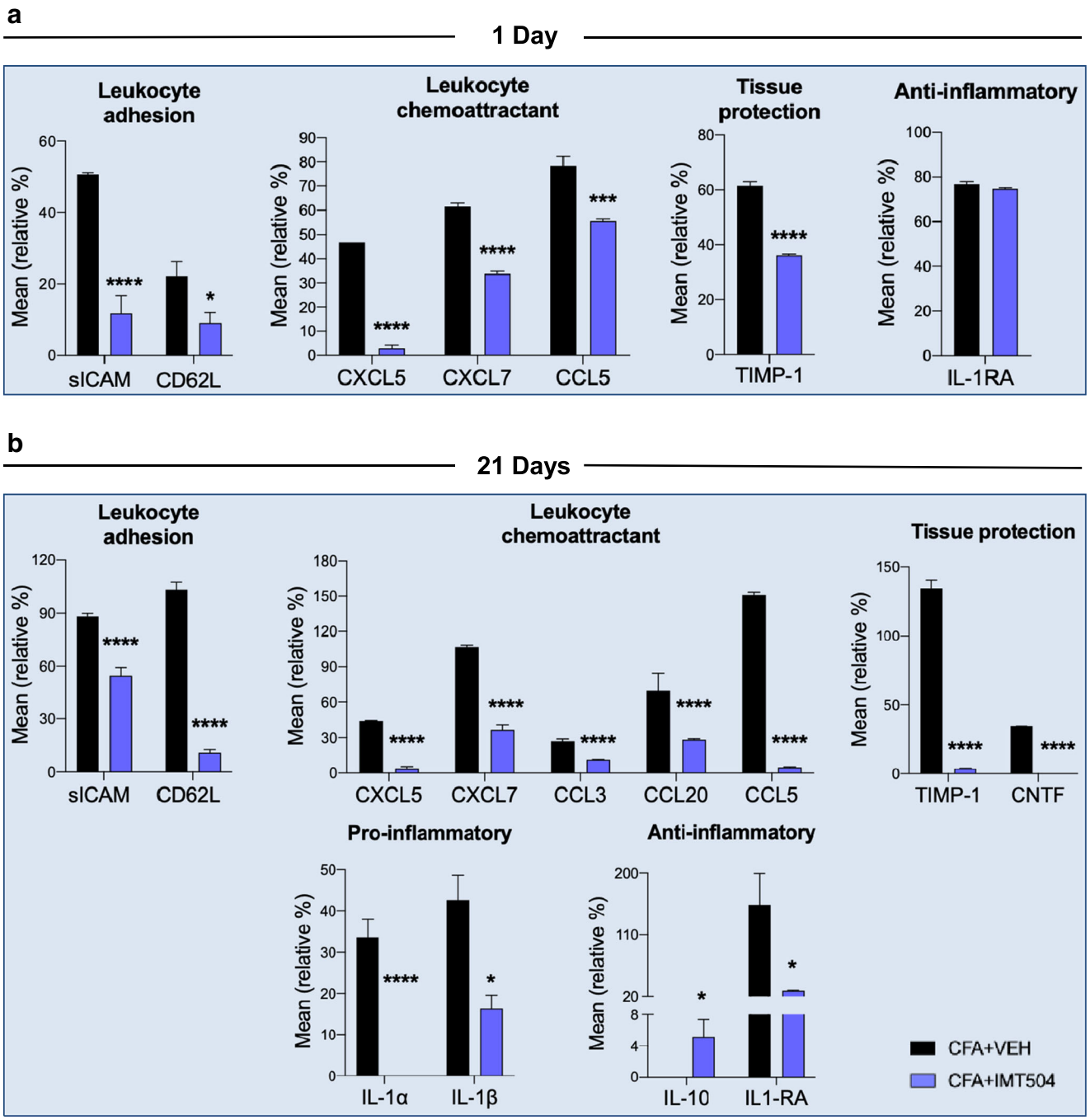


Fig. 4 Single s.c. IMT504 administration modulates the expression of various cytokines and chemokines in the hindpaw of CFA-injured rats. **a** One day after treatment, CFA-IMT504 rats show significantly decreased expression levels of sICAM, CD62L, CXCL5, CXCL7, CCL5 and TIMP-1. **b** The downregulating effect of IMT504 is maintained 21 days after treatment, and includes downregulated expression of CCL3, CCL20, CNTF, IL-1α, IL-1β and IL1-RA, and upregulation of IL-10 (n = 5)

T cells (Sitte et al. 2007; Maddila et al. 2017) have all been implicated as sources of endogenous opioid peptides at different stages of CFA inflammation. The downregulation of β-endorphin in CFA-IMT504 rats was surprising, as the opposite was expected in rats receiving this anti-allodynic ODN. However, and interestingly, the recruitment of β-endorphin-expressing immune cells in the inflamed hindpaw of rat appears to be regulated by the leukocyte adhesion molecules sICAM-1 and CD62L (Mousa et al. 2000, 2010; Machelska et al. 2002), both found downregulated in IMT504-treated rats. This effect, possibly influencing the reductions in infiltrating cell numbers, particularly of macrophages and B cells, may also explain the decreased expression levels of β-endorphin in the inflamed hindpaw of IMT504-treated rats.

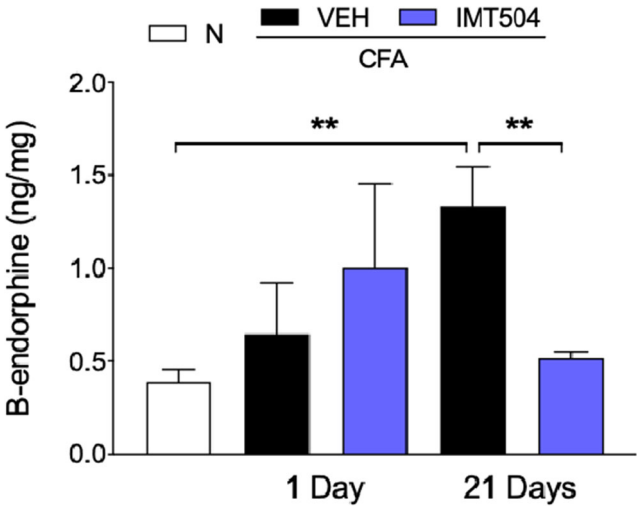


Fig. 5 Single s.c. IMT504 administration downregulates the expression of β-endorphin in the hindpaw of CFA-injured rats. One day after treatment, both CFA-VEH and CFA-IMT504 showed a slight, although not significant, tendency towards upregulated levels of β-endorphin. Twenty-one days after treatment, CFA-VEH rats exhibited significantly increased levels of ß-endorphin, in contrast to CFA-IMT504 rats, showing values comparable with N rats (n = 3) (Hedges'g CFA-VEH: vs CFA-IMT504: 3.16, vs. N: 3.53)

However, this hypothesis does not refute a possible potentiating effect of IMT504 over other endogenous opioid peptides. Even though lymphoid cells represent a minority of infiltrating cells (see Ghasemlou et al. 2015; Laumet et al. 2019 and current study), it appears that T cells are relevant in the resolution of inflammatory pain (Basso et al. 2016; Laumet et al. 2020), and at least in part through the synthesis and release of high amounts of enkephalins (Boué et al. 2011). Accordingly, an impaired recovery from mechanical allodynia and thermal hyperalgesia, plus of endogenous opioid signaling, particularly of Met-enkephaline, has been shown in $CD8^+$ T cell KO mice (Baddack-Werncke et al. 2017). And in rats with CFA-induced inflammation, presence of pro-enkephalin-synthesizing infiltrating cells has been previously detected in the hindpaw tissue of inflamed rats (Przewlocki et al. 1992), and the local inhibition of enkephalin degradation has been show to enhance anti-nociception (Schreiter et al. 2012). Here we show that numbers of $CD4^+$ and $CD8^+$ T-cells remain upregulated or are even potentiated in IMT504-treated rats, thus potentially representing a source of enkephalin. However, if IMT504 induces a switch from β-endorphin- to enkephalin-dependent endogenous analgesia, it remains to be established.

While the anti-allodynic and anti-inflammatory actions of IMT504 in rats undergoing inflammatory pain reported here are considerably clear, they seem to contrast with the previously reported immune stimulating effects observed in naïve rats. In fact, during the initial characterization of PyNTTTTGT ODNs, we formerly reported that IMT504 has an immune stimulating profile by acting on B cells and PDCs, promoting cellular proliferation and pro-inflammatory interleukins release (Elias et al. 2003, 2005; Rodriguez et al. 2006; Hernando-Insúa et al. 2007; Montaner et al. 2011). However, in a more recent study, we also reported that treatment with IMT504 in neutropenic rats undergoing fatal *Pseudomonas aeruginosa* bacteriemia and sepsis results in remarkable survival advantages, associated with reductions in the plasmatic levels of the pro-inflammatory IL-6 (Chahin et al. 2015). It is thus possible that IMT504 exerted different types of immune modulation, depending on the presence or absence of a pre-existing pathologic condition. In addition, we also previously reported that IMT504 induces MSC expansion in bone marrow and blood, both in vitro and in vivo (Hernando-Insúa et al. 2007). Besides their progenitor cell properties, MSCs are also known to interact with constituents of the immune system, exhibiting anti-inflammatory or pro-inflammatory properties depending on the milieu composition (Le Blanc and Mougiakakos 2012; Prockop and Oh 2012; Keating 2012). In general, MSCs first adopt a pro-inflammatory phenotype (MSC1) during early microbial invasion or trauma, when the concentration of pro-inflammatory cytokines in the milieu is relatively low. As inflammation proceeds, pro-inflammatory cytokines accumulate up to a critical level that switches differentiation of MSCs to an anti-inflammatory phenotype (MSC2). A hypothesis could thus be advanced where IMT504 induced a switch towards anti-inflammatory states involving MSCs and potentially also immune cells, thus facilitating reconstructive responses (Zorzopulos et al. 2017). In the present study, we focused on the analysis of certain components of the immune system, and therefore it remains to be established if IMT504 in fact facilitates the mobilization of MSCs towards the inflamed paws of injured rats, where they could influence other migrating cells.

Finally, while most of the analysis in the present study relates to effects at the site of injury, IMT504 could also modulate peripheral/circulating immune cells and other neuroimmune interactions influencing nociception at distant sites, such as DRGs and the spinal cord (Pinho-Ribeiro et al. 2017). In support, the injection of IMT504 by the s.c route has been shown to result in cellular proliferation and increased pro-inflammatory interleukins detection in peripheral blood (Elias et al. 2005; Hernando-Insúa et al. 2007; Montaner et al. 2011). Future studies will be needed to further determine the local and systemic mechanisms by which IMT504 modulates inflammatory pain.

Conclusions

Collectively, our study shows for the first time that the non-coding non-CPG, ODN IMT504 locally modulates immune cells in the innate and adaptive systems during an inflammatory insult, favoring a switch from pro- to anti-inflammatory

conditions, as also revealed by influences on the expression of several leukocyte adhesion proteins, chemokines and cytokines. Importantly, the identification of actions upon macrophages and $CD8^+$ T cells suggests newly found potential cell targets of IMT504. The multiple actions exposed in the present study seem to underlie the potent and long-lasting, antinociceptive and anti-inflammatory effects of IMT504, evident even after a single systemic administration, and support its potential use against chronic inflammatory pain.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11481-020-09971-2.

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Author's Contribution All authors state to have discussed the results and commented on the manuscript. Candelaria Leiguarda and Drs. Luis Constandil, Alejandro Montaner, Marcelo J. Villar and Pablo R. Brumovsky discussed and designed the project. Candelaria Leiguarda performed most experimental and analytical work, in collaboration with Drs. Constanza Potiliski (molecular array), Julia Rubione (flow cytometry), Pablo Tate (histology) and Veronica Bisagno (open-field test). The manuscript was written by Candelaria Leiguarda and Pablo Brumovsky, with assistance from all other authors (through discussion of all results and the strategy for its presentation in manuscript format).

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Data Availability Available if requested.

Compliance with Ethical Standards

Competing Interests None.

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