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Oligonucleotide IMT504 reduces neuropathic pain after peripheral nerve injury

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ABSTRACT

We have recently shown that the administration of bone marrow stromal cells (MSCs) prevents the development of mechanical and thermal allodynia in animals subjected to a sciatic nerve injury. Furthermore, exogenously administered MSCs have been shown to participate in the repair and regeneration of damaged tissues in a variety of animal models. However, some limitations of this therapeutic approach, basically related to the *ex vivo* cell manipulation procedure, have arisen. IMT504, the prototype of the PyNTTTTGT class of immunostimulatory oligonucleotides, stimulates MSC expansion both *in vitro* and *in vivo*. In this study, we evaluated the effect of IMT504 systemic administration on the development of mechanical and thermal allodynia in rats subjected to a sciatic nerve crush. Animals were treated with IMT504, MSCs or saline either immediately after performing the lesion or 4 days after it, and were evaluated using the von Frey and Choi tests at different times after injury. Control animals developed both mechanical and thermal allodynia. Animals receiving either IMT504 or MSCs immediately after injury did not develop mechanical allodynia and presented a significantly lower number of nociceptive responses to cold stimulation as compared to controls. Moreover, injury-induced allodynia was significantly reduced after IMT504 delayed treatment. Our results show that the administration of IMT504 reduces neuropathic pain-associated behaviors, suggesting that IMT504 could represent a possible therapeutic approach for the treatment of neuropathic pain.

Adult stem cells are being evaluated for their possible application in cell therapy [2,9,17,23,32]. Mammalian bone marrow (BM) contains two populations of adult stem cells, the hematopoietic stem cells, responsible for maintaining lifelong production of blood cells, and the variously termed mesenchymal stem cells or BM stromal cells (MSCs), with a well-documented role in providing the microenvironment that supports the tightly regulated process of hematopoiesis [2,30].

We have recently shown that MSC administration prevents the development of mechanical and thermal allodynia in animals subjected to a sciatic nerve injury [24]. In this model, MSCs injected into the ipsilateral lumbar 4 (L4) dorsal root ganglia (DRG) selectively migrate to the other lumbar ganglia affected by the lesion, where they acquire a characteristic perineuronal localization resembling glia/satellite cells [8]. This particular localization, acquired in an active and time-dependent fashion, suggests an association with a specific role in the injured nervous tissue. In fact, MSCs can attenuate the changes in neuropeptide expression (unpublished data), thus modifying pain neurotransmission and preventing the development of neuropathic pain-associated behaviors [24].

MSC implantation in the distal stump of the transected rat sciatic nerve also promotes motor recovery, as assessed by the walking track test [10]. Furthermore, MSC administration contributes to the functional recovery of animals with different types of lesion of the central nervous system, such as spinal cord injury [7,25], cerebral ischemia [5] or traumatic brain injury [20,21]. Finally, exogenously administered MSCs have been shown to participate in the process of repair and regeneration in a variety of animal models of tissue damage, including osteochondral defects [1,31], myocardial infarction [26,27] and acute renal failure [19]. As a consequence, MSCs are being evaluated for their clinical application in tissue repair therapies [2,3,17,23]. However, one important limitation for cell therapy is the complex process of cell isolation, *in vitro* expansion and cell delivery [23].

IMT504 is the prototype of the PyNTTTTGT class of immunostimulatory oligodeoxinucleotides (ODNs) [11]. These ODNs are synthetic molecules that stimulate cells of the immune system, such as B cells and plasmocytoid dendritic cells, inducing their activation, proliferation, differentiation, secretion of immunoglobulins

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and expression of costimulatory molecules [11,12]. For this reason, ODNs have been assayed as adjuvants in vaccines [12] and as medicines in the therapy of allergy and cancer [29]. Moreover, it has been recently shown that IMT504 is also a potent stimulatory signal for MSC expansion both *in vitro* and *in vivo* [16]. In fact, when rat BM mononuclear cells are cultured with IMT504, the mean number of MSC colonies is increased about three times [16]. Furthermore, rats inoculated with IMT504 have a significantly higher number of stromal progenitors, both in BM and peripheral blood [16]. These observations suggest that systemic treatment with this ODN can stimulate the animals own MSCs, inducing their expansion and mobilization, and thus avoiding *ex vivo* cell manipulation.

In this study, we evaluated the effect of IMT504 administration on the development of mechanical and thermal allodynia in animals subjected to a sciatic nerve crush. Different doses and treatment schemes were evaluated and compared to MSC administration.

Animals: Adult Sprague–Dawley male rats (200–300 g, Fucal, Argentina) were kept in a 12 h light-cycle, with water and food *ad libitum*. All the experiments performed in this study were approved by the local Ethical Committee of the Department of Bioethics of the School of Biomedical Sciences from Austral University, and were carried out in accordance to the policy of the Society for Neuroscience and the International Association for the Study of Pain for the use of animals in pain research.

Isolation of MSCs: Rats were anesthetized by intraperitoneal (i.p.) injection of a mixture of ketamine (50 mg/kg) and xylazine (5 mg/kg) and euthanized for BM harvesting. After removing epiphyses and gaining access to the marrow cavities, whole BM plugs were flushed out from femoral bones using a 1 ml syringe with α-minimal essential medium (MEM; GIBCO, USA) supplemented with 100 IU/ml gentamicine and 2.5 μg/ml amphotericine. The cell suspension was centrifuged at 400 × *g* for 10 min, and the pellet was suspended in fresh medium. This procedure was repeated twice. Cell concentration was evaluated by microscopic cell counting using a Neubauer hemocytometer using samples treated with 3% acetic acid. Cellular viability was determined using the trypan blue staining method. The cells were then suspended at a concentration of 1×10^6 cells/ml in Dulbecco's modified Eagle's medium (DMEM; GIBCO) supplemented with 100 IU/ml gentamicine, 2.5 μg/ml amphotericine, 2 mM L-glutamine and 20% fetal calf serum (GIBCO), and expanded by culture in 25 cm^2 tissue culture flasks. After 24 h of culture, non-adherent cells were discarded and cultures continued until confluence, renewing the medium every 7 days. Cells were harvested with 0.25% trypsin–1 mM EDTA (GIBCO), washed with phosphate buffered saline (PBS) and suspended at a concentration of 3×10^6 cells/ml in PBS. Immediately after, MSCs were injected intravascularly to animals subjected to a sciatic nerve crush.

ODN: IMT504 sequence is 5′-TCATCATTTTGTCATTTTGTCATT-3′ (Property of Immunotech SA, Argentina). The HPLC-grade phosphorothioate ODN (Oligos ETC, USA) was suspended in sterile saline (10 mg/ml), assayed for LPS contamination using the Limulus test and injected subcutaneously to the animals with a sciatic nerve crush.

Nerve injury model: Rats ($n = 62$) were anesthetized with chloral hydrate (350 mg/kg, i.p.) and their sciatic nerve was exposed and dissected free from the surrounding tissue. The nerve was then crushed for 3 s at the mid-thigh level using jeweller's forceps. Eight rats were subjected to the sciatic nerve crush alone while 54 animals were lesioned and treated as follows.

IMT504 treatment: Immediately after the surgery, a group of rats ($n = 10$) was subcutaneously injected with 20 mg/kg of the ODN IMT504 dissolved in saline. These rats received the same dose of the ODN, once daily, for the next four consecutive days (immediate IMT504 treatment, 20 mg/kg/dose). A second group of animals ($n = 10$) was treated with five subcutaneous injections of the ODN (20 mg/kg/dose), but starting 4 days after performing the lesion (delayed IMT504 treatment, 20 mg/kg/dose). Finally, a third group of animals ($n = 10$) received a lower dose of the ODN (5 mg/kg) following the delayed treatment scheme of administration (delayed IMT504 treatment, 5 mg/kg/dose).

MSC treatment: Another group of animals ($n = 10$) received MSC intravascular administration: a suspension of MSCs (1.5×10^6/500 μl PBS) was injected into the tail vein immediately after the surgery (immediate MSC treatment).

Control groups: (1) Animals subjected to the sciatic nerve crush alone. (2) Animals with a sciatic nerve crush receiving five subcutaneous injections of saline, once daily, starting immediately after performing the lesion. (3) Animals with a sciatic nerve crush receiving five subcutaneous injections of saline, once daily, starting 4 days after performing the lesion. (4) Animals with a sciatic nerve crush receiving one intravascular administration of PBS immediately after performing the lesion. Eight animals were included in each of these control groups.

Behavioral assessment: Behavioral testing was performed during daytime (9.00–18.00) in all animals before surgery (day 0) and at different time points after the sciatic nerve crush. The animals were placed in their acrylic testing chambers for 15 min for adaptation, and mechanical sensitivity was assessed with von Frey hairs (Stoelting, USA). The hairs were applied in ascending order (1, 2, 4, 6, 8, 10, 15, and 26 g) onto the plantar surface of both ipsilateral and contralateral hindpaws [4]. Each hair was delivered three times with 5 s intervals. The lowest force at which application elicited a paw withdrawal was taken as the mechanical response threshold. A paw withdrawal reflex obtained with 6 g or less was considered as an allodynic response. Cold sensitivity of the hindpaw to acetone (Choi test) [6] was quantified by foot withdrawal frequency. Thus, 100 μl of acetone was applied to the plantar surface of the paw using a plastic tubule connected to a 1 ml syringe. Acetone was applied five times to each paw at an interval of at least 5 min. The number of brisk foot withdrawals was recorded. Only rats showing normal responses to mechanical and thermal stimulation before surgery were included in the experiments.

Statistical analysis: It was carried out by applying one-way analysis of variance (ANOVA) and the Newman–Keuls multiple comparison post-test. Paw withdrawal thresholds to the von Frey filaments and the number of brisk foot withdrawals elicited upon the application of acetone was expressed as mean ± S.E.M. p values are presented as follows: ns, $p > 0.05$; $^{*}0.05 > p > 0.01$; $^{**}0.01 > p > 0.001$ and $^{***}p < 0.001$.

Animals receiving either IMT504 or MSC treatment immediately after nerve crushing did not develop mechanical allodynia and presented a significantly lower number of nociceptive responses to cold stimulation when compared to control rats. Moreover, injury-induced mechanical and thermal allodynia was significantly reduced after IMT504 delayed treatment.

Animals with a sciatic nerve crush alone: All animals showed normal pain thresholds on both hindpaw footpads before surgery and also on the contralateral footpad after surgery. The sciatic nerve crush induced the development of both mechanical (Fig. 1a) and thermal (Fig. 1b) allodynia in the ipsilateral hindpaw. These animals showed guarding behaviors and changes in the posture of the affected hindpaw, including plantar flexion and toe-clenching. Three days after performing the lesion, a marked decrease in paw withdrawal threshold was observed in almost all rats after application of the von Frey filaments, with 70% of these animals showing an allodynic response to mechanical stimulation (painful responses elicited with filaments of 6 g or less) (Fig. 1a). These allodynic responses were also detected at days 7 and 10 after injury (Fig. 1a).

At day 14, an increase in the mechanical threshold was observed, reaching almost normal values at day 21 (Fig. 1a). A similar behavioral pattern was detected when we evaluated thermal sensitivity using the Choi test: there was a clear increase in the number of positive nociceptive responses starting 1 day after the surgery, with the highest number of allodynic responses detected 3 days after the lesion and maintenance of thermal painful responses over time until day 14 (Fig. 1b).

Animals with a sciatic nerve crush and saline or PBS administration: These animals showed behavioral patterns similar to those observed in animals subjected to the sciatic nerve lesion alone (described above). No statistically significant differences were observed between any of these control groups at any of the time points evaluated (not shown). Therefore, and in order to facilitate the visualization of the results, only lesioned animals without any treatment were included in the graphs (Figs. 1 and 2).

Animals with a sciatic nerve crush and immediate IMT504 or MSC treatment: Both treatments prevented the development of mechanical allodynia (Fig. 1a) and reduced the number of nociceptive responses to cold stimuli (Fig. 1b). When evaluated using the von Frey test, a slight decrease in the mechanical paw withdrawal threshold was observed in both treated groups from day 1 after injury, with no generation of allodynia-like behaviors even at days 3, 7 or 10 after injury (Fig. 1a). In both groups of animals this non-allodynic pattern persisted until day 21 (Fig. 1a). Cold stimulation of the hindpaw also elicited similar responses in animals treated with either IMT504 or MSCs (Fig. 1b). A significantly lower number of positive nociceptive responses to cold stimuli were detected in animals treated with IMT504 at days 3 and 7 after injury, when compared to animals subjected to the lesion alone (Fig. 1b). Animals treated with MSCs also showed a lower number of allodynic responses to cold stimulation when compared to controls, although not reaching statistically significant values at any of the time points evaluated (Fig. 1b).

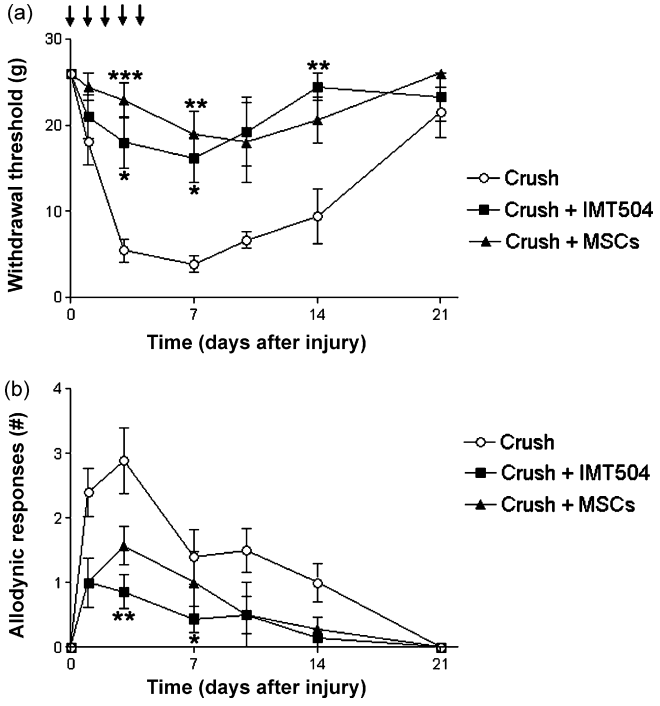

Fig. 1. Effect of either IMT504 or MSC immediate treatment on the development of mechanical (a) and thermal (b) allodynia in animals subjected to a sciatic nerve crush. The nociceptive behavior of lesioned animals without any treatment is also shown. These control animals showed a behavioral pattern similar to that of animals receiving either saline or PBS after the sciatic nerve lesion (not shown). (a) The sciatic nerve crush induced a significant decrease in paw withdrawal threshold to the von Frey filaments in control animals. Nociceptive responses in the allodynic range were detected 3, 7 and 10 days after the lesion. It is noticeable that the administration of either IMT504 or MSCs prevented the development of mechanical allodynia. Animals receiving either treatment showed similar behavioral responses at all the evaluated time points. (b) A significant increase in the number of allodynic responses to cold stimuli was induced in the ipsilateral hindpaw footpad after the sciatic nerve crush. Note that the administration of IMT504 significantly reduces the number of nociceptive responses to cold stimulation, 3 and 7 days after the lesion. MSC administration also results in fewer painful responses when compared to control animals. Values show mean ± S.E.M. Only statistically significant differences between treated and control animals are stated in the graphs, using the following symbols to represent p values: $^{*}0.05 > p > 0.01$; $^{**}0.01 > p > 0.001$ and $^{***}p < 0.001$. Arrows indicate the time points of IMT504 administration.

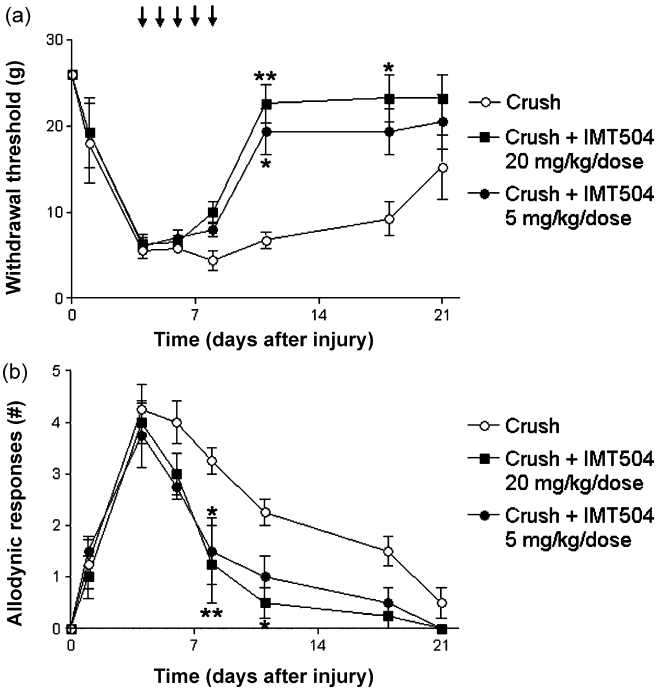

Fig. 2. Effect of IMT504 delayed administration on the development of mechanical (a) and thermal (b) allodynia in animals subjected to a sciatic nerve crush. Animals treated with two different doses of the ODN (5 and 20 mg/(kg dose)) were evaluated. The nociceptive behavior of animals with a sciatic nerve lesion without any treatment is also shown. These control animals showed a behavioral pattern similar to that of animals receiving saline after the lesion (not shown). (a) The sciatic nerve crush induced a significant decrease in paw withdrawal threshold to mechanical stimuli, with detection of allodynic values from day 4 till day 10 in control animals. IMT504 delayed administration (from day 4 till day 8) induced an increase in mechanical threshold, with an earlier recovery of non-allodynic values. Treated animals showed normal pain thresholds from day 11. (b) The number of allodynic responses to cold stimulation was significantly increased after the sciatic nerve crush. IMT504 delayed administration resulted in a decrease in the number of painful responses, with a more rapid recovery of non-allodynic behaviors. Values show mean ± S.E.M. Only statistically significant differences between treated and control animals are stated in the graphs, using the following symbols to represent p values: $^{*}0.05 > p > 0.01$ and $^{**}0.01 > p > 0.001$. Arrows indicate the time points of IMT504 administration.

Animals with a sciatic nerve crush and delayed IMT504 treatment: These animals developed both mechanical (Fig. 2a) and thermal (Fig. 2b) allodynia before treatment. A decrease in paw withdrawal threshold to the von Frey filaments was already observed 24 h after nerve crushing, with detection of allodynic values at days 4 and 6 after injury (Fig. 2a). Administration of either 5 or 20 mg/kg/dose of IMT504 (from day 4 till day 8) resulted in a marked increase in the mechanical withdrawal threshold: non-allodynic responses were detected from day 8 and thereafter, and a more rapid recovery of control values was detected when compared to control animals (Fig. 2a). The injury-induced increase in

the number of allodynic responses to cold stimulation was also attenuated after IMT504 delayed administration (Fig. 2b). The number of allodynic responses elicited by paw stimulation with acetone significantly decreased from day 8 and thereafter in animals treated with either 5 or 20 mg/kg/dose of IMT504, when compared to control animals (Fig. 2b).

The present study shows that IMT504 administration reduces mechanical and thermal allodynia in animals subjected to a sciatic nerve crush.

We have previously observed that MSC administration also results in a reduction of neuropathic pain-associated behaviors in animals with a sciatic nerve lesion [24]. MSCs injected into the ipsilateral L4 DRG specifically migrate to the other lumbar ganglia affected by the nerve lesion [8], induce changes in neuropeptide expression in primary afferent neurons and prevent the development of mechanical and thermal allodynia [24]. Considering the lack of effective therapies for the treatment of patients with neuropathic pain [13,14], these results seem promising. However, one important limitation of this experimental model is the route of administration of the cells. The intraganglionic injection is a technically complex procedure that could not be applied in patients. Therefore, in the present work we have evaluated if the same antinociceptive effects could be achieved in animals with MSC intravascular administration. Our results show that systemic administration of MSCs is effective in reducing neuropathic pain-associated behaviors, indicating the feasibility of using this administration route.

MSCs have been shown to participate in the regeneration process that is activated following different types of injury of the nervous system [5,7,20,21,25] and other tissues and organs [1,19,26,27,31]. Therefore, MSC administration has been proposed as a possible therapeutic strategy for tissue repair therapies [2,3,17,23]. However, some limitations of this therapeutic approach, basically related to the *ex vivo* cell manipulation procedure, have arisen [23]. MSC-based therapies would require taking a biopsy from the patient and then isolating and expanding the cells in culture. These procedures are expensive, laborious and may not generate enough cells. Furthermore, there are still certain unresolved questions regarding cell therapies, such as methodological, medical and ethical issues [15,22].

Systemic treatment with IMT504 has the advantage of avoiding the complex process of cell isolation, *in vitro* expansion and cell delivery. We have recently shown that rats inoculated with IMT504 have a significantly higher number of MSC progenitors, both in BM and in peripheral blood [16], suggesting that this ODN can induce MSC proliferation and mobilization. We have also observed that when rat BM mononuclear cells are cultured in the presence of IMT504, the number of MSC colonies is significantly increased [16]. This same effect was observed when culturing BM aspirates from human origin [16], suggesting that IMT504 administration to humans could also result in MSC expansion.

Considering a possible clinical application, and as part of preclinical studies, the toxicity profile of IMT504 was established in mice, rats and monkeys. Using the doses and schemes of inoculation here reported, no toxic, mutagenic or theratogenic effects were observed (manuscript in preparation). In the last years, the potential use of oligonucleotides as therapeutic agents has elicited a great deal of interest [28]. Several preclinical and clinical studies are being carried out in order to evaluate the potential use of different ODNs for the therapy of various conditions such as cancer, diabetes, hypertension, autoimmune, and cardiovascular diseases [18].

The present results suggest that IMT504 could represent a possible therapeutic approach for the treatment of neuropathic pain. Immediate IMT504 administration prevents the development of mechanical allodynia and reduces the number of allodynic responses to cold stimuli in animals subjected to a sciatic nerve crush. Furthermore, once mechanical and thermal allodynia have developed as a consequence of peripheral nerve injury, delayed IMT504 treatment is still beneficial since reduces these neuropathic pain-associated behaviors. Therefore, IMT504 not only prevents the development of pain in animals subjected to a sciatic nerve crush, but also reverses the effect of the lesion in animals showing painful responses prior to IMT504 administration.

The alleviation of pain induced by IMT504 is even stronger than that achieved after MSC administration. Further studies should be carried out in order to elucidate the mechanisms involved in IMT504-induced analgesia. However, the promptness of the regeneration process suggests that both an increase in the number of MSC precursors or tissue specific stem cells, and/or modifications in the immuno-chemical microenvironment at the site of nerve damage should be considered.

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