# Mesenchymal Cells Modulate Neuroma Microenvironment In Rats And Humans And Prevents Postamputation Pain

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## BACKGROUND AND AIM

By 2050, an estimated 3.6 million people in the United States alone will be living with amputated limbs. Persistent postamputation pain in these individuals is highly debilitating, often accompanied by non-painful sensory abnormalities. Current neuron-targeted pharmacological and interventional therapies treatments have shown limited efficacy. Recent research indicates that non-neuronal pain mechanisms play pivotal roles in the onset and persistence of chronic pain. Therefore, targeting these mechanisms presents promising avenues for innovative therapeutic approaches.

IMT504, an immunomodulatory non-CpG oligodeoxynucleotide, has demonstrated prolonged reduction of pain-like behaviors in rat models of inflammatory, neuropathic, and postoperative pain. In all cases, recruitment of mesenchymal stem cells (MSCs), as well as the promotion of an anti-inflammatory state at injured sites, appear as landmark mechanistic associations behind the anti-allodynic and anti-inflammatory actions of IMT504.

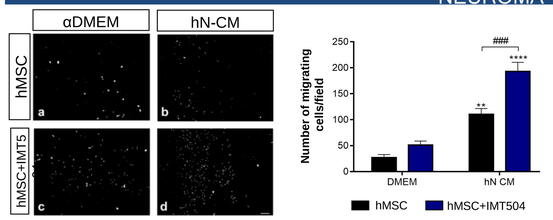
We examined the hypothesis that engaging MSCs, either by their direct use, or after *in vitro* exposure, or *in vivo* treatment with the oligodeoxynucleotide IMT504, would foster local neuroimmune interactions, leading to a potential reduction in postamputation pain. We included in the analysis three different sites in the pain neuroaxis, two in the periphery (the neuroma and corresponding dorsal root ganglia (DRG)), and one centrally located, the dorsal horn of the lumbosacral spinal cord.

## METHODS

MSC samples from 4 different donors undergoing hip replacement, and 1 neuroma sample from a patient suffering postamputation pain of over a year duration, were utilized for *in vitro* and *ex vivo* analyses of pro-migratory and anti-inflammatory phenotype-inducing actions of IMT504 over MSCs. Migration assays, plus ELISA and qRT-PCR analysis of MSCs, neuroma and conditioned media resulting from culturing experiments were carried out. *In vivo* studies included use of male, 8-week-old Sprague Dawley rats undergoing full axotomy of the right sciatic nerve and receiving two consecutive subcutaneous doses of IMT504 (6 mg/kg) on days 0 and 1 after injury. Rats were scored during 28 days, observing for signs of autotomy (self-mutilation behavior). Flow cytometry of injured nerves and DRGs was employed to address MSC tissue homing. Immunofluorescence and ELISA analysis of the spinal cord, neuromas and DRGs was employed to address changes in glial reactivity and the immune microenvironment. Migration assay, RT-qPCR, ELISA, flow cytometry and immunofluorescent expression of GFAP and CD11b/c were statistically analyzed using one-way ANOVA followed by Dunnett's post-hoc test for comparisons with the control group and for multiple comparisons between groups one-way ANOVA followed by Sidak's post-hoc test. Autotomy score was statistically analyzed using Kruskal-Wallis with Dunn's multiple comparisons tests. In all experiments data is represented with mean ± SEM. Alpha value was set at P<0.05 (*P< 0.05, **P< 0.01, ***P< 0.001, ****P< 0.0001).

## RESULTS

### IMT504 PROMOTES THE MIGRATION OF hMSCs TOWARDS INJURED PAINFUL HUMAN NEUROMA

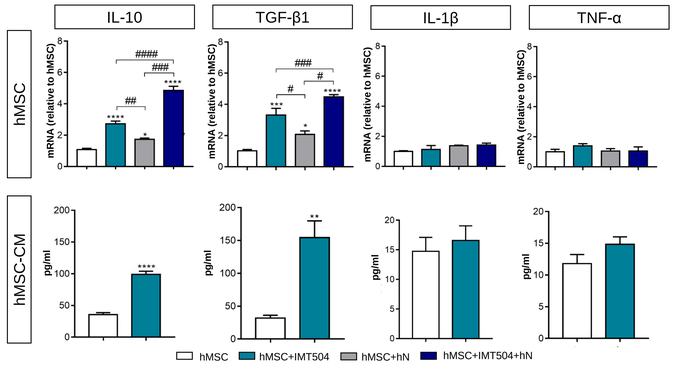






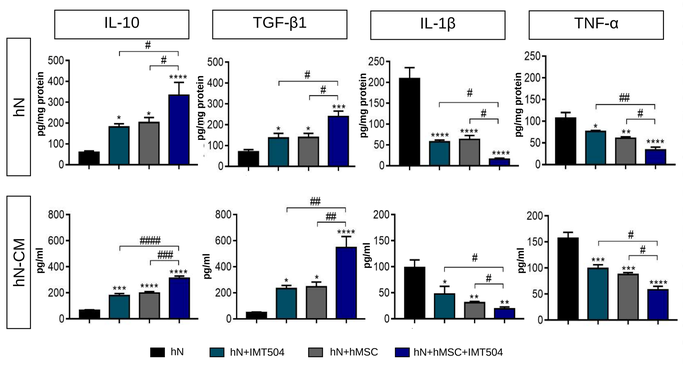
**(A)** Representative photomicrographs of hMSCs attached to polycarbonate filters and stained with DAPI, after running migration assays in different conditions. Scale bar : 10 µm. **(B)** Quantification of DAPI-positive hMSC attached to polycarbonate filters per field (n=4 per group). Using human tissues, we observed that hMSCs effectively migrated towards conditioned media from a human patient amputee painful neuroma (hN-CM). Notably, we found that hMSCs exposed to IMT504 migrated towards hN-CM at a two-fold higher rate.

### IMT504 MODULATES THE EXPRESSION OF ANTI-INFLAMMATORY MEDIATORS BY hMSCs



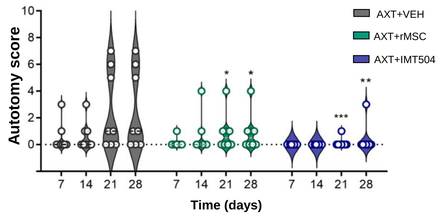

Quantification of anti- (IL-10 and TGF-β1) and pro-inflammatory (IL-1β and TNF-α) cytokines, detected by qRT-PCR, in human MSC (hMSC) **(first row)** incubated under a variety of conditions and the quantification of those, detected by ELISA, in the conditioned media (hMSC-CM) **(second row)** resulting from the incubations in the preceding experiment (n=4 per group). IMT504 induced the transcript expression of the anti-inflammatory factors *IL-10* and *TGF-β1* in hMCSs and was more pronounced upon exposure of hMCSs to IMT504 plus hN. We did not observe changes in the pro-inflammatory genes *IL-1β* and *TNF-α* in any of the conditions studied. Accordingly, these IMT504's molecular effects were phenocopied at the protein level (second row).

### IMT504 AND IMT504-ESTIMULATED hMSCs PROMOTE AN ANTI-INFLAMMATORY ENVIRONMENT IN PAINFUL HUMAN NEUROMA



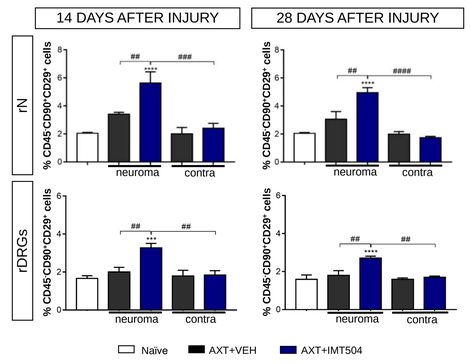

Quantification of anti- (IL-10 and TGF-β1) and pro-inflammatory (IL-1β and TNF-α) cytokines, detected by ELISA, in a sample of human neuroma (hN) **(first row)** and in the conditioned media (hN-CM) **(second row)** resulting from the incubations in the preceding experiment (n=4 per group). The neuroma displayed a marked pro-inflammatory phenotype (first row). The exposure of the neuroma to either IMT504 or hMSCs reversed its molecular phenotype as IL-10 and TGF-β1 levels were significantly increased, and IL-1β and TNF-α concentrations were significantly reduced. Moreover, the incubation of the neuroma with IMT504-treated hMSCs produced a more robust anti-inflammatory effect. We further confirmed these effects by measuring these factors in the conditioned media (second row).

### IMT504 PREVENTS AUTOTOMY BEHAVIOR IN RATS



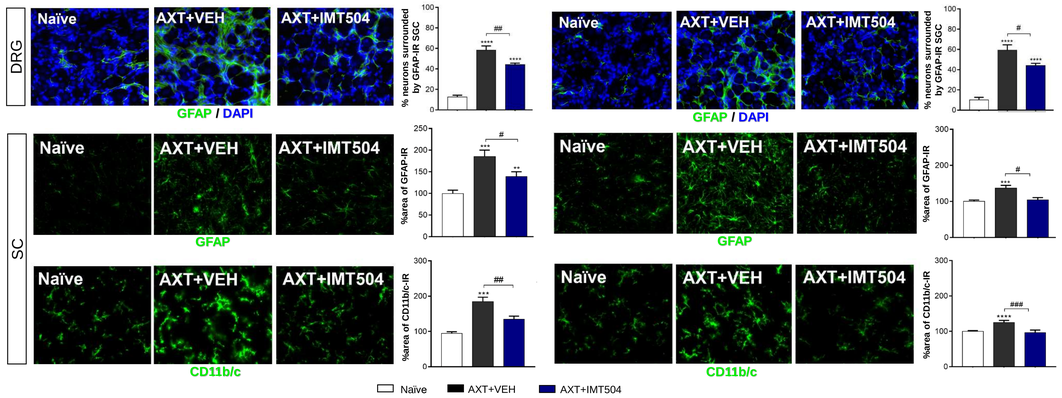

Characterization of the autotomy score at different survival times after injury, in injured rats receiving VEH, rat MSC (rMSC) or IMT504 (n=8 per group). In injured rats receiving intravenous rMSCs (AXT+rMSC), autotomy increased at a lower rate and was significantly less severe over time than in the AXT+VEH group. Then we sought to engage endogenous MSC in rats with sciatic nerve axotomy by treating the animals with IMT504 (6 mg/kg, on the day and 1 day after axotomy). Notably, in this rats (AXT+IMT504), only one animal exhibited autotomy, which started at later stages (day 21 after axotomy) and was significantly less severe than in AXT+VEH or AXT+rMSC groups.

### IMT504 PROMOTES rMSCs MIGRATION TOWARDS RAT NEUROMA AND DRGs



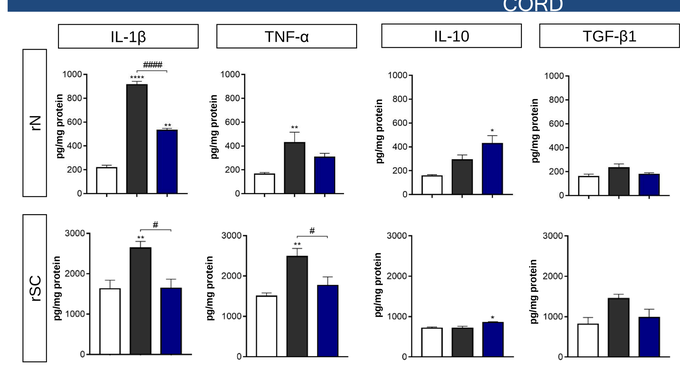

Percentages of MSCs detected by flow cytometry in sciatic nerves (rN) **(first row)** and L4-L6 DRGs (rDRG) **(second row)** of rats were measured at 14- and 28-days post-injury in naïve, AXT+VEH, and AXT+IMT504 groups (n=5 per group). The AXT+IMT504 group showed significantly higher numbers of rMSCs in the neuroma (first row) compared to both naïve and AXT+VEH groups. Moreover, rMSC counts in neuroma-associated DRGs (second row) were elevated in the AXT+IMT504 group compared to all control groups. The number of rMSCs in contralateral nerve and DRGs in rats receiving VEH or IMT504 remained comparable to the naïve group.

### IMT504 REDUCES DRG AND SPINAL GLIOSIS




Representative photomicrographs of ipsilateral L4-L6 DRGs **(first row)**, from naïve, AXT+VEH or AXT+IMT504 rats, incubated with GFAP antiserum (satellite glial cells (SGCs) marker), 14 or 28 days after injury, followed by the percentage of DRG neuron surrounded by GFAP-IR SGCs (n=6 per group). Representative photomicrographs of the dorsal horn, at the lumbar enlargement of the spinal cord (SC) (laminae I-II) of naïve, AXT+VEH or AXT+IMT504 rats, incubated with GFAP (astrocytes marker) **(second row)** or CD11b/c (microglial marker) **(third row)** antisera, 14 or 28 days after injury followed by the quantification of GFAP or CD11b/c mean fluorescent intensities per percentage area (laminae I-V), respectively (n=6 per group) Reactive gliosis in the injury-associated DRGs after peripheral nerve injury has been shown to contribute to pain-related behaviors in rodents. This peripheral effects alter key spinal cord mechanisms that depend upon nociceptive input, namely hyperactivity of microglia and astrocytes. Interestingly, L4-6 DRGs in the AXT+IMT504 group displayed a significant reduction of neurons with at least 50% encapsulation by SGCs with GFAP immunoreactivity compared to AXT+VEH rats at 14 and 28 days after injury (first row). Notably, spinal cords from AXT+IMT504 group displayed reduced glial marker immunoreactivities, both for astrocytes and microglia at 14 and 28 days after injury (second and third row). These results indicate that IMT504 exerts anti-nociceptive effects by modulating immunoreactive mechanisms in both the injured peripheral and spinal cord nociceptive axis.

### IMT504 REDUCES THE RELEASE OF PRO-INFLAMMATORY MEDIATORS IN RAT NEUROMA AND SPINAL CORD




Quantification of pro- (IL-1β and TNF-α) and anti-inflammatory (IL-10 and TGF-β1) cytokines, detected by ELISA, in sciatic nerves (first row, n=5 per group) and in the lumbar enlargement of the spinal cord (rSC) (second row, n=3 per group) from naïve, AXT+VEH and AXT+IMT504 rats, 28 days after injury.

Notably, in both neuroma and lumbar spinal cords, the systemic administration of IMT504 reduced the synthesis of IL-1β and TNF-α when compared to tissue obtained from AXT+VEH rats. Synthesis of IL-10 and TGF-β1 were not significantly modified by IMT504 treatment, although a certain tendency towards upregulated expression of IL-10 was detected in both neuroma and dorsal horn of rats receiving the oligodeoxynucleotide.

## CONCLUSIONS

The present study suggests that IMT504-dependent recruitment of endogenous MSCs within severely injured nerves and DRGs may prevent post-amputation pain by modifying the inflammatory scenario at relevant sites in the pain pathway. Reinforcing data in rat and human tissues supports the potential therapeutic value of IMT504 in patients suffering postamputation pain

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