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THERAPEUTIC EFFECT OF HUMAN ADIPOSE-DERIVED STEM CELLS AND THEIR SECRETOME IN EXPERIMENTAL DIABETES: FOCUS ON NEUROPATHIC PAIN

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Riassunto

Il diabete mellito è una delle più comuni e gravi patologie croniche al mondo. Anche se il numero di farmaci/ composti disponibili per gestire il diabete continua ad espandersi rapidamente, il trattamento delle complicazioni diabetiche, come la neuropatia, rimane una sfida sostanziale. La neuropatia diabetica periferica è infatti una delle complicanze più frequenti del diabete mellito [Aring *et al.*, 2005]. La patofisiologia della neuropatia diabetica è molto complessa e non è ancora completamente chiarita; è caratterizzata da meccanismi multipatogenici che causano una diversità di sintomi fisici, tra cui: allodinia, iperalgesia, intorpidimento e ulcerazione cutanea [Vinik *et al.*, 1995]. Il dolore neuropatico persistente interferisce in modo significativo con la qualità della vita dei pazienti, compromettendone il sonno e il benessere emotivo. Recenti progressi nei meccanismi coinvolti nel dolore neuropatico hanno dimostrato che le citochine pro- ed anti-infiammatorie prodotte da cellule immunitarie, nonché da glia e microglia a livello dei nervi, dei gangli della radice dorsale (DRG) e del midollo spinale sono denominatori comuni nel dolore neuropatico [Sacerdote *et al.* 2013; Old *et al.*, 2015]. Le citochine iniziano infatti una cascata di eventi correlati alla neuro-infiammazione che possono mantenere e peggiorare la lesione originale, partecipando alla generazione del dolore e alla sua cronicizzazione [Valsecchi *et al.*, 2011; Sommer and Kress, 2004; Austin and Moalem-Taylor, 2010]. In generale, l'attivazione della cascata infiammatoria, l'up-regolazione pro-infiammatoria delle citochine e le vie di comunicazione neuro-immuniche svolgono un ruolo fondamentale nel danno strutturale e funzionale dei nervi periferici che conducono alla neuropatia periferica diabetica. Purtroppo, la maggior parte dei farmaci analgesici disponibili sembra essere relativamente inefficace nel controllo del dolore neuropatico diabetico, sia per insufficienza di efficacia che per gli effetti collaterali [Galer *et al.*, 2000; Kapur 2003]; pertanto, vi è una chiara necessità di nuovi approcci terapeutici che vadano a modificare la patologia.

Le cellule staminali/ stromali mesenchimali (MSC) potrebbero offrire una nuova opzione terapeutica per il trattamento della neuropatia diabetica. Le MSC sono in grado di modulare l'ambiente lesionato del sistema nervoso e promuovere la riparazione in quanto in grado di secernere molecole anti-infiammatorie, anti-apoptotiche e fattori trofici che sostengono la crescita assonale, l'immunomodulazione, l'angiogenesi, la rimielinizzazione e la protezione dall'apoptosi [Ma *et al.*, 2014]. Quindi, le MSC trapiantate inoltre non solo differenziano direttamente nelle cellule endogene ma secernono anche un'ampia gamma di fattori biologicamente attivi, generalmente definiti come secreto MSC o secretoma; infatti anche se inizialmente le MSC sono state proposte per la terapia cellulare basata sul loro potenziale di differenziazione, la mancanza di correlazione tra il miglioramento funzionale e l'assunzione o la differenziazione cellulare nel sito di lesione ha portato a supporre che le MSC esercitino i loro effetti non attraverso il potenziale di differenziazione ma attraverso i prodotti secreti [Makridakis, 2016; Blaber *et al.*, 2012].

Per queste ragioni, utilizzando un modello preclinico di diabete di tipo I, nel presente studio confrontiamo l'effetto terapeutico di hASC (cellule adipose umane / stromali) e del loro medium

condizionato (CM, secretoma), sull'allodinia, sull'iperalgia, sull'espressione di citochine pro- e anti-infiammatorie nelle principali stazioni nervose coinvolte nella trasmissione del dolore e nelle risposte immunitarie periferiche. Il diabete di tipo 1 è stato indotto nei topi mediante iniezione intraperitoneale (i.p.) di basse dosi di streptozotocina (STZ, 80 mg / kg, una volta al giorno per tre giorni consecutivi), mentre gli animali controllo sono stati iniettati con veicolo (buffer citrato). In tutti i gruppi sperimentali, l'allodinia meccanica è stata valutata con il test di Von Frey prima dell'induzione del diabete ed ogni settimana dopo STZ fino alla fine del protocollo (14 settimane da STZ). Quando l'allodinia si è instaurata (2 settimane dopo STZ) gli animali sono stati trattati per via endovenosa (i.v.) con 10^6 hASC o con il secretoma ottenuto da 2×10^6 hASC (CM-hASC), cellule e secretoma prima di essere iniettati sono stati risospesi in PBS con eparina al 2,5%. Gli animali controllo sono stati iniettati con il veicolo. I nostri dati hanno dimostrato che i trattamenti con hASC e CM-hASC erano in grado di ridurre l'allodinia, anche se l'effetto delle hASC era significativamente superiore a quello ottenuto dal CM-hASC. L'effetto di entrambi i trattamenti è stato molto veloce, infatti una significativa riduzione dell'allodinia meccanica era già evidente 3 ore dopo l'iniezione; mentre il massimo effetto antiallodinico è stato raggiunto tra la prima e la seconda settimana dopo il trattamento. Inoltre l'effetto osservato è estremamente duraturo, infatti 12 settimane dopo un unico trattamento l'effetto antiallodinico risulta ancora evidente.

Successivamente, 4 settimane dopo il primo trattamento (6 settimane dopo STZ) abbiamo deciso di ritrattare un gruppo di animali diabetici con hASC e CM-hASC; il trattamento ripetuto con hASC non è stato in grado di migliorare ulteriormente allodinia; mentre poche ore dopo la seconda iniezione il trattamento ripetuto con CM-hASC ha potenziato significativamente l'effetto antiallodinico. Al fine di capire se i trattamenti con hASC e CM-hASC potessero essere efficaci anche in una fase più avanzata della patologia, 6 settimane dopo l'induzione del diabete un gruppo di animali STZ è stato trattato per la prima volta con hASC o CM-hASC. Anche in questa situazione entrambi i trattamenti sono stati efficaci nel fornire un effetto antiallodinico veloce ed irreversibile. Inoltre, per verificare se la staminalità fosse un prerequisito fondamentale per ottenere sollievo dal dolore, un gruppo di animali STZ è stato trattato con il secretoma ottenuto da 2×10^6 fibroblasti umani (CM-hF). La somministrazione di CM-hF non ha esercitato alcun effetto sull'allodinia meccanica, dimostrando che solo il secretoma derivante da cellule staminali è biologicamente attivo. Molto importante risulta essere anche la metodica di preparazione del secretoma stesso, perché il CM-hASC se liofilizzato non è in grado di fornire nessun sollievo dal dolore, questo ci fa ipotizzare che durante il processo di liofilizzazione dei fattori bioattivi essenziali vengano persi.

Poiché nei pazienti le alterazioni sensoriali associate alla neuropatia diabetica sono molteplici abbiamo valutato l'iperalgia termica ed abbiamo iniziato a valutare l'allodinia termica, rispettivamente mediante plantar test e test dell'acetone. Gli animali STZ sono sensibili all'allodinia termica ed entrambi i trattamenti sono stati in grado di ridurre in modo significativo questa ipersensibilizzazione. Negli

animali diabetici abbiamo osservato che l'iperalgia termica in risposta a stimoli caldi è presente fino a 3 settimane dopo la somministrazione di STZ e che successivamente i topi diabetici sono caratterizzati da ipoalgia, entrambi i trattamenti sono stati in grado di evitare il passaggio da iper- a ipo- algia. Questi test comportamentali dimostrano la capacità delle cellule staminali e del loro secreto di alleviare l'ipersensibilità a diversi tipi di stimoli, caratteristici del dolore diabetico.

Allo scopo di valutare l'impatto dei trattamenti sulle citochine pro- e anti- infiammatorie, gli animali sono stati sacrificati in momenti diversi: 2 settimane dopo STZ, cioè 3 ore dopo trattamento con hASC /CM-hASC; 3 settimane dopo STZ, cioè 1 settimana dai trattamenti e 14 settimane dopo STZ, cioè 12 o 8 settimane dai trattamenti. Da ogni animale è stato prelevato il nervo sciatico, i gangli della radice dorsale, il midollo spinale e gli splenociti. I livelli delle citochine IL-1 β , TNF- α , IL-6, IL-10 sono stati valutati nei tessuti nervosi mediante saggio ELISA. Tre settimane dall'induzione della neuropatia, negli animali diabetici, le citochine pro-infiammatorie IL-1 β , TNF- α e IL-6, risultano over-esprese sia a livello del sistema nervoso periferico (nervo sciatico e DRG) che a livello del sistema nervoso centrale (midollo spinale). Entrambi i trattamenti sono stati in grado di ripristinare i livelli delle citochine pro-infiammatorie, infatti dopo una settimana dai trattamenti sono tornate ai livelli basali; mentre i livelli della citochina anti-infiammatoria IL-10, in tutti i tessuti nervosi, sono apparsi significativamente ridotti negli animali diabetici, e sia il trattamento con hASC che con CM-hASC hanno significativamente aumentato le concentrazioni di IL-10. Quattordici settimane dopo STZ, i livelli di IL-1 β , TNF- α e IL-6 nel midollo spinale erano ancora significativamente elevati e i livelli di IL-10 erano ridotti rispetto ai topi non diabetici, indicando la persistenza della neuro-infiammazione. Come osservato per l'effetto anti-allodinico, anche la modulazione delle citochine indotta da hASC e CM-hASC è duratura, infatti 12 settimane dopo i trattamenti effettuati a 2 settimane dalla STZ, i livelli di IL-1 β , TNF- α e IL-6 erano ancora significativamente ridotti negli animali trattati con hASC e CM-hASC, mentre solo negli animali trattati con hASC abbiamo osservato una significativa normalizzazione dei livelli di IL-10. Effetti simili sono stati osservati anche nel doppio trattamento (2 e 6 settimane dopo STZ) ed entrambi i trattamenti hASC/CM-hASC sono stati efficaci anche quando le citochine sono state valutate in animali trattati in uno stadio avanzato della patologia (6 settimane dopo STZ).

Inoltre, per valutare il possibile effetto acuto dei trattamenti, i livelli di IL-1 β e IL-10 sono stati valutati dopo 3 ore dalla somministrazione di hASC e CM-hASC (2 settimane da STZ). Entrambi i trattamenti sono in grado di modulare positivamente tali livelli, alterati dalla patologia.

I livelli proteici di CGRP sono stati misurati nei DRG e nel midollo spinale e abbiamo osservato che entrambi i trattamenti sono in grado di normalizzare i livelli di CGRP che risultano elevati negli animali diabetici. E' stata valutata la perdita di fibre nervose e lo spessore della pelle a livello delle zampe posteriori, 1 e 12 settimane dopo una singola somministrazione di hASC/CM-hASC. Entrambi i

trattamenti sono stati in grado di contrastare la perdita di fibre nervose e di prevenire l'assottigliamento della pelle, anche se il trattamento con hASC si è dimostrato più efficace.

Il protocollo utilizzato, ovvero la somministrazione di basse dosi ripetute di STZ, è in grado di sviluppare una risposta autoimmune contro il tessuto pancreatico sostenuto da un modello di attivazione T-helper 1. Abbiamo quindi studiato se una polarizzazione di T-helper fosse presente negli splenociti dei topi diabetici e se il trattamento con hASC o con il loro secretoma fosse in grado di esercitare un'attività immunomodulatoria. Due settimane dopo la somministrazione di STZ, gli splenociti stimolati con Con-A hanno rilasciato livelli più elevati di IFN- γ mentre il rilascio di IL-10 è stato significativamente ridotto; entrambi i trattamenti con hASC e CM-hASC, 3 ore dopo la somministrazione sono stati in grado di aumentare i livelli di IL-10. Tre settimane dopo STZ, lo sbilanciamento Th1/Th2 appare molto più evidente ed entrambi i trattamenti hASC e CM-hASC sembrano in grado di ristabilire i corretti livelli di IFN- γ e IL-10. Quattordici settimane dopo STZ, è presente un chiaro passaggio verso un profilo Th1, caratterizzato da elevati livelli di IFN- γ e IL-2 e bassi livelli di IL-4 e IL-10; il trattamento con hASC o CM-hASC è stato in grado di normalizzare i livelli di tutte le citochine in esame. Nel complesso, i dati indicano che entrambi i trattamenti sono in grado di bloccare la polarizzazione Th1 che si sviluppa in questo modello sperimentale di diabete. Inoltre nel corso dell'intero esperimento sono stati monitorati i livelli di glucosio nel sangue ed il peso corporeo degli animali. Gli animali trattati con STZ sono caratterizzati da una graduale perdita di peso, che diventa significativa (vs. animali controllo-non diabetici) 3 settimane dopo la STZ; la somministrazione di hASC o CM-hASC, 2 settimane dopo l'induzione del diabete ha significativamente impedito la perdita di peso corporeo. Né il trattamento con hASC né quello con CM-hASC è stato in grado di modificare i livelli di glucosio nel sangue che rimangono sempre elevati. Per meglio approfondire questo punto abbiamo eseguito anche un test di tolleranza del glucosio.

Inoltre, gli animali STZ proprio come i pazienti sono caratterizzati anche da nefropatia ed entrambi i trattamenti sono stati in grado di migliorare tale condizione, indicando che i trattamenti sono efficaci anche nel contrastare anche altre complicazioni diabetiche.

I nostri risultati dimostrano che il trattamento con hASC può controllare complicazioni diabetiche come il dolore neuropatico, agendo su diversi meccanismi periferici e centrali coinvolti nello sviluppo e nel mantenimento di questa condizione, sia a livello neurale che immunitario. Inoltre, i risultati positivi osservati anche con il secretoma suggeriscono l'effetto delle hASC è probabilmente mediato dai loro prodotti secreti.



Summary

Diabetes mellitus is one of the most common and serious chronic disease in the world. Although the number of available agents to manage diabetes continues to rapidly expand, treatment of diabetes complications, such as neuropathy that is one of the most frequent complication of diabetes mellitus, remains a substantial challenge [Aring *et al.*, 2005]. Pathophysiology of diabetic neuropathy is complex and not fully elucidated; it has multipathogenic mechanisms that cause a diversity of physical symptoms: allodynia, hyperalgesia, numbness and cutaneous ulceration [Vinik *et al.*, 1995]. Persistent Neuropathic Pain (NP) interferes significantly with quality of life, impairing sleep, and emotional well-being, and is a significant causative factor for anxiety, loss of sleep, and non-compliance with treatment. Recent advances in the mechanisms involved in NP have demonstrated that pro- and anti-inflammatory cytokines produced by immune cells as well as by glia and microglia in nerve, dorsal root ganglia (DRG) and spinal cord are common denominators in neuropathic pain [Sacerdote *et al.*, 2013; Old *et al.*, 2015]. These start a cascade of neuroinflammation-related events that may maintain and worsen the original injury, participating in pain generation and chronicization [Valsecchi *et al.*, 2011; Sommer and Kress, 2004; Austin and Moaelem-Taylor, 2010]. Activation of inflammatory cascade, pro-inflammatory cytokines upregulation, and neuroimmune communication pathways play a vital role in structural and functional damage of the peripheral nerves leading to the diabetic peripheral neuropathy.

Unfortunately, most of the available analgesic drugs appear to be relatively ineffective in controlling diabetic neuropathic pain, both for insufficient efficacy and side effects [Galer *et al.*, 2000; Kapur, 2003]. Thus, there is a clear need for new disease-modifying therapeutic approaches.

Mesenchymal stem/stromal cells (MSCs) may offer a novel therapeutic option to treat diabetic neuropathy. MSCs modulate the nervous system injured environment and promote repair as they secrete anti-inflammatory, anti-apoptotic molecules, and trophic factors to support axonal growth, immunomodulation, angiogenesis, remyelination, and protection from apoptotic cell death [Ma *et al.*, 2014]. Transplanted MSCs not only directly differentiate into endogenous cells on administration, but also secrete a broad range of biologically active factors, generally referred to as the MSCs secretome; in fact even if initially MSCs were proposed for cell therapy based on their differentiation potential, the lack of correlation between functional improvement and cell engraftment or differentiation at the site of injury has led to the proposal that MSCs exert their effects not through their differentiation potential but through their secreted products [Makridakis, 2016; Blaber *et al.*, 2012].

For these reasons in the present study we analyze in a Streptozotocin mouse model of type 1 diabetes the therapeutic effect of hASC (human adipose stem/stromal cells) and their conditioned media (CM-hASC/ secretome) on allodynia and hyperalgesia, on pro- and anti-inflammatory cytokines expression in the main tissue stations involved in nociception transmission as well as in peripheral immune responses. Type 1 diabetes was induced in mice by intraperitoneal (i.p.) injection of moderate low doses of Streptozotocin (STZ, 80 mg/kg, daily for three consecutive days) while control mice were injected with

vehicle (citrate buffer). In all groups, mechanical allodynia was evaluated by Von Frey test before diabetes induction and every week after STZ until the end of protocol (14 weeks after STZ). When allodynia was established (2 weeks after STZ) animals were treated with 10^6 hASC that have been mechanically dissociated to a single cell suspension in PBS solution with 2.5% heparin; CM-hASC from 2×10^6 cells was also re-suspended in PBS solution with 2.5% heparin and both hASC and CM-hASC were intravenously injected in the tail vein to mice. Animals injected with vehicle only were considered as controls. Our data demonstrated that hASC and CM-hASC treatments were able to reduce allodynia, although the effect of hASC was significantly higher than that elicited by CM-hASC. The effect of both hASC and their secretome was very fast, since a significant reduction of mechanical allodynia was evident already 3 hours after the injection. The antiallodynic effect was maximal between 1 and 2 weeks after treatments and it was extremely long lasting: a significant reduction of allodynia was still present 12 weeks after a single hASC and CM-hASC treatment.

Moreover, 4 weeks after the first hASC/CM-hASC treatment (6 weeks after STZ) we decided to treat again a group of diabetic animals with hASC or CM-hASC; repeated hASC treatment did not further ameliorate allodynia. On the other hand, already few hours after the second CM-hASC injection, the antiallodynic effect was significantly potentiated and it completely mimicked the effect evoked by hASC. In order to discover whether hASC and CM-hASC treatments were effective also in a more advanced stage of the disease, when a severe loss of nerve function is reported, we treated animals 6 weeks after diabetes induction. Also in this situation both treatments were efficacious in providing a fast and irreversible antiallodynic effect. Furthermore, in order to verify whether stemness is a fundamental prerequisite for obtaining pain relief a group of STZ-mice was treated with CM obtained from 2×10^6 human fibroblasts (CM-hF). CM-hF did not exert any effect on mechanical allodynia, demonstrating that only secretome from stem cell cultures is biologically active. It is very important also to consider preparation method of secretome, because lyophilized CM-hASC was unable to provide pain relief, suggesting that during the lyophilization process some essential bioactive factors may be lost.

Moreover, since in patients sensory alterations associated to diabetic neuropathy are often diverse in order to ascertain whether the effects of hASC and CM-hASC were limited only to mechanical allodynia, we evaluated thermal hyperalgesia (hot stimuli) and thermal allodynia (cold stimuli) by plantar test and acetone test, respectively. In STZ-mice cold allodynia was present and both treatments were able to significantly reduce it. As regards to heat hyperalgesia, it was present in diabetic mice until 3 weeks from STZ administration, but subsequently we observed hypoalgesia appearance and both treatments were able to avoid hypoalgesia development; these results demonstrate the ability of stem cells and their secretome to relieve and prevent the typical diabetic hypersensitivity in response to different types of stimuli.

In order to evaluate the impact of treatments on pro- and anti-inflammatory cytokines, animals were sacrificed at different time points: 2 weeks after STZ, i.e. 3 hours after hASC/CM-hASC treatment; 3 weeks after STZ, i.e. 1 week from treatments and 14 weeks after STZ, i.e. 12 or 8 weeks from treatments. From each animal, sciatic nerves, dorsal root ganglia, spinal cord and spleens were collected. IL-1 β , TNF- α , IL-6 and IL-10, were evaluated as protein in nervous tissues by ELISA assay.

Three weeks after neuropathy induction pro-inflammatory cytokines IL-1 β , TNF α and IL-6 resulted overexpressed in peripheral (sciatic nerve and DRG) and central (spinal cord) nervous system of diabetic mice, both hASC and CM-hASC were similarly able to restore pro-inflammatory cytokine levels that 1 week from treatments were back to basal levels; while in all nervous tissues IL-10 levels appeared instead significantly reduced in diabetic animals and both hASC and CM-hASC significantly increased IL-10 concentrations, reaching physiological levels in DRG and spinal cord, while it exceeded basal levels in the sciatic nerve, indicating a switch towards an anti-inflammatory environment in all these tissues. Fourteen weeks after STZ, spinal cord IL-1 β , TNF- α and IL-6 levels were still significantly elevated and IL-10 levels reduced in comparison to non diabetic mice, indicating the persistence of neuroinflammation. As observed for the antiallodynic effect, also cytokine modulation induced by hASC and CM-hASC was long lasting. Twelve weeks after treatments performed 2 weeks from STZ, IL-1 β , TNF- α and IL-6 levels were still significantly reduced by hASC and CM-hASC treatments, while hASC-treated mice showed a significant normalization of IL-10 levels. Similar effects were observed also in double treatments (2 and 6 weeks after STZ) and both treatments were effective in modulating cytokine levels also when they were administered in an advanced pathological state (6 weeks after STZ). Moreover, to investigate the timing of cytokines modulation exerted by both treatments IL-1 and IL-10 levels in sciatic nerves, DRG and spinal cord were measured. Two weeks after diabetic induction, STZ mice were characterized by pro-inflammatory profile and only 3 hours after hASC and CM-hASC administration, both treatments were able to modulate cytokines levels.

To further demonstrate the modulation of treatments on pain-related mediators we demonstrated the ability of hASC and CM-hASC to normalize calcitonin gene related peptide level (CGRP), that was elevated in DRG from diabetic animals. Moreover, we evaluated loss of nerve fibers and skin thickness 1 and 12 weeks after a single hASC/CM-hASC administration at 2 weeks after STZ. Both treatments were able to contrast loss of nerve fibers and skin thickness, although hASC treatment was more effective.

Since STZ multiple low-doses protocol that we utilized is able to develop an autoimmune response against pancreatic tissue sustained by a T-helper 1 pattern of activation, we studied whether a T-helper polarization was present in splenocytes from diabetic mice and whether hASC or their secretome did exert any immunomodulatory activity.

Two weeks after STZ, Con-A stimulated splenocytes released higher levels of IFN- γ , while IL-10 release was significantly reduced; both hASC and CM-hASC treatments 3 hours after administration were already able to augment IL-10 levels. Th1/Th2 cytokines imbalance was more evident 3 weeks after STZ and both treatments appeared able to reestablish a correct IFN γ /IL-10 balance. When cytokine levels were measured at longer time from diabetes induction, i.e. 14 weeks after STZ, a clear shift toward a Th1 pattern, characterized by higher IFN- γ and IL-2 secretion and lower levels of IL-4 and IL-10, was present and both hASC/CM-hASC treatments were able to normalize cytokine levels. In the whole, the data indicate that both hASC and CM-hASC treatments are able to block Th1 polarization that develops in this experimental model of diabetes. Moreover, throughout the experiment, blood glucose levels and weight were monitored. In respect to non-diabetic control animals, a significant body weight loss was observed in diabetic mice, that started to be significant 3 weeks after STZ. In STZ-mice the administration of hASC or CM-hASC, 2 weeks after diabetes induction significantly prevented the loss of body weight. Neither treatments did modify blood glucose levels that were elevated in STZ-mice nor glucose tolerance test response.

Moreover both hASC and CM-hASC did ameliorate nephropathy that was present in diabetic animals, indicating that the treatments may be useful for treating also other diabetes complications.

Our results demonstrated that hASC can control diabetic complications such as neuropathic pain, acting on several peripheral and central mechanisms involved in development and maintenance of this condition, such as neural and immune elements. Moreover the significant new positive results observed also with hASC conditioned medium strongly suggest that their effect is likely to be mediated by their secreted products.

Introduction

1.1 Anatomy and physiology of pain

In the 1979, the International Association for the Study of Pain (IASP) defined pain as “an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage” [Merskey, 1979]. This definition takes into consideration two important concepts concerning the phenomenon of pain: its subjectivity and its manifestation also in absence of real tissue damage.

Pain experience is in fact complex and highly variable between individuals. It involves an intricate neurobiological apparatus consisting of dynamic excitatory and inhibitory nervous circuits, deputed to the transmission and elaboration of painful perception. Moreover the presence of modulatory pathways controlling the emotional state (fear, anxiety, attention and distraction) and the cognitive functions (past experiences, memories of pain) can in turn either enhances or diminishes the individual pain experience [Ossipov et al., 2010].

Pain can last for a short (acute pain) or long (chronic pain) period, it can be caused by several conditions and its processing can imply different neuronal mechanisms. Given the complexity and the heterogeneity of pain manifestation, the distinction of the pathways and molecules implicated in the several types of pain processing is fundamental in order to target the treatments at the mechanisms responsible.

According to Woolf classification, pain can be broadly divided into three main types: nociceptive, inflammatory and pathological pain [Woolf, 2010].

Nociceptive pain is protective and needful for maintaining body integrity as it represents a primitive defence mechanism evolved for warning us about the presence of potential dangerous stimuli and for minimizing tissue injury. It follows that the absence of nociceptive pain sensitivity has a negative impact on health; congenital insensitivity to pain due to mutations of genes encoding for channels or molecules involved in pain processing or lacking the ability of pain perception due to a sensory damage for instance, typically results in bone fractures, self-mutilation, maintaining of unrealized infections and a reduction of the life span [Axelrod and Hilz, 2003; Raouf et al., 2010].

Also the inflammatory pain is physiological and protective. This kind of pain takes place in presence of tissue injuries or infections and is associated to an inflammatory state caused by the activation of the immune system. However the increased pain hypersensitivity is functional because it protects the injured tissue site until healing occurs, by minimizing contact with stimuli potentially able to provoke other pain.

Usually, both nociceptive and inflammatory pain trigger adaptive responses and last until the resolution of the initial injury. The switch from a physiological to a pathological condition occurs in presence of damage (neuropathic pain) or an abnormality functioning (dysfunctional pain) of the nervous system. In these cases pain is maladaptive, not protective and is maintained for a long period ranging from weeks

to years. In other words, pathological pain is not a symptom of some disorders but rather a disease of the nervous system [Woolf, 2010].

1.1.1 Pain pathways

Nociception is the neuronal process that allows the people to experience pain. Four distinct steps occur along the pain pathways:

- I. Transduction of noxious stimuli (mechanical, thermal or chemical stimuli) in neural electrical activity by the activation of ion channels, including transient receptor potential channel subtypes (TRPA, TRPM and TRPV), sodium channel isoforms (Nav), potassium channels subtypes (KCNK) and acid-sensing ion channels (ASICs), clustered on nociceptors [Grace et al., 2014]; moreover, kinin peptides, in particular bradykinin, are released from kininogen precursors at the site of tissue injury and inflammation by protease kallikreins and kininases and act on constitutively expressed B2 and injury-induced B1 G-protein-coupled receptors (GPCRs) expressed on primary sensory neuron peripheral terminals [Prado et al., 2002], to contribute to nociception [Rueff and Dray, 1993; Couture et al., 2001].
- II. Transmission of nerve impulses from the site of transduction to spinal cord, brainstem, thalamus, and central structures in the brain;
- III. Modulation of nociceptive signals through endogenous mechanisms of pain control (spinal inhibitory interneurons and descending pain modulatory circuits);
- IV. Perception of pain as the end result of nociceptive stimulus travelling through the entire nervous system, including the supra-spinal structures involved in memory, cognition and emotion, which contribute to the neuronal network of conscious experience of pain [Fields and Basbaum, 1999].

Nociceptive ascending pathway begins in periphery with the activation of the nociceptors, sensory neurons able to detect dangerous or potentially damaging stimuli for the organism.

Nociceptors are pseudounipolar neurons with a single axon that forks into peripheral and central processes. The afferent branch projects to skin, mucosa, blood vessels and connective tissue of visceral organs, while the other runs to Central Nervous System (CNS). Specialized structures at the terminal end of nociceptors, once coming in contact with noxious stimuli, convert painful messages in electrical signals that propagate along this axonal pathway from periphery to the spinal cord or hindbrain. From here the signals reach the supraspinal structures and, finally the cortical and subcortical regions, where painful information is interpreted and perceived.

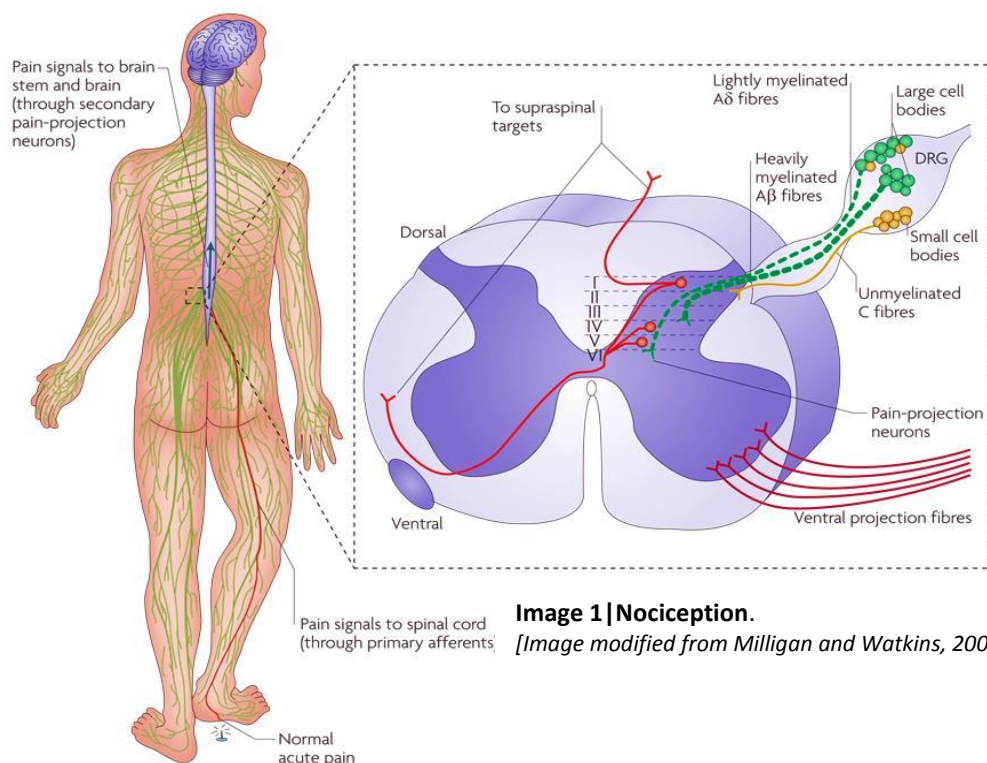
Unlike to other classes of sensory fibers, nociceptors have high activation thresholds; in fact in normal conditions, they can be excited only by stimuli enough intense to cause a real tissue damage, but not by

innocuous stimulations such as light touch, vibrations or warning. High threshold stimuli for nociceptors activation include extreme temperatures, intense pressure and irritant chemicals [Dubin and Patapoutian, 2010].

The main primary afferent fibers that convey noxious stimuli to CNS arise from nociceptive neurons having a small or medium-diameter cellular body located in the dorsal root ganglia (DRG). They comprehend the thinly myelinated, mostly mechanosensitive $A\delta$ fibres and the unmyelinated, polymodal, i.e. able to detect mechanical, thermal and chemical stimuli, C fibers [Julius and Basbaum, 2001].

All $A\delta$ fibres respond to intense mechanical stimulations but depending on their responsiveness to noxious heat, they can be divided in type I and type II fibres. The first type includes capsaicin-insensitive fibres responding to high temperatures (52-56 °C), while the type II population comprehends capsaicin-sensitive fibers responsive to noxious heat of 40-45 °C [Giordano, 2005].

Unmyelinated C fibers represent the majority of the primary afferents fibers and, according to ability of synthesizing neuropeptides, mostly Substance P (SP) and Calcitonin Gene Related Peptide (CGRP), are broadly divided in two main populations, the peptidergic and the non-peptidergic fibers.



$A\delta$ and C nociceptive fibers differ also in terms of conduction velocity. When activated, $A\delta$ fibers transmit nociceptive potentials with a velocity ranging to 12-30 m/s leading to a rapid pain sensation, while C fibers propagate noxious information more slowly (0.5-2 m/s) inducing a second, delayed response to pain. The different pattern of signal propagation results in two successive and qualitatively

distinct pain sensations: brief, pricking, and well localized “first pain” and a longer-lasting, burning, and less well localized “second pain” [Ploner et al., 2002].

Another group of nociceptors is represented by C nociceptive fibers known as “silent” or “sleeping” nociceptors which respond to noxious stimuli only when sensitized by tissue injury or inflammation. Peripheral primary afferents also include myelinated, fast conducting (30-100 m/s) A β fibers arising from DRG large-diameter neurons, responsive to mechanical innocuous stimuli. Normally they don't contribute to pain sensation but in some cases they begin to signal pain in response to non-noxious stimuli.

Information transmit by A β fibers can be in fact greatly altered during disease conditions or after tissue damage has resolved, leading to abnormal pain signalling [Milligan and Watkins, 2009].

The information collected in periphery by nociceptive fibers reach the spinal cord via the dorsal roots. Here (spinal cord) their central processes form synapses with different populations of second-order neurons mostly distributed in the superficial layers of the dorsal horns (Rexed laminae I and II). Nociceptors also synapse in some deeper laminae such as Rexed laminae V and X. Dorsal horns neurons include Nociceptive Specific (NS) neurons, exclusively responsive to nociceptive input conveyed by A δ and C fibres, Wide Dynamic Range (WDR) neurons that respond to both nociceptive and non-nociceptive stimuli coming from A δ , C and A β central axons and Non-Nociceptive (N-NOC) neurons responding to innocuous stimulations mostly propagated by A β fibers [Almeida et al., 2004].

Nociceptive fibers transmit the nociceptive message through the release of glutamate, as the primary neurotransmitter, and of neuropeptides (SP, CGRP), which have the potential of exciting second-order nociceptive projection neurons [Grace et al., 2014].

The excitatory amino acid binding to postsynaptic glutamate AMPA and kainate receptors is responsible for a fast synaptic transmission and a rapid, short-term depolarization of second-order neurons. On the contrary a slow and long-term synaptic transmission is mediated by SP and CGRP release.

Spinal projection neurons relay the nociceptive signals received to higher centres of the CNS following the ascending spinal tracts. Secondary afferents decussate and pass up the spinal cord to midbrain via the spinothalamic, spinoreticular and spinomesencephalic tracts to the thalamus and to sensory cortex, but also have other links, such as to reticular formations, limbic and hippocampal areas [Farquhar-Smith, 2008].

Cortical and supra-spinal areas form the “pain matrix”, i.e. a collection of brain regions involved in neurological functions, including cognition, emotion, motivation and sensation, as well as pain, which acting together in pain modulation context lead to the conscious experience of pain [Tracey and Johns, 2010; Ossipov et al., 2010].

As mentioned above the three main ascending pathways linking the dorsal horns to brain are:

- *Spinothalamic tract (STT)*

This tract originates from spinal WDR, NS and N-NOC neuron types propagating noxious and innocuous signals that are related to pain, temperature and touch. Before ascending, secondary axons decussate transversely through the anterior commissure of the spinal cord. During its passage through the brain stem, STT originates collateral branches destined to reticular formation of the medulla, pons and midbrain, periaqueductal grey matter (PAG), hypothalamus and medial and intralaminar thalamic nuclei [Almeida et al., 2004].

- *Spinoreticular tract (SRT)*

This tract mostly originates from spinal WDR and NS neurons. It presents two components in the brain stem; one is directed at the precerebellar nucleus in the lateral reticular formation involved in motor control, while the other is directed to the medial pontobulbar reticular formation involved in mechanism of nociception [Millan, 1999]. SRT is an important pathway for the modulation of the nociceptive segmental pathway by activating brain stem structures responsible for descending suppression [Almeida et al., 2004].

- *Spinomesencephalic tract (SMT)*

As SRT, this tract is highly involved in the mechanisms of pain modulation. This tract originates from spinal WDR and NS neurons and projects to different regions of PAG. The projections to the midbrain PAG matter from spinal neurons are functionally distinct; those that reach the PAG in the portion more dorsal to the limiting sulcus have excitatory characteristic in afferent nociceptive transmission, while those that project more ventral to the limiting sulcus activate mechanisms responsible for the inhibition of the afference of this same pathway [Almeida et al., 2004].

1.1.2 Pain modulation

The sensory experience of pain extends beyond the activation of nociceptors as it involves an intricate neuronal network consisting of dynamic excitatory and inhibitory nervous circuits which modulate pain experience enhancing or inhibiting pain sensation.

In the dorsal horn of spinal cord local GABAergic and glycinergic interneurons have a functional role of inhibition of pain information processing. As described by *Melzack and Wall's in the Gate Control Theory of Pain* [1978], these inhibitory interneurons function as a gate in spinal cord regulating the transmission of pain message from primary afferents to the spinal second-order projection neurons. A β non-nociceptive fibers activity inhibits (or "closes") the gate inducing the activation of local inhibitory

interneurons, while the activity of small nociceptive C and A δ fibres facilitates (or “opens”) the gate [Moayedi and Davis, 2013]. The balance between the inputs from nociceptive and non-nociceptive primary afferents which is controlled by the complex spinal inhibitory interneuron circuits determines the status of this gate, i.e. whether and how strong the nociceptive signal will be transmitted, via secondary projection neurons, to the higher brain centers [Guo and Hu, 2014]. Descending fibers originating from supra-spinal regions which project to the spinal dorsal horns are also implicated in the modulation of this gate [Moayedi and Davis, 2013].

Pain modulation exists in fact also in the form of descending inhibitory and facilitatory pain pathways which involve different regions within CNS, including cortex, thalamus and brainstem.

The midbrain periaqueductal grey region (PAG) and the rostral ventromedial medulla (RVM) are particularly important in the endogenous modulation of pain. These regions are known to be involved in endogenous pain control through PAG-RVM-spinal cord descending inhibitory pain pathway [Fields, 2006].

These supra-spinal sites exert influences on the perception of pain either directly, sending projection neurons to the spinal cord, or indirectly, sending projection neurons to other regions in the brainstem that send projections to the spinal cord. These modulatory effects are predominantly mediated by descending pathways that utilize serotonin, norepinephrine and endogenous opioids.

Monoamines and endogenous opioids modulate the release of neurotransmitters from nociceptive afferents and the excitability of dorsal horn neurons by binding to different receptor subtypes.

Stimulation of PAG and RVM was found to cause release of serotonin in spinal cord [Cui *et al.*, 1999]; at this level serotonin has either inhibitory or facilitatory role of pain transmission which is dependent on the receptor subtype activated [Suzuki *et al.*, 2004; Dogrul *et al.*, 2009].

Norepinephrine also strongly contributes in anti-nociceptive mechanisms associated to descending inhibition. PAG and RVM communicate with an important noradrenergic site to pain modulation, the locus coeruleus (LC), which is one of the major source of direct noradrenergic projections to spinal cord involved in the inhibition of presynaptic and post-synaptic spinal pain transmission neurons [Fields *et al.*, 2005; Proudfit, 1992].

Descending pain inhibition from PAG was also observed after direct microinjection of opioids [Fang *et al.*, 1989; Ossipov *et al.*, 2010].

“On-cells” and “off-cells” are two classes of pain modulatory neurons identified in the RVM and PAG [Fields *et al.*, 1991; Mason, 1999]. “Off-cells” are excited by opioids and inhibit ascending noxious stimuli from the periphery by triggering descending inhibition. On the contrary, “on-cells” are thought to trigger descending facilitation [Fields and Basbaum, 1999; Mason, 1999; Fields, 2000].

1.1.3 Mechanisms involved in the transition from physiological to pathological pain processing

1.1.3.1 Peripheral sensitization

Tissue injury and nerve damage produce pain hypersensitivity inducing molecular and cellular changes in the primary afferent neurons. This neuronal plasticity manifests through an increased responsiveness and reduced activation threshold for thermal and mechanical stimuli of nociceptors and is referred as peripheral sensitization.

These electrophysiological phenomena correlate to behavioral phenomena which include spontaneous pain, hyperalgesia (increased responses to noxious stimuli) and allodynia (nociceptive response to innocuous stimuli) [Cheng and Ji, 2008].

The capacity to produce increases in sensitivity after injury is functional and protective in physiological pain; however peripheral sensitization can be a leading cause for the development of persistent pathological pain. Increases in pain sensitivity are mediated by the local release of inflammatory mediators (collectively referred as “inflammatory soup”) from primary afferents terminals and different non-neuronal cells, including fibroblasts, mast cells, neutrophils, monocytes and platelets. After nerve damage, these mediators can also be released by Schwann cells and damaged neurons [Campana, 2007]. “Inflammatory soup” includes prostaglandins E2 (PGE2), bradikinin, ATP, protons, nerve growth factor (NGF) and pro-inflammatory cytokines (TNF- α and IL-1 β) [Julius and Basbaum, 2001].

These mediators stimulate/activate nociceptors directly (e.g. protons, ATP and 5-hydroxytryptamine (5-HT)) or by increasing sensitivity to subsequent stimuli (e.g. bradikinin, prostaglandins, leukotrienes and NGF) via receptor-mediated second messenger action.

Cyclic AMP, Protein Kinase A (PKA) and specific subtypes of Protein Kinase C (PKC) such as PKC ϵ are important second messenger and effectors involved in nociceptor sensitization, which may be at least in part caused by changes to ion channels. Ion channels, including voltage-gated sodium ion channels, acid-sensing ion channels and the heat and capsaicin receptor, the Transient Receptors Potential channel V1 (TRPV1), give a significant contribution to activation, sensitization and consequently hyperalgesia and allodynia development, and their sensitivity is strongly regulated by inflammatory mediators [Farquhar-Smith, 2008].

Beyond to activate PKC and PKA pathways, the inflammatory mediators released after injury also activate MAPK signalling in nociceptive primary sensory neurons. Activation of this pathway results in the regulation of transcriptional and translational factors which ultimately lead to increased synthesis of gene encoding for ion channels (such as TRPV1, TRPA1, tetrodotoxin-resistant (TTX-R) sodium and calcium channels), neuromodulators (BDNF, SP and CGRP) and pro-inflammatory cytokines TNF- α and IL-1 β . Persistent increase in the synthesis of these pro-nociceptive mediators in primary sensory neurons maintains hypersensitivity of these neurons and persistent pain [Cheng and Ji, 2008].

In addition, nociceptors activation not only results in the transmission of pain message to spinal cord (and from here to brain), but is also responsible for the initiation of neurogenic inflammation.

The antidromic release of peptides and neurotransmitters, notably substance P and CGRP, from activated nociceptors induces in fact vasodilation, plasma extravasation and activation of immune cells, which in turn contribute additional elements to the inflammatory soup [Julius and Basbaum, 2001].

1.1.3.2 Central sensitization

Several chemical signals in the spinal cord trigger pain transmission in response to incoming noxious stimuli, including SP, glutamate, CGRP and many others neuromodulators. Under normal, non-pathological condition, low-frequency activation of A δ and C fibre nociceptors leads to glutamate release from the central presynaptic afferent nerve terminals in the spinal cord dorsal horn, where the excitatory amino acid induces a short-term activation of glutamate AMPA/kainate receptor subtypes. Although glutamate also binds the NMDA ionotropic glutamate receptor (NMDAR) present on the pre- and postsynaptic neurons, the receptor remains silent, i.e. it does not lead to changes in membrane potential and subsequent pain-projection neuron excitation, because it is plugged by Mg²⁺ [Milligan and Watkins, 2009].

Intense, repeated and sustained activity of primary sensory neurons elicits changes in neuronal and biochemical processing at central synapses and descending projections, transitioning these sites into a pain facilitatory state [Basbaum and Scherre, 2009; Ossipov et al., 2010]. In the spinal dorsal horn, these changes are collectively known as central sensitization and windup.

These processes involve the phosphorylation of a range of receptors, including AMPA and/or kainate receptors, which increases synaptic efficacy by altering channel opening time, increasing burst firing, removing the Mg²⁺-mediated channel blockade at the NMDA receptor, and promoting trafficking of receptors to the synaptic membrane [Latremoliere and Woolf, 2009].

As a consequence of central sensitization, spinal dorsal horn neurons increase ongoing activity, expand their receptive field and increase their responsiveness by lowering of excitation thresholds [Farquhar-Smith, 2008].

During this time, altered low-threshold non-nociceptive A β sensory fibers activate spinal pain-projection neurons, contributing to development of allodynia. SP and CGRP released from primary afferent neurons also contribute to central sensitization by the activation of the NMDA receptor in persistent pain states. Neurokinin A and B acting on neurokinin receptors may influence the NMDA receptors directly by inducing a slow depolarization (by decreased potassium ion conductance).

Nerve growth factor (NGF) induces stimulation of neuropeptide formation and release, which contribute to the development of central sensitization and hyperalgesia. Brain-derived neurotrophic factor (BDNF) is produced by NGF-dependent nociceptors, and its synthesis is increased with inflammation. BDNF

augments spinal neuron excitability by phosphorylation-mediated stimulation of the NMDA receptor [Farquhar-Smith, 2008].

Sustained nociceptor activation caused by peripheral nerve injury and inflammation leads to central sensitization, where enhanced responsiveness of neurons in the spinal dorsal horn is thought to underlie chronic hyperalgesia and allodynia [Chapman et al., 1998; Kidd and Urban, 2001].

1.2 Neuropathic pain

Neuropathic pain is a pathological pain that occurs secondarily to injury of the central and/or peripheral nervous system. The *International Association for the Study of Pain (IASP)* has recently modified its definition as “pain caused by a lesion or disease of the somatosensory system” to emphasize that the injury in the nervous system has to be within the somatosensory system [Treede et al., 2008].

Neuropathic pain is an emerging pathology affecting million people in the world and can be classified as an incurable disease for the lack of valid treatments. In fact it is partially unresponsive to classical analgesics and its treatment with adjuvant drugs, i.e. antidepressant or anticonvulsive, provides only a temporary relief of pain in a small percentage of patients.

This chronic pain is highly invalidating because it directly impacts on lifestyle of sufferers who very often are forced to leave their work influencing the social life and psychological and emotional condition characteristic of the individual. This obviously poses enormous costs to society in terms of healthcare and social care.

For these reasons, nowadays, the development of new and efficacious therapies to contrast painful neuropathy represents a priority.

Neuropathic pain arises from lesions to both the central and peripheral nervous system and in the human many etiologies have been recognized, including [Sacerdote et al., 2013]:

- mechanical nerve injuries/compression;
- spinal cord injuries;
- metabolic diseases (e.g. diabetes);
- viral diseases (e.g. herpes zoster, HIV);
- inflammatory/immunological mechanisms (e.g. Multiple sclerosis);
- alcoholism (vitamin B12 deficiency);
- iatrogenic: cancer chemotherapy(e.g. cis-platinum), anti-viral treatments (AIDS) or anti-tuberculosis medications (isoniazide)
- vascular lesions of the hypothalamus;
- congenital (e.g. Charcot-Marie-Tooth);
- aging.

Whatever the cause, neuropathic patients typically exhibit a mixture of sensory loss with ongoing spontaneous pain and enhanced sensitivity to innocuous stimuli, i.e. mechanical allodynia. Mechanical and thermal hyperalgesia (increased pain response to painful stimuli) are also frequent and classical symptoms [Treede et al., 2008; Zimmermann, 2001; Colleoni and Sacerdote, 2010].

Much of the initial research on the pathophysiological basis of neuropathic pain focused on the plasticity properties of neurons following a nerve injury, leading to the proposal of both peripheral and central sensitisation as important disease mechanisms [Sacerdote et al., 2013].

Peripheral and central amplification is mediated by changes in the expression and distribution of ion channels (sodium and calcium channel); expression of receptors and neurotransmitters; increased neuronal excitability and ectopic generation of action potentials; axonal atrophy, degeneration or regeneration (Wallerian degeneration); damage to small fibers; neuronal cell death and reorganization of central nociceptive circuits [Costigan et al. 2009; Latremoliere and Woolf, 2009]. Loss of spinal inhibitory control and changes in the balance of facilitation/inhibition within descending pain modulatory pathways are other mechanisms which contribute to neuropathic pain development [Tesfaye et al., 2013].

Recently it has emerged that neuropathic pain pathogenesis and maintenance also involve a pathological interaction between neurons, inflammatory immune cells and glia cells, as well as a wide cascade of pro- and anti-inflammatory cytokines [Austin and Moalem Taylor, 2010; Calvo et al. 2012]. Neuronal injury therefore, not only results in profound modifications of the activity of sensory neurons and their central projection pathways, but is also coupled to a sustained immune-inflammatory response at different anatomical locations associated to chronic pain processing, i.e. nerve, DRG, spinal cord and brain [Calvo et al., 2012].

1.2.1 Neuropathic pain triad (neurons, immune cells and glia)

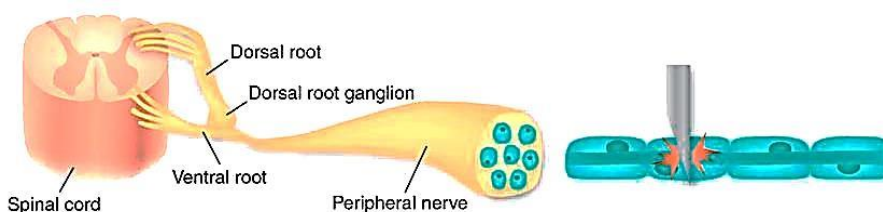


Image 2 | Peripheral nerve injury. [Image modified from Scholz & Woolf, 2007]

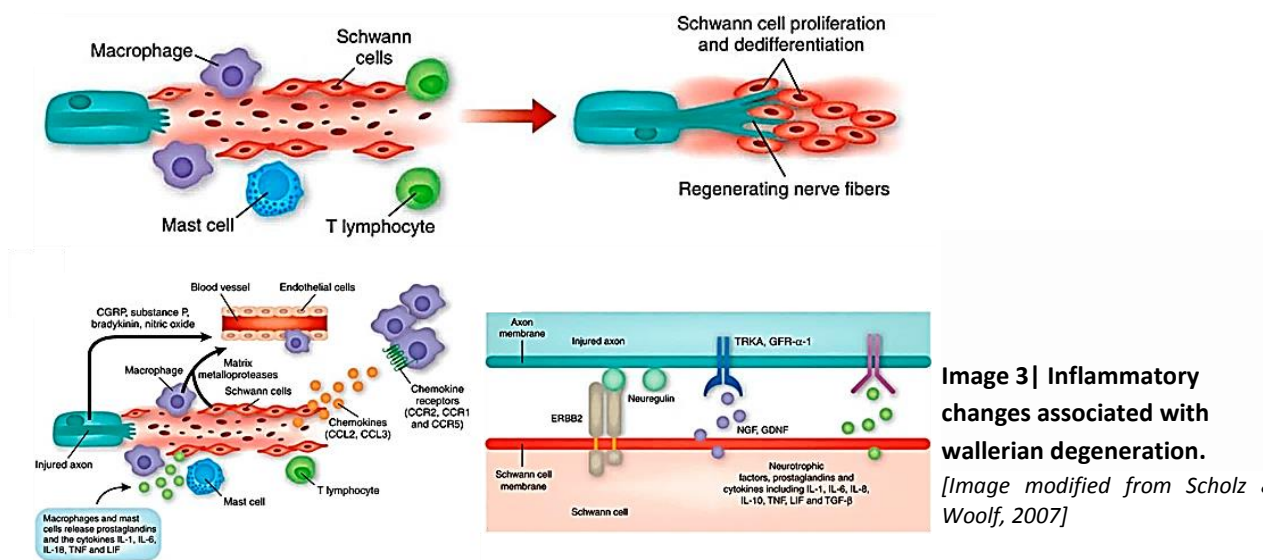
Peripheral nerve injury provokes the recruitment of immune cells as well as the activation of resident cells at the site of injured nerve, in the DRG, and in the spinal

cord. Communication among immune cells and immune-like glial cells along the way of pain transmission is driven by a plethora of immune cell-derived inflammatory cytokines and chemokines, which are crucial mediators for the development and maintenance of persistent pain state.

1.2.1.1 Neuroimmune interactions in injured nerve

Macrophages act as pivotal mediators in the peripheral inflammatory reactions to nerve lesion. Immediately after nerve injury, resident macrophages rush to the lesion site like a rapid-response team [Mueller *et al.*, 2001].

Neutrophils also participate in the very early immune response to nerve injury, particularly during the first 24 hours after injury, reinforcing macrophage recruitment through the release of chemoattractants and cytokines [Perkins and Tracey, 2000].



Activated macrophages and Schwann cells produce matrix metalloproteases that interrupt blood-nerve barrier [Shubayev *et al.*, 2006]. In addition, vasoactive mediators including CGRP, SP, bradykinin and nitric oxide released from injured axons promote further invasion of monocytes and lymphocytes at site of injury by increasing vascular permeability. As results of these vascular changes, two days after injury, a dense cellular infiltrate predominantly composed by macrophages, T lymphocytes and mast cells, forms at the lesion site [Scholz and Woolf, 2007].

Resident and infiltrating immune cells as well as Schwann cells release pro-inflammatory cytokines, such as TNF- α , IL-1 β , IL-6 that contribute to axonal damage, and several other factors including chemokines, PGs and NGF, responsible for the initiation and maintenance of sensory abnormalities after injury.

Macrophages and Schwann cells are also implicated in the Wallerian degeneration of axotomized nerve fibers distal to nerve lesion. Macrophages remove by phagocytosis dead or dying remnants of injured Schwann cells, axotomized axons and myelin debris [Bruck, 1997], thus facilitating the reorganization of Schwann cells in order to activate axonal repair.

Schwann cells, in turn, release chemical signals, such as NGF and GDNF, that promote axonal growth and remyelination [Esper and Loeb, 2004]. However, these growth factors induce pain initiation directly activating and sensitizing nociceptors [Malin *et al.*, 2006].

The inflammatory responses to nerve injury driven by resident and infiltrating cells, particularly by macrophages, directly contribute to pain hypersensitivity. Hyperalgesia is delayed in genetically manipulated WLDs mice, in which Wallerian degeneration and the recruitment of macrophages in response to nerve injury are delayed [Myers *et al.*, 1996; Araki *et al.*, 2004]. Moreover, systemic depletion of macrophages reduces mechanical hypersensitivity after peripheral nerve injury in animal models, revealing their crucial role in the generation of neuropathic pain [Liu *et al.*, 2000].

Uninjured fibers also contribute to the development of pain. Crossing the degenerating environment they change their biologic properties with increased spontaneous activity and up-regulate nociceptive molecules like TRPV1, cytokines and chemokines. They also develop an enhanced responsiveness to pro-inflammatory cytokines.

1.2.1.2 Neuroimmune interactions in DRG

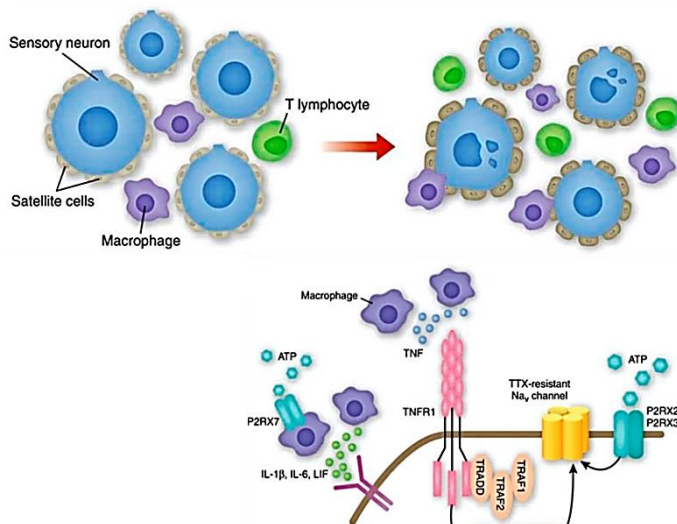


Image 4 | Immune response in DRG.

[Image modified from Scholz & Woolf, 2007]

Normally, macrophages and few T lymphocytes reside in DRG. Their numbers increase after nerve damage. In parallel satellite glia cells begin to proliferate.

These resident immune and glia cells strongly react to nerve injury and their response is reinforced by invading macrophages and T cells. Injury-induced macrophage invasion appears to be triggered by the release of chemokines from DRG neurons [Zhang and

DeKoninck, 2006]. The accumulation of pro-inflammatory cytokines (TNF- α , IL-1 β , IL-6) and chemokines in the DRG after injury contributes to the sensitization of sensory neurons [Levin *et al.*, 2008; Uceyler *et al.*, 2007].

TNF- α acts to increase the density of tetrodotoxin-resistant (TTX-R) sodium channel currents within DRG neurons [Jin and Gereau, 2006], while other cytokines such as IL-6 regulate the synthesis of neuropeptide transmitters. The resultant changes in the phenotype of sensory neurons is likely to alter the efficacy of their synaptic input to the spinal cord [Scholz and Woolf, 2007].

Moreover, it was found that deletion of IL-1 β receptor or IL-1RA overexpression inhibit the development of spontaneous sensory neuron firing, and blocking IL-1 β or IL-6 mediated signalling attenuates neuropathic pain-like behavior [Arruda *et al.*, 2000; Wolf *et al.*, 2006].

1.2.1.3 Neuroimmune interactions in spinal cord

Spinal cord glia activation is a common underlying mechanism that leads to development and maintenance of chronic pain. Microglia and astrocytes have in fact a well-documented role in pain facilitation, modulating neuronal synaptic function and excitability by various mechanisms [Halassa et al., 2007; Pocock and Kettenmann, 2007]. In spinal cord and supra-spinal sites within CNS microglia predominate in the early glial response, subsequently followed by activation and proliferation of astrocytes. CGRP, SP, glutamate and ATP released from the presynaptic terminals of the primary afferents after nerve injury determine the activation of spinal microglia and astrocytes.

Once become activated, these cells release immune mediators which diffuse and bind to receptors on presynaptic and postsynaptic terminals in the spinal dorsal horn to modulate excitatory and inhibitory synaptic transmissions, resulting in nociceptive hypersensitivity.

The release of inflammatory mediators, such as TNF- α , IL-1 β , IL-6, nitric oxide, ATP and prostaglandins initiates in fact a self-propagating mechanism of enhanced cytokines expression by microglial cells. The production and subsequent release of pro-inflammatory cytokines from activated microglia cells leads to further activation of neighboring astrocytes [Watkins and Maier, 2003]. The activation of astrocytes results in the prolongation of a pain state [Dinarello, 1999].

TNF- α , IL-1 β and IFN- γ , chemokines and reactive oxygen species (ROS) directly modulate excitatory synaptic transmission at central terminals by enhancing glutamate release. Their effect is partly due to the activation of transient receptor potential channel subtypes (TRPV1 and TRPA1), and the functional coupling between IL-1 β receptor and NMDAR [Grace et al., 2014].

TNF- α and IL-1 β from astrocytes increase neuronal excitability and synaptic strength by increasing the conductivity of glutamate AMPA and NMDA receptors, as well as by increasing the trafficking and surface expression of glutamate AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) receptors, which render neurons vulnerable to excitotoxicity [Beattie et al., 2002; Stellwagen and Malenka, 2006]. IL-1 β also induces the phosphorylation of specific NMDA receptor subunits (i.e. NR1 and NR2A or NR2B subunits) leading to its activation [Stellwagen et al., 2005; Zhang et al., 2008; Viviani et al., 2003]. NMDA receptor channel opening leads to the influx of calcium and to increased production of NO and PGE2, which are involved in amplifying the excitability of pain-projection neurons [Milligan and Watkins, 2009].

Excitatory synaptic transmission is further indirectly enhanced as the spinal astrocyte glutamate transporters, Excitatory Amino Acid Transporter 1 (EAAT1) and EAAT2 are persistently downregulated after peripheral nerve injury, leading to excitotoxicity and nociceptive hypersensitivity [Xin et al., 2009; Ramos et al., 2010].

Cytokines, chemokines and ROS also decrease GABA and glycine release from interneurons and inhibitory descending projections, leading to spinal cord pain circuits disinhibition, phenomenon known

to be implicated in the genesis of central sensitization and chronic pain [Latremoliere and Woolf, 2009]. Brain-derived neurotrophic factor (BDNF) released as a consequence of microgliosis, also contributes to pain inhibition/ disinhibition by causing a depolarization shift that inverts the polarity of currents activated by the inhibitory neurotransmitter GABA in spinal second-order nociceptive projection neurons [Milligan and Watkins, 2009].

It is also important to highlight that either in peripheral nerve and DGR or in CNS a complex network of regulatory circuits controls immune signalling after neuronal insult. These mechanisms include the production of anti-inflammatory mediators and the polarization of specialized immune and glia cells with an anti-inflammatory phenotype to prevent uncontrolled inflammation [Grace et al., 2014].

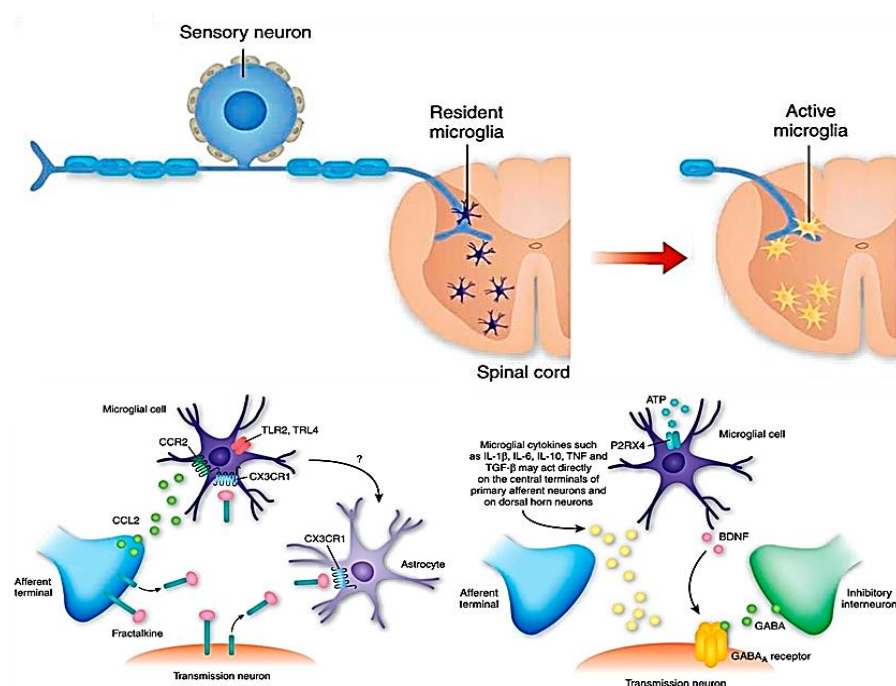


Image 5 | Recruitment and activation of spinal microglia and astrocytes.
Image modified from Scholz & Woolf, 2007]

Alternatively activated microglia (also known as M2 macrophages), sub-populations of T lymphocytes (i.e. Th2 and Treg cells) as well as macrophages contribute to the resolution of nociceptive hypersensitivity after nerve injury, by releasing naturally anti-inflammatory mediators which include IL-10, IL-4 and IL-1RA.

IL-10 and IL-4 cytokines indirectly inhibit the synthesis of pro-inflammatory/pro-nociceptive cytokines and chemotactic factors by microglia, T-cells and macrophages regulating and promoting the differentiation of immune-like cells towards an anti-inflammatory profile.

Intrathecal IL-1RA administration and the elevation of IL-10 and IL-4 levels in spinal cord result in attenuated nociceptive hypersensitivity associated to gliosis [Watkins et al., 1997; Leger et al., 2011].

1.2.2 Pharmacological considerations: present and future

Pain processing involves multi-pathways and dynamic systems in the periphery, spinal cord and brain which expose potential pharmacological targets for analgesia. Some of these are already exploited in clinic by existing analgesics [Farquhar-Smith, 2008].

Opioids are among the most powerful analgesics. Their efficacy can be explained by the large distribution of opioids receptors in the spinal cord and in areas of brain associated to descending pain modulatory pathways as locus coeruleus and PAG. Thus, opioids induce analgesia acting both on transmission and pain perception.

The fact that most of pain inhibitory pathways are noradrenaline/serotonin-based pathways indicate why the antidepressants (inhibitor of noradrenaline/serotonine re-uptake) may be effective in controlling pain.

Anticonvulsants induce analgesia acting on neuron excitability through the blocking of sodium and calcium channels; similarly capsaicin exerts its analgesic effect binding to TRPV1 channel. Non-steroidal anti-inflammatory drugs (NSAID), that prevent the formation of prostaglandins by inhibiting cyclooxygenase (COX), are generally not efficacious in neuropathic pain treatment .

Moreover, considering the key role of the neuroimmune interface in chronic pain there is great interest in targeting immune and glia functions for pain management. Several promising strategies to target the neuroimmune interface include the direct inhibition of pro-inflammatory signalling, the stimulation of local protective anti-inflammatory mechanisms and inhibition of specific immune mediators [Grace et al., 2014].

In this direction, a better knowledge of the mechanisms underlying neuropathic pain could lead to the identification of novel promising targets for the development of more efficacious analgesic therapy.

1.2.3 Classical pharmacological treatments

Pain has a very complex nature. Nowadays there are no drugs for neuropathic pain treatment acting in a complete and definitive way.

Currently, lidocaine, lamotrigine, acetaminophen, dextromethorphan, carbamazepine, gabapentin, valproic acid, opioid analgesics, tramadol hydrochloride, and tricyclic antidepressants are used for the classical pharmacological treatment of neuropathic pain; but neuropathic pain responds poorly to classical analgesics (acetaminophen, NSAIDs and opioids) and the reference treatments are only partially effective. Three molecules have specific authorization in this indication: calcium channels $\alpha_2 \delta$ subunit ligands, gabapentin and its successor pregabalin; and duloxetine, a serotonin and norepinephrine reuptake inhibitor (SNRI).

1.3 Diabetes

Diabetes mellitus (DM) is a group of metabolic disorders characterized by chronic hyperglycaemia with impaired metabolism of carbohydrate, fat and proteins as a result of endogenous insulin deficiency and/or resistance [Davey *et al.*, 2014].

Polydipsia, polyphagia, polyuria, blindness, weight loss or gain, burning and tingling sensation are some of distinct symptoms associated to diabetic status and are common for the two main forms of diabetes: type 1 (DMT1) and type 2 (DMT2) diabetes.

Both types of diabetes are characterized by a progressive failure of pancreatic β -cells but the mechanisms leading to pancreatic cell death are quite different in the various forms of the disease [Cnop *et al.*, 2005]. In type 1 diabetes β -cell destruction arises from an autoimmune assault against pancreatic cells by autoreactive T lymphocytes resulting in chronic pancreatic inflammation (process known as insulinitis) which culminates with an absolute insulin deficiency. During insulinitis, invading immune cells, including Th1 (CD4 and CD8) lymphocytes, macrophages and dendritic cells, participate to destruction of pancreatic β -cells by directly triggering cytotoxic processes or releasing pro-inflammatory cytokines.

IL-1 β , IFN- γ and TNF- α are important pro-inflammatory mediators. These cytokines induce β -cell apoptosis via the activation of pancreatic cell gene networks under the control of different transcription factors, including Nf-kB. The activation of Nf-kB leads to nitric oxide and chemokines production and depletion of endoplasmic reticulum calcium, which ultimately contribute to pancreatic cell destruction. Parallel, anti-inflammatory cytokines, such as IL-4 and IL-10, produced by activated Th2 lymphocytes, prevent β -cell destructive insulinitis, indicating that an imbalance between pro- and anti-inflammatory cytokines could be essential for the development of type 1 diabetes [Amirshahrokhi and Ghazi-Khansari, 2012].

The pathogenesis of type 2 diabetes is more variable than that of type 1 diabetes as it is linked to a combination of genetic and lifestyle factors that result in different degrees of insulin resistance and deficiency. Chronic exposure to elevated glucose and free fatty acids causes β -cell dysfunction and may induce β -cell apoptosis in type 2 diabetes [Cnop *et al.*, 2005]. Islet cell inflammation as result of altered immune activation has since long time been recognized in type 1 diabetes, and now it is increasingly implicated in the pathogenesis of type 2 diabetes leading to defects in β -cell secretion (Das and Mukhopadhyay, 2011).

Inflammation and alteration in immune system thus result as common underpinning mechanisms in the pathophysiology of type 1 and type 2 diabetes as well as of their complications [Agrawal and Kant., 2014].

Elevation of systemic inflammatory mediators was found in patients with type 1 and type 2 diabetes [Agrawal and Kant, 2014; Davey *et al.*, 2014].

The establishment of a persistent hyperglycaemic state in diabetes causes a series of physiological dysfunctions in the organism which, over time, turn into very serious complications responsible of the high rate of morbidity and mortality in diabetes sufferers.

These complications are associated to a progressive, dramatic failure and dysfunction of the vascular system. They are grouped in “macro-vascular complications” and “micro-vascular complications” depending on the vascular district which is affected.

Macro-vascular complications result from damage to arteries that supply the heart, brain, and lower extremities and include accelerated cardiovascular disease, peripheral arterial disease, myocardial infarction, stroke and limb amputation [Hofmann and Brownlee, 2004; Forbes and Cooper, 2013].

The complications resulting from damage to small blood vessels are grouped as microvascular complications. Persistent chronic hyperglycemia resulting in the development of diabetes-specific microvascular complications in the retina, renal glomerulus, and peripheral nerves are characteristic of all forms of diabetes. Microvascular complications are classified into retinopathy, nephropathy, and neuropathy [Davey et al., 2014].

1.3.1 Diabetes complications

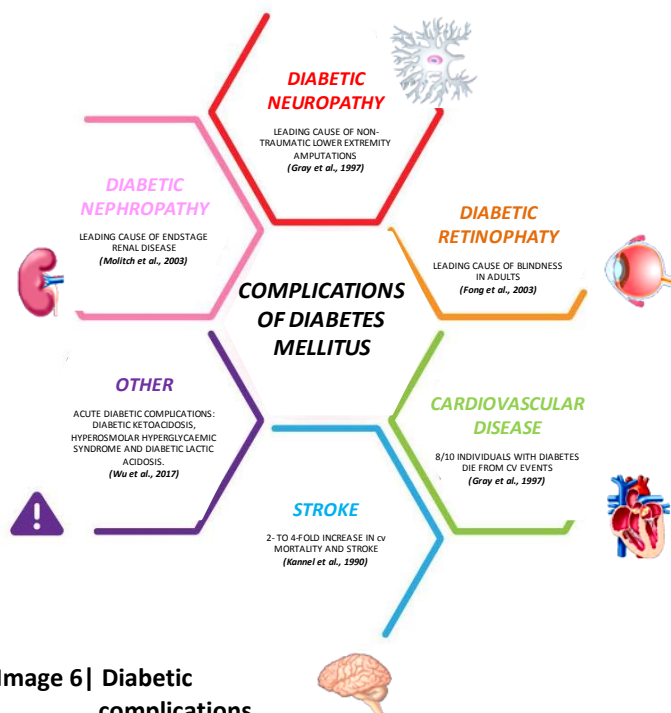


Image 6 | Diabetic complications

As reported above, diabetes mellitus (DM) is a complex metabolic disorder that is associated with insulin resistance (IR), impaired insulin signalling, β -cell dysfunction, abnormal glucose levels, altered lipid metabolism, sub-clinical inflammation and increased oxidative stress [Testa et al., 2016]. Acute diabetic complications occur in the early stage of diabetes and include hyperglycaemia, diabetic ketoacidosis, hyperosmolar hyperglycaemic syndrome and diabetic lactic acidosis. Chronic diabetic complications that occur in the later

developmental stage of diabetes include cerebral vascular disease, diabetic coronary artery disease (CAD), diabetic peripheral neuropathy (DPN), diabetic retinopathy (DR), diabetic nephropathy (DN), lower extremity vascular disease and diabetic foot disease .

1.3.1.1 Painful diabetic neuropathy

Diabetic neuropathy is one of the most frequent long-term complications of diabetes. It affects about 60% of diabetic population and is a source of morbidity and mortality in diabetic patients [Aslam *et al.*, 2014]. This type of peripheral nervous disorder is characterized by a progressive neuronal death, demyelination and suppression of the nerve regeneration mechanisms, resulting in an impaired nerve functioning.

Nerve damage can involve both the autonomic and the sensorimotor divisions of the nervous system, so every nerve fiber in the body is potentially vulnerable.

Diabetic neuropathic patients typically experience lack of sensibility in several areas of the body and lose the capacity to perceive the temperature or even painful stimuli, with a consequent impairment of the quality of life. Further, the progressive loss of innervations can lead to atrophy of essential pedal muscles, resulting in deformities that predispose the patient to ulceration and in the more severe cases to lower extremities amputation [Duby *et al.*, 2004]. Severity of symptoms increases gradually over time and correlate with the degree of hyperglycaemia [Han *et al.*, 2013].

There are many types of diabetic neuropathy. Depending on the organ systems the types of nerves affected and the entity of the nerve damage, which can be diffused or well localized, diabetic neuropathies are classified in: diffuse somatic neuropathy, autonomic neuropathy and focal neuropathy. Among these, the sensorimotor neuropathy or distal symmetrical polyneuropathy (DSPN) is the most common form.

DSPN affects both large and small sensory fibres resulting in a mixture of symptoms and sensory loss. The onset of the neuropathy is usually gradual and insidious and is heralded by sensory symptoms that start from the most distal extremities of the limbs (toes and fingers) and progress proximally in a symmetrical “glove and stocking” distribution [Tesfaye *et al.*, 2013]. The syndrome involves initially the lower limbs.

Approximately 50% of patients with DSPN exhibit neuropathic pain symptoms, which include uncomfortable tingling in the lower limbs (dysesthesia), spontaneous and evoked pain and numbness. Unusual sensations such as feeling of swelling of the feet or severe coldness of the legs when clearly the lower limbs look and feel fine, odd sensations on walking likened to “walking on pebbles” or “walking on hot sand” are other characteristic clinical manifestations of the disease [Tesfaye *et al.*, 2013]. The pain is often worse at night and interferes with normal sleep causing tiredness during the day. The constant unremitting pain negatively impacts on the quality of life of diabetic patients resulting in form of depressions; in some cases patients experience loss of appetite and body weight known as “diabetic neuropathic cachexia” [Aslam *et al.*, 2014].

The exact pathophysiology of diabetic neuropathic pain is not fully understood. However, the abnormalities of pain processing in the peripheral and central nervous system which are supposed to

contribute to the development and maintenance of neuropathic pain could be related to hyperglycaemia, as this is the classic metabolic abnormality of diabetes [Aslam *et al.*, 2014].

1.3.1.1.1 Pathogenesis of diabetic neuropathy

Despite decades of intensive researches, the pathogenesis of the diabetic neuropathy has not been yet fully elucidated. Hyperglycaemia is considered to be one of the major pathophysiological determinants of the disease. However, the cause of this syndrome is more complex than dysregulated glucose levels alone [Han *et al.*, 2013]. Several factors have been postulated to participate in the diabetic neuropathy pathogenesis, including microcirculatory dysfunction, impaired insulin signalling, growth factor deficiency and inflammation.

All these abnormalities are intertwined through numerous competing or parallel pathways and are supposed to contribute to a pathological self-perpetuating cycle of oxidative stress, inflammation and cellular dysfunction ultimately resulting in the progression of neurovascular disease associated to loss of nerve fibers.

Polyol pathway, advanced glycation end-product (AGE) production, poly-ADP ribose polymerase (PARP) over activation, protein kinase C (PKC) and altered Na⁺/K⁺ ATPase activity are some of the molecular mechanisms underlying diabetic neuropathy [Sandireddy *et al.*, 2014].

- **Hyperglycaemia**

Prolonged hyperglycaemic state contributes to cell damage and thus to neuropathy, through the generation of oxidative stress, as the final result of increased glycolysis, polyol pathway activity and generation of AGE products. Excess of glycolysis overloads the mitochondrial electron transport chain promoting the production of reactive oxygen species (ROS), while the abnormal high rate glucose “flux” through the polyol pathway leads to NADPH depletion causing oxidative stress.

Accumulation of reactive oxygen species (ROS) increases lipid, DNA and protein peroxidation, induces cellular apoptosis, reduces nerve blood flow (NBF) and induces impairment of vasodilation of epineural blood vessels, which results in ischemia to the neural tissue. Oxidative stress also leads to deterioration of Schwann cells, which play a key role as a provider of insulation for neurons, immunologic perineurial blood nerve barrier, and effector of nerve regeneration [Han *et al.*, 2013].

The production of AGEs can impair cellular function by altering the structure and so the biological function of essential proteins. Moreover, binding to receptors (RAGE), AGEs can trigger an inflammatory cascade that involves the activation of MAPK and PKC pathways and that ultimately generate oxidative stress [Duby *et al.*, 2004].

- **Impaired insulin signalling**

Insulin is essential for general neuronal function as it promotes neuronal growth and survival. Insulin receptors are present in DRG sensory neurons and in peripheral axons sustaining epidermis and after physical injury of peripheral nerves they increase [Guo *et al.*, 2011].

In diabetic mice, local or intranasal insulin administrations improve sensory nerve fiber density in the plantar foot pad and mechanical sensation [Francis *et al.*, 2009].

In DMT1 patients, the reduction of C-peptide, marker for pancreatic β -cells functionality, contributes to nerve dysfunction by reducing the activity of Na^+/K^+ ATPase and eNOS and the endoneurial blood flow.

Treatment with C-peptide can slow down the progression of neuropathy [Ekberg and Johansson, 2008]. Tight glucose control with insulin supply can also reduce neuropathy in DMT1 patients. However, the reduction of glucose levels is not enough to block the vicious cycle of oxidative stress, inflammation and cellular damage triggered by hyperglycaemia [Han *et al.*, 2013].

- **Vascular and growth factor deficiency**

Various studies report a major pathophysiological role of vascular and neurotrophic supply in diabetic neuropathy.

Maintaining adequate blood supply to nerves is crucial for maintaining nerve structure and function [Han *et al.*, 2013]. Deficiency in the nerve blood perfusion resulting in ischemic hypoxia largely contributes to pathogenic mechanisms of diabetic neuropathy as it determines malnourishment of nerve and thus neuronal dysfunction.

Pathologies of nervous and vascular system are highly intertwined in diabetes. As the disease progresses, neuronal dysfunctions correlate closely with the development of vascular abnormalities. Diabetic foot is a common complication for diabetic people associated with epidermal thickness alteration, recent studies demonstrated that in pure diabetic patients the average epidermal thickness of plantar skin was increased (capillary basement membrane thickening and endothelial hyperplasia) while was decreased in people with diabetic neuropathy [Chao *et al.*, 2011; Doby *et al.*, 2004]. Impaired vasodilation in diabetic epineurial arterioles (caused by ROS) decreases nerve conduction velocity.

Neurotrophic support has also an important role in diabetic neuropathy. Reduced neurotrophic supply in experimental diabetes was found to contribute to nerve malnourishment and neuronal dysfunction [Ekberg and Johansson, 2008].

Many growth factors exert both neurotrophic and angiogenic effects. In ischemic tissues, VEGF induces angiogenesis by stimulating the proliferation and migration of endothelial, thus improving tissue ischemia. It also promotes axonal outgrowth and survival of neurons and Schwann cells in

DRG. Like VEGF, IGF induces vessel remodelling and has neurotrophic effect. It also stimulates Schwann cell mitogenesis and myelination. NGF, a well-known neurotrophic factor, promotes survival and differentiation of sensory and sympathetic neurons [Han et al., 2013]. Moreover it provides neuroprotective and repair functions. In addition to these neurotrophic effects, NGF directly induces angiogenesis [Kim et al., 2012].

- **Neuroinflammation**

Oxidative stress in combination with the activation of the classic metabolic pathways mentioned above, especially the MAPK signalling and the increased production of AGEs, can directly or indirectly initiate and progress the production of inflammatory mediators leading to neuroinflammation and thus nerve damage.

Activation of RAGE on microglia and macrophages initiates an inflammatory cascade through the activation of the transcriptional factor Nf- κ B, a potent inducer of the inflammatory processes [Yan et al., 1994]. The activation of this transcriptional factor results in the up-regulation of genes encoding for pro-inflammatory cytokines (IL-1 β , TNF- α and IL-6) and chemokines, induction of neuronal apoptosis, and suppression of antioxidant genes with a consequent weakening of the innate antioxidant defence [Ganesh et al., 2013].

A persistent hyperglycaemic state also induces neuroinflammation by affecting the structural features of neurons; the glycosylation of myelin proteins alters their antigenicity and causes the infiltration of monocytes, macrophages, neutrophils from the blood circulation as well as the activation of glia cells [King, 2001; Shi et al., 2013].

Moreover, NADP depletion mediated by PPARs (Peroxisome proliferator-activated receptors) overactivation leads to bioenergetic failure driving the cells towards necrosis. This mechanism contributes to neuroinflammation as the release of cellular debris by necrotic cells determines the recruitment of further inflammatory cells to injury site which enhance the local inflammatory response [Szabò, 2003].

Inflammatory cytokines released by either resident or infiltrating cells mediate damages to myelin sheets and increase nerve excitability leading to edema and neuroinflammation. Moreover, inflammatory cells have a vicious positive feedback loop for increasing further production of inflammatory mediators thus potentiating nerve derangement [Sandireddy et al., 2014].

Hypoxia and ischemia created in diabetes aggravate the neuroinflammation by inducing iNOS activity, which is responsible for the release of nitric oxide (NO), another important mediator of inflammation.

1.3.1.2 Diabetic nephropathy

In the diabetic milieu, metabolic derangements and hemodynamic alterations, particularly activation of the renin–angiotensin system, trigger a number of cell signaling cascades, including the MAPKs (p38 and JNK) and PKC- β , which mediate a cellular response through activation of key transcription factors such as NF- κ B. In response to such signals, renal cells such as tubular epithelial cells, podocytes, and mesangial cells can produce chemokines, growth factors, and profibrotic cytokines. CSF-1 and MCP-1 function as chemotactic molecules and promote the recruitment of monocytes from the circulation. Upregulation of ICAM-1 on endothelial cells – a key leukocyte adhesion molecule - facilitates infiltration of circulating mononuclear cells into the kidney. CSF-1 also promotes monocyte/macrophage differentiation, proliferation, and activation. MIF functions to retain macrophages at sites of inflammation and has counter-regulatory functions against the anti-inflammatory actions of glucocorticoids. Activated macrophages can produce proinflammatory and profibrotic cytokines, reactive oxygen species, and antiangiogenic factors and contribute to a cycle of inflammation, oxidative stress, cellular injury, progressive fibrosis, and loss of glomerular filtration rate. Podocyte loss, endothelial dysfunction, alterations in the tubular injury contribute to increasing proteinuria during the development and progression of diabetic nephropathy. Diabetic nephropathy or diabetic kidney disease is a syndrome characterized by the presence of pathological quantities of urine albumin excretion, diabetic glomerular lesions, and loss of glomerular filtration rate (GFR). This diabetes complication is characterized by structural and functional changes: in glomeruli, there is mesangial expansion, thickening of the basement membrane, and, characteristically, nodular glomerulosclerosis (Kimmelstiel–Wilson nodules). Initially tubular hypertrophy is present but eventually interstitial fibrosis with tubular atrophy develops, along with arteriolar hyalinosis. In advanced cases, there is an infiltrate of macrophages and T-lymphocytes. Ultrastructurally, there is podocyte loss and reduced endothelial cell fenestration [Toyoda *et al.*, 2007]. Functionally, there is early glomerular hyperfiltration and increased albumin excretion; and with advancing nephropathy, increasing proteinuria and declining GFR. Pathways that promote this pathology are many, complex, overlap and interact with one another and enhance one another's biophysiological effects.

- **Hemodynamic factors**

There is an imbalance in afferent and efferent arteriolar resistance, resulting in increased glomerular hydrostatic pressure and hyperfiltration. Activation of the renin–angiotensin system (RAS) increases angiotensin II levels, leading to efferent arteriolar vasoconstriction and production of proinflammatory and profibrotic molecules through multiple mechanisms. High angiotensin converting enzyme (ACE) levels are associated with greater albuminuria and nephropathy in diabetic mice and humans [Huang *et al.*, 2001]. Increased levels of endothelin-1 and urotensin II also

contribute to vasoconstriction. Various dysregulation of nitric oxide and nitric oxide synthase has been described in DN. Nitric oxide mediates endothelium-dependent vasodilatation, and is formed from L-arginine by endothelial nitric oxide synthase. Diabetic endothelial nitric oxide synthase knockout mice develop more severe glomerular lesions and proteinuria compared to wild-type mice [Kanetsuna et al., 2007].

- **Metabolic factors**

Oxidative stress and generation of reactive oxygen species (ROS) damage DNA and protein, or function as signaling amplifiers to activate cellular stress pathways such as PKC, MAPK, and NF- κ B [Haneda et al., 1997; Ha and Lee, 2000]. Activation of the polyol pathway, with aldose reductase converting excess glucose to sorbitol, and subsequent conversion to fructose by sorbitol dehydrogenase contributes to oxidative stress by increasing the NADH/NAD⁺ ratio [Srivastava et al., 2005; Williamson et al., 1993]. A recently described novel mechanism of injury also involves endogenous fructose production with activation of fructokinase in the proximal tubule [Lanaspa et al., 2014]. The formation of advanced glycation end-products (AGE) by nonenzymatic binding of glucose to proteins, lipids, and nucleic acids can lead to alteration of protein structure and function, oxidative stress, and expression of proinflammatory cytokines and growth factors [Sheetz and King, 2002].

- **Growth factors/cytokines**

Activation of TGF- β and its downstream cytokine, CTGF (Connective tissue growth factor), induce extracellular matrix formation and fibrosis. In kidney biopsies, glomerular expression of TGF- β 1 and CTGF were higher in diabetics compared to controls, and correlated with albuminuria. PDGF expression is also increased in DN, which can modulate chemotaxis, vascular tone, and platelet aggregation. VEGF is crucial in angiogenesis but also mediates vasodilatation and leukocyte trafficking in DN [Lim, 2014].

- **Cell signaling and transcription factors**

Increased renal gene transcription of PKC- β showed a strong relationship with glycemic control [Langham et al., 2008]. PKC activation has wide ranging effects, including enhancing angiotensin II actions, nitric oxide dysregulation, endothelial dysfunction, and activation of MAPK and NF- κ B [Noh and King, 2007; Derubertis and Craven, 1994]. MAPKs are intracellular kinases which integrate cell signaling into cellular responses. MAPKs activate a number of nuclear transcription factors, including NF- κ B, which then regulates the gene expression of various cytokines, chemokines, and adhesion molecules. The activation of p38 α isoform of the p38 MAPK pathway is most strongly associated

with renal inflammation and DN [Sakai *et al.*, 2005; Adhikary *et al.*, 2004]. There may also be a role for toll-like receptors (TLR2, TLR4) and B7-1 costimulatory signaling in modulating inflammation and injury in DN [Fiorina *et al.*, 2014; Mudaliar *et al.*, 2013]. Finally, transcription factors bind to the promoter regions of genes and modulate transcription of messenger RNA. NF- κ B has been the best studied in DN. Activation of NF- κ B in both human peripheral blood mononuclear cells and kidney biopsies correlate with severity of proteinuria and glycemic control [Schmid *et al.*, 2006; Hofmann *et al.*, 1998]. A review of transcription factors in DN is provided by Sanchez and Sharma [Sanchez and Sharma, 2009].

- **Inflammation**

In diabetic nephropathy, there is recruitment and activation of innate immune cells and elaboration of proinflammatory cytokines [Lim and Tesch, 2012]. Macrophages and T-lymphocytes are prominent in early diabetic glomeruli while an interstitial infiltrate develops later. Strategies impairing kidney leukocyte recruitment, proliferation, or activation have demonstrated that macrophages mediate DN [Chow *et al.*, 2007; Lim *et al.*, 2009]. In humans, kidney macrophage accumulation is associated with the severity of glomerulosclerosis [Furuta *et al.*, 1993]. Accumulation of interstitial macrophages correlated strongly with proteinuria, interstitial fibrosis, and GFR decline [Nguyen *et al.*, 2006]. The role of lymphocytes is less clear. A higher circulating level of activated T-cells is associated with DN [Bending *et al.*, 1988]. A kidney T-cell influx is common in early type 1 diabetes, and correlates with renal function and albuminuria [Moriya *et al.*, 2004]. However, absence of lymphocytes did not prevent fibrosis and declining renal function in experimental DN [Lim *et al.*, 2010]. Recent attention has focused on the subset of regulatory T-cells (Treg), which may play a protective role in DN. Treg numbers are increased in diabetic mice [Lim *et al.*, 2010]. Treg depletion in diabetic mice exacerbated albuminuria and hyperfiltration, while adoptive transfer of Treg improved DN [Eller *et al.*, 2011]. In type 2 diabetics, the number of Tregs as determined by flow cytometry showed an inverse correlation with albuminuria, particularly in patients with macroalbuminuria [Xu *et al.*, 2009]. Treg also demonstrated an anti-inflammatory function, which reduces the metabolic abnormalities and insulin resistance in a mouse model of type 2 diabetes [Ilan *et al.*, 2010]. The main proinflammatory cytokines implicated in DN are TNF- α , MCP-1, ICAM-1, IL-1, IL-6, and IL-18. These cytokines are increased in diabetic patients and show correlation with albuminuria and glomerular pathology [Lim and Tesch, 2012].

1.4 Rodent models of painful diabetic neuropathy

Although intensive researches over the last decades have contributed to make light of some of molecules, receptors, channels and pathways mainly associated to diabetic neuropathy, a clear comprehension of the mechanisms underlying the development and maintenance of neuropathic pain is unfortunately far. Important advances in the study of neuropathic pain have been achieved using animal models, excellent systems to simulate the clinical pain conditions observed in human and to test novel therapeutic agents for contrasting this intractable pain. Even if most of animal models of neuropathic pain were initially generated in rats as preferred species, now the majority of pain models have been transposed in mice for the availability of genetically characterized or manipulated inbred strains in which specific proteins or signal transduction components have been altered throughout genetic knockout technology [Colleoni and Sacerdote, 2010].

According to the huge etiology of human diabetic neuropathy different types of well-characterized animal models have been developed over time in order to resemble as closely as possible the heterogeneity of the pain manifestations.

1.4.1 Models of diabetes-induced peripheral neuropathic pain

Experimental models of diabetes exhibit behavioural responses similar to those present in diabetic neuropathic patients, including spontaneous pain, decrease in mechanical nociceptive thresholds and/or hypoalgesia characterized by decreased responses to mechanical and thermal stimuli [Colleoni and Sacerdote, 2010].

Experimental rodent models of diabetes include [Leither and Herrath, 2004; Roep et al., 2004]:

- **Spontaneous diabetes:** T1DM insulinopenic BB /Worcester rats, T2DM hyperinsulinemic BBZDR / Worcester rats, NOD mice, LETL rats, Akita mice spontaneous T1DM;
- **Diabetic animals obtained by dietary manipulations:** overeating, fasting, shift from a high fat diet to a high carbohydrate diet and High-fat diet-fed Mice;
- **Diabetic animals obtained by genetic manipulations:** Zucker diabetic fatty rat, Obese leptin-deficient (ob/ob) Mice, Leptin receptor deficient (db/db) mice, nonobese diabetic mice (NOD);
- **Chemo-induced pancreatic toxicity:** streptozotocin (STZ) and alloxan (ALX) - induced diabetes.

1.4.1.1 Experimental Models of T1DM Induced by Chemical Pancreatectomy

Two agents can be used to induce chemical pancreatectomy, both are glucose analogs: ALX, a pyrimidine derivative (synthesized in 1938) and STZ, an alkylating and antimicrobial agent. Chemical properties of these compounds are crucial for their ability to induce diabetes [Lenzen, 2008]. Both are

hydrophilic and cannot cross plasma membrane. They use the glucose transporter GLUT2, which is expressed by the pancreatic β -cells. Cytotoxic effects of ALX are due to its reduced reaction product, dialuric acid, and to the production of reactive oxygen species (ROS) [Szkudelski, 2001] while STZ exerts its toxicity through DNA alkylation [Szkudelski, 2001]. Protein glycosylation is an additional deleterious factor. STZ induces ADP polymerase over-stimulation leading to a decrease in NAD⁺ as well as in ATP concentration and leads to the activation of apoptotic program that destroys β -cells and all the cells expressing the GLUT2 transporter (including cells from the kidney and liver). By performing a bibliography research using the database MEDLINE (PUBMED) and the following keywords: “diabetes” and “alloxan” or “streptozocin” and “neuropathy” during the last 30 years (i.e. 1982 to 2012), 298 studies used the antimicrobial agent STZ and only 48 used ALX to induce diabetes. During the last 10 years (i.e. 2002 to 2012) the ratio ALX:STZ was 9:139 probably due to the poor specificity of alloxan compared to STZ against pancreatic β -cells. Indeed STZ generally produces greater cytotoxicity due to its conversion to anionic radicals. STZ is more commonly used because of its greater stability and relative lack of extrapancreatic toxicity [Lee *et al.*, 2010]. Thus, we focused on STZ-induced diabetic neuropathy in mice.

1.4.1.1.1 Clinical signs of STZ-induced diabetes in experimental models

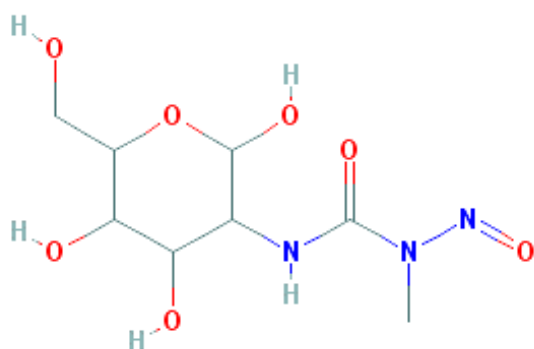


Image 7 | Streptozotocin 2D structure [PubChem].

After STZ administration, hyperglycemia and hypoinsulinemia appear in the first days and persist, attesting to an irreversible toxicity. A halt in weight growth or a weight loss are also observed, moreover sometimes decreased weight of the internal organs (such as kidney, liver and pancreas) has been observed [Brini *et al.*, 2017; Courteix *et al.*, 1994; Zafar and Naeem-ul-hassan Naqvi, 2010].

Hyperglycemia is concomitant with polydipsia (water intake 10-times higher), polyuria and polyphagia [Rondon *et al.*, 2010]. While most morphological, histological and electrophysiological studies show that diabetic neuropathy is accompanied by nerve structural changes (segmental demyelination and axonal degeneration) and functional changes (assessed by nerve conduction velocity) in diabetic patients [Said, 2007], structural changes are rarely reported in STZ-induced diabetic mice or appear slowly and later. Tibial nerve biopsies from diabetic rats [Walker *et al.*, 1999] reported the lack of abnormal nerve tissue regarding the distribution of unmyelinated axons, diameter of myelinated axons, fascicular area, absence of Wallerian degeneration. However, abnormalities in the structure of endoneurial capillaries presented increased luminal surface and decreased endothelial cells size, related to impairment in vaso nervorum.

1.4.1.1.2 Hypersensitivity in STZ-induced diabetic models

Behavioral studies assessed in STZ-diabetic model often focus on their response to nociceptive or non-nociceptive stimuli, because of the absence of quantifiable signs of spontaneous pain. These tests consist in measuring time latency or withdrawal thresholds of an animal whose paw or tail is exposed to a thermal, mechanical or tactile stimulation.

Among these behavioural tests, an interesting one consists in placing the animal on plates where the animal can spontaneously choose between two temperatures [Moqrich *et al.*, 2005]. This test presents the advantage of getting rid of animal handling, therefore allowing the assessment of spontaneous behavior towards a range of thermal stimuli, leaving the animal free to stand on one of the two plates of different temperatures.

The use of this test has allowed to reveal the presence of a thermal hypersensitivity/hyperalgesia (for a temperature > 45°C) in STZ animals [Pichon *et al.*, 2010]. However, thermal hyperalgesia to high temperatures is not a common painful symptom in diabetic patients, which makes difficult the extrapolation of these results toward clinic [Gold *et al.*, 2006]. Some authors, using the thermal ramp test that consists in placing the animal on a surface where the temperature increases of 1°C/sec from 30°C to 50°C, have observed hypersensitivity during the first few weeks of diabetes, but a transformation into a hypoalgesia 1 to 3 month later, signing an evolution of neuropathy toward an insensitive neuropathy to hot stimuli, similarly to what can be found in humans [Guy *et al.*, 1985], and consistent with the loss in thermal nociceptors that was reported in diabetic patients [Kennedy and Wendelschafer-Crabb, 1996].

Altered perception of tactile stimuli (light touch) and mechanical (pressure) are principal symptoms in diabetic patients. In STZ mice, the application of a von Frey filament, producing a light static touch, causes a paw withdrawal induced by the inappropriate activation of A δ and C fibers, signing a tactile allodynia. Cotton swabs or brushes also have been used to measure dynamic tactile allodynia by caressing the plantar surface of the hind paw of the animal, which evokes a paw withdrawal if A β fibers are impaired. The comparison of the two different symptoms reveals that dynamic allodynia has a later onset than static allodynia and both painful symptoms worsen over time in STZ rats [Brini *et al.*, 2017; Castelli *et al.*, 2016; Fields and Basbaum, 1999]. The search for a chemical sensitivity (such as formalin) in diabetic mice, that could, at best, mimic human inflammatory hypersensitivity observed in clinic, revealed an increase in the tonic response while the phasic response is not altered by the chemical agent [Calcutt *et al.*, 1996]; moreover diabetic patients are hypersensitive to cold stimuli [Ziegler *et al.*, 1988] and recent studies demonstrated that also this parameter may be to evaluated in STZ-mice by acetone test, since this behavioural test showed that STZ-mice had elevated sensitivity threshold to the cold stimuli [Flatters and Bennett, 2004; Brini *et al.*, 2017].

1.4.1.1.3 Pathophysiology of STZ-induced neuropathic pain

Pain associated with nerve damage from diabetes initially involves peripheral mechanisms causing sensory fibers hypersensitivity, which secondarily leads to central rearrangements responsible for central nociceptive system hyperexcitability. In this section, we discuss the main peripheral and central mechanisms of diabetic neuropathic pain in STZ-mice model [Lim, 2014].

- **Peripheral changes:**

Involvement of voltage-dependent calcium channels Cav. The T-type Cav channels (“LVA” low voltage activated) or CaV 3.1, 3.2 and 3.3 are localized in cell bodies and dendrites of primary afferent fibers, and play an important role in modulating the neuronal excitability [Cao, 2006]. Their involvement in the pathophysiology of neuropathic pain has also been demonstrated, particularly in models of diabetes and traumatic neuropathies by sciatic nerve ligation, where current density of type T is greatly increased [Jagodac et al., 2007]. The “knockdown” strategy by CaV3.2 isoform antisense but not the CaV3.1 or CaV3.3 isoforms, suppresses thermal (Hargreaves test) and mechanical hypersensitivity (applying a von Frey filament # 4.93) in STZ-diabetic model. Electrophysiological recording from small cells (C fibers) of dorsal root ganglia (DRG) and spinal cord (whole cell voltage-clamp) shows that the same strategy inhibits the “up-regulation” of T-type currents induced by diabetes [Messinger et al., 2009]. Finally, over-expression of the $\alpha 2\delta$ subunit of L-type calcium channels belonging to the family of “HVA” (high activation threshold) in the DRG of diabetic mice is contemporary with the development of tactile allodynia appreciated by the test of von Frey filaments [Yusaf et al., 2001]. This $\alpha 2\delta$ subunit is also the pharmacological target of certain antiepileptic drugs such as gabapentin and pregabalin.

- **Involvement of voltage-dependent sodium channels NaV:**

Peripheral nerve injury can alter the expression and function of NaV channels α subunits which results in a change in neuronal excitability [Dickenson and Bee, 2010]. It has been showed that four weeks after induction of diabetes by STZ, the NaV currents sensitive (S) and resistant (R) to tetrodotoxin (TTX) increased in small diameter DRG [Hong et al., 2004]. Quantification by Western blotting of different types of sodium channels showed an increased expression of NaV1.3 and NaV1.7 (TTX-S) channels and a decreased expression of NaV1.6 (TTX-S) and NaV1.8 (TTX-R) channels in DRG of diabetic mice (four weeks post-STZ). These authors also reported that phosphorylation of Thr / Ser residues of NaV1.8 and NaV1.6 channels, and Tyr residues of NaV1.7 and NaV1.3 channels is increased by diabetes. Sensitive or resistant TTX Nav channels play an important role in the pathophysiology of neuropathic pain of all etiologies, including diabetic, by changing the electrical properties of the membrane, thus contributing to the genesis of ectopic discharges. These channels

are also the target of different molecules (tricyclic antidepressants, anticonvulsants, local anesthetics etc.) whose therapeutic efficacy in the treatment of neuropathic pain is established.

- **Involvement of Transient Receptor Potential (TRP) channels:**

Thermal sensitivity observed in STZ-treated animals [Pichon *et al.*, 2010] is probably due to the sensitization of cutaneous nociceptors associated with A δ and C fibers. TRPV1 channel (Transient Receptor Potential Vanilloid type 1), is a major actor in thermal sensitivity, predominantly present in C fibers and, to a less extent, in A δ fibers [Kamei *et al.*, 2001; Pabbidi *et al.*, 2008b; Rashid *et al.*, 2003]. TRPV1 is a non-selective calcium/sodium-permeable channel activated by temperatures up to 43°C, capsaicin (extracted from red pepper), protons (pH < 5.9), metabolites of arachidonic acid etc; TRPV1 can be sensitized by phosphorylation, by prostaglandins, bradykinin, glutamate, histamine, serotonin, ATP or NGF. Any change in TRPV1 expression, associated with changes in intracellular signal transduction, may lead to spontaneous neuronal activity induced by normal body temperature; this is the case if the response threshold of TRPV1 is lowered below 38°C [Biggs *et al.*, 2008]. The expression of TRPV1 channels in A δ and C fibers of STZ-treated mice was increased in those presenting hyperalgesia, and reduced in hypoalgesic mice. The same team also showed that thermal hypersensitivity developed by diabetic wild-type mice is abolished when the gene coding for TRPV1 channel is disabled (TRPV1^{-/-} mouse). Finally, treatment with anti-vanilloid VR1 receptor antiserum abolishes thermal hyperalgesia in STZ-treated mice [Kamei *et al.*, 2001]. In physiological conditions, it was shown that insulin positively modulates the activity and expression of TRPV1 channels via protein kinase C (PKC) [Pabbidi *et al.*, 2008a]. It is therefore possible that the sudden decrease in insulin levels induced by STZ is indirectly responsible for a decrease in TRPV1 activity, which would lead to a compensatory increasing of the expression of these channels. Another hypothesis suggests a direct action of STZ onto sensory neurons, involving the ROS-p38 MAPkinase pathway, thereby altering expression and function of TRPV1 [Pabbidi *et al.*, 2008b].

A second TRP channel, TRPA1 (Transient Receptor Potential Ankyrin type 1) seems to be involved in DPN, since some studies showed that TRPA1 antagonists changed mechanical thresholds in STZ-treated animals [Koivisto *et al.*, 2012; Wei *et al.*, 2009]. Moreover, the TRPA1 channel can be activated in sensory neurons by ROS, alkenyl aldehydes and 15-deoxy-prostaglandin J₂, which are generated during oxidative stress leading to intracellular calcium rise [Andersson *et al.*, 2008; Nishikawa *et al.*, 2000]. Hence, TRPA1 receptor through indirect activation by metabolites from oxidative stress seems to be an important molecular protagonist in mechanical hypersensitivity of diabetic neuropathy.

- **Involvement of HCN channels:**

Described for the first time in pacemaker cells of the heart sinus node, HCN (hyperpolarization activated cyclic nucleotide-gated cation) channels were discovered in neurons and responsible for I_h currents (Hyperpolarization-activated current) [Jiang *et al.*, 2008]. HCN channels open when the membrane is hyperpolarized (-60 to -50 mV, i.e. to the rest potential) and generate a mixed Na⁺, K⁺ cationic current. Four genes coding for HCN channels have been identified (HCN1-4). The most abundant in neurons of DRG are the HCN1/2 type. The I_h current generated is of greater amplitude, faster and more frequent in neurons of large and medium diameter (type A) than in small diameter neurons (type C). The administration of a HCN channel blocker, the ZD7288, suppresses tactile allodynia in STZ-induced diabetic rats (three weeks post-STZ) and reduces mechanical hypersensitivity as well [Wattiez *et al.* 2011], whereas ivabradine, a more selective blocker of HCN channels, suppresses cold allodynia in a model of toxic neuropathy induced by oxaliplatin [Descoeur *et al.*, 2011].

- **Central changes**

Involvement of N-Methyl-D aspartate (NMDA) receptors: dizocilpine, memantine or D-CPP, NMDA receptor non-competitive and competitive antagonists respectively induce analgesic effects in vivo, on mechanical hypersensitivity in STZ rats [Chen *et al.*, 2009; Courteix *et al.*, 2007; Malcangio and Tomlinson, 1998] NMDA receptor phosphorylation would be involved in the development of tactile allodynia, mechanical and thermal hypersensitivity [Rondon *et al.*, 2010]. It has been also demonstrated the importance of the specific activation of certain isoforms of MAPKinases in painful hypersensitivity in STZ animals, as well as the need for NMDA receptor activation for the phosphorylation activity of these kinases [Wattiez *et al.*, 2011; Daulhac *et al.*, 2006], opening new prospects for a more targeted drug therapy, thus better tolerated for diabetic neuropathic pain.

Alteration of descending systems: One of the pathophysiological mechanisms involved in the pathogenesis of chronic pain including neuropathic pain is a loss of the inhibitory role of serotonin on persistent pain, as evidenced by (i) the nearly ineffectiveness of selective serotonin reuptake inhibitors (SSRIs) in neuropathic pain patients [Finnerup *et al.*, 2010] and, (ii) results obtained in STZ-induced diabetic rats showing an alteration of spinal 5-HT_{2A} receptor-mediated analgesic effect, usually involved in the analgesic effect of serotonin [Pichon *et al.*, 2010]. These receptors have the particularity to be associated with specific multiprotein complexes, consisting in part of proteins containing PDZ domains, which can modulate signal transduction of receptors to which they are associated [Becamel *et al.*, 2004]. In STZ animals, administration of a cell-penetrating peptidyl mimetic of the 5-HT_{2A} receptor C-terminus ending, which disrupts their interaction with

PDZ proteins, induces antihyperalgesic effect per se and enhanced the analgesic effect of fluoxetine, a SSRI [Pichon *et al.*, 2010].

Most of peripheral and central abnormalities in the transmission and modulation of nociception that have been described in STZ mice were also found in other neuropathic pain models, especially traumatic peripheral nerve injury (CCI or SNL) showing the lack of specificity of the model.

It would be over simplistic to associate a pathophysiological mechanism to an etiology because the same mechanism can be found in neuropathies of different etiologies [Baron *et al.*, 2010], and a given injury may involve several mechanisms.

1.5 Mesenchymal Stem Cells (MSCs)

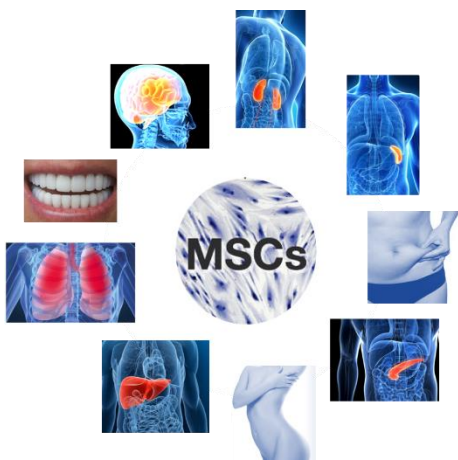


Image 8 | MSCs human niche.

The first isolation from bone marrow of stromal multilineage progenitor cells giving rise to mesodermal cells was carried out by Friedenstein more than 50 years ago, opening a field of research in continue expansion [Friedenstein *et al.*, 1976]. Successively, Caplan named them “Mesenchymal Stem Cells” (MSCs) because of their ability of self-renewal and differentiation [Caplan, 1991].

Nowadays, MSCs can be isolated nearly from all adult human tissues and organs: liver, kidney, spleen, pancreas, lungs, brain, adipose tissue, dermis, dental pulp and periodontal

ligaments. Interesting sources of MSCs are discarded tissues, such as fat from liposuctions, deciduous teeth, placenta and umbilical cord blood [de Girolamo *et al.*, 2013]. Because of their broad distribution in the body, MSCs are thought to play a central role in maintaining tissue homeostasis.

Isolated MSCs share common features: fibroblastoid morphology, clonogenic ability (Colony Forming Unit- Fibroblasts, CFU-F), self-renewal and multipotential differentiation. However, a considerable variability exists among MSC populations [Dominici *et al.*, 2009]. For this reason, these progenitor cells are now generally termed “multipotent Mesenchymal Stromal Cells”, while the definition of “Mesenchymal Stem Cells” should be applied only if specific criteria are satisfied. In 2006, the International Society for Cellular Therapy (ISCT) proposed three minimal criteria to define Mesenchymal Stem Cells:

- i) purification by plastic adherence,
- ii) differentiation towards adipogenic, osteogenic and chondrogenic lineages under standard *in vitro* specific stimuli,

iii) antigen surface expression of CD105 (endoglin, SH2), CD73 (ecto-5'-nucleotidase) and CD90 (Thy1), while absence of the hematopoietic markers CD45, CD34, CD14 or CD11b, CD19 or CD79 α and HLA-DR [Dominici *et al.*, 2006].

Despite these common markers, MSC populations are phenotypically characterized by the expression of variable set of surface proteins [Mafi *et al.*, 2011].

The variability in surface antigen expression, biological functions and activities is not surprising, since MSC populations derive from different tissues and each tissue possesses its own unique progenitor niche. Recently it has been proposed that surface marker expression could be influenced not only by the source but also by the method of isolation and stage of culture. This observation highlights the possibility that MSCs express markers *in vitro* which not necessarily correspond to their *in vivo* patterns [de Girolamo *et al.*, 2013].

MSC populations can be isolated from almost all adult vascularized tissues, where they are localized in a vascular niche probably in the wall of large and medium-size vessels. This localization originates during embryogenesis and persists in adult body presumably with the role of controlling vessel integrity by secreting factors that promote vasculogenesis, thus stabilizing endothelial tissue and immune system homeostasis during physiological and pathological conditions. For these reasons, some authors have proposed that MSCs could represent a subset of precursor cells in close relationship with pericytes or even that MSCs identify with them [Caplan and Correa, 2011].

1.5.1 Adipose-derived Stem/Stromal Cells (hASCs)

Like bone marrow, adipose tissue has a mesenchymal origin. It is composed by different cell populations at various stages of differentiation, mainly mature adipocytes, pre-adipocytes, fibroblasts, vascular smooth muscle cells, endothelial cells and Adipose-derived Stem/Stromal Cells (ASCs). Adipose tissue represents an ideal source of MSCs because it can be obtained by discarded lipoaspirates from aesthetic surgery, with the advantages of large availability, easy accessibility and minimal discomfort for the patient [Yu *et al.*, 2011]. ASCs can be isolated by a simple collagenase digestion, followed by centrifugation to separate floating adipocytes from ASC-containing stromal vascular fraction (SVF). ASCs are then purified by plastic adherence. The frequency of stem cells in SVF is approximately 2500-fold that of bone marrow (up to 0.002%) [Fraser *et al.*, 2008].

ASCs share the main properties of MSCs: fibroblastoid morphology, self-renewal and clonogenic ability, differentiative potential towards several mesodermal lineages (adipocytes, osteoblasts, chondrocytes) but also trans-differentiative potential towards endodermic (i.e. hepatocytes and endocrine pancreatic cells) and ectodermic lineages (i.e. epithelial and neuronal-like cells) [Zuk *et al.*, 2001].

Despite these general properties, ASCs show slight differences in surface antigen expression respect to other MSCs. For example, the hematopoietic marker CD106 is expressed by BMSCs (Bone Marrow-

derived Stem Cells) while it is absent in ASCs. SVF-isolated populations are often a non-uniform subset of stem and precursor cells lacking an unique marker that allows the identification of ASCs without ambiguity. Furthermore, it is known that ASC phenotype has a dynamic profile that changes during cell culture and overall in different *in vitro* conditions. Donor characteristics are another important source of variability (i.e. age, body mass index, gender and health conditions) [Baer, 2014].

In 2013, International Fat Applied Technology Society (IFATS) published a revised statement to point out the minimal phenotypic criteria to define Adipose-derived Stem/Stromal Cells: “in the SVF, cells are identified phenotypically by the following markers: CD45-, CD235a-, CD31-, CD34+. The fibroblastoid colony-forming unit assay permits the evaluation of progenitor frequency in the SVF population. In culture, ASCs retain markers in common with other mesenchymal stromal/ stem cells (MSCs), including CD90, CD73, CD105, and CD44 and remain negative for CD45 and CD31. They can be distinguished from bone marrow-derived MSCs by their positivity for CD36 and negativity for CD106. The CFU-F assay is recommended to calculate population doublings capacity of ASCs. The adipocytic, chondroblastic and osteoblastic differentiation assays serve to complete the cell identification and potency assessment” [Bourin et al., 2013].

1.5.2 MSCs as therapeutic tool

MSCs are thought to be an excellent candidate for cell therapy because they exert many beneficial effects, are available in large amounts, can be cryopreserved and their use arises fewer ethical concerns than that of embryonic stem cells (ESCs). In recent years it has become evident that their therapeutic effect is related not only to their stemness features of self-renewal and differentiation. Indeed, MSCs, in response to the cross-talk with micro-environmental cells, act mainly through the secretion of a wide set of molecules that have both paracrine immunomodulatory and trophic activities [Figure 1]. Recently, Caplan suggested to modify the acronym of MSC in “Medicinal Signaling Cells”, highlighting that the peculiar ability of these cells is to produce bioactive factors [Caplan, 2010]. In 2001, the European Directive 2001/83/CE declared that products for advanced medicinal therapy (AMT, i.e. gene therapy, cell therapy and tissue engineering) are considered drugs because of their pharmacologic, metabolic and immunologic activities, useful in the potential treatment of many disorders. For this reason, stringent requirements of safety and efficacy are applied to AMT cellular products, which should be manipulated according to Good Manufacturing Practices (GMP) and tested in approved clinical trials before being commercialized [European Commission, 2011]. The design of appropriate clinical trials needs to consider many important factors: source (i.e. tissue origin), isolating methods, expansion culture conditions (e.g. medium and oxygenation), cryopreservation procedures, quality controls, doses and administration routes. Furthermore, cells have to be administered at early passages in culture (<4) to avoid the risk of cellular senescence or the accumulation of genetic instability [Keating, 2012].

Safety represents a significant barrier to the successful translation of MSCs into an acceptable clinical therapeutic tool. Preclinical studies on immuno-suppressed mice have highlighted the possibility of long-term risks associated to MSCs therapy, which are mainly related to immunosuppression and tumor formation. However it is not sure that these effects could appear also in immune-competent hosts [Huang *et al.*, 2013]. In recent years efficacy and safety of MSCs are under investigation in many clinical trials of cardiovascular, neurological and immunological disease with encouraging preliminary results. (It has been reported no apparently association between MSC treatment and the development of acute infusional toxicity, organ system complications, infections, malignancy or death [Lalu *et al.*, 2012]. For this reason, MSCs transplantation is considered safe, even if continuous and rigorous reporting of adverse events is required to further define their safety profile.

Nowadays the use of allogeneic rather than autologous MSCs is considered preferable because the host immune system would be able to eliminate possible transformed cells, avoiding the risk of tumor formation.

1.5.3 Current clinical trials

In recent years, MSCs have been worldwide applied to treat a broad spectrum of human diseases, including bone-articular, immune, neurological, cardiovascular, gastrointestinal and blood pathologies [Murphy *et al.*, 2013]. Entering the keyword “stem cells”, the NIH website on November 2014 produced a lists of 4714 stem cell-based current clinical trials, 440 of them employing MSCs [<http://clinicaltrials.gov>]. The clinical appeal of MSCs is due to their wide biological functions, mainly differentiation ability, immunoregulatory properties and production of multiple paracrine trophic factors. Moreover, MSCs are tested in a wide variety of experimental applications, such as myocardial infarction, acute kidney failure, liver fibrosis, amyotrophic lateral sclerosis, cerebral palsy, traumatic brain injury, stroke and inherited metabolic disorders [Daley, 2012]. Giving their ability to mitigate autoimmunity, MSCs have been tested as cellular approach for graft-versus-host disease (GVHD) after allogeneic transplantation and autoimmune conditions (e.g. Crohn’s disease, multiple sclerosis and systemic lupus) [Shi *et al.*, 2011]. MSCs are also being tested for orthopedic applications (e.g. bone fracture, joint cartilage repair, osteoporosis, osteoarthritis and rheumatoid arthritis) because of their *in vitro* potential to form bone and cartilage [Griffin *et al.*, 2011].

Among MSCs, ASCs are largely applied in different medical fields, such as plastic, orthopedic, oral maxillofacial and cardiac surgery, mainly thanks to their ability of soft-tissue regeneration and wound healing [Tobita *et al.*, 2011]. Collectively, ASC-based clinical trials are focused on digestive, autoimmune, cardiovascular, skeletal and neurologic pathologies and disorders [Gir *et al.*, 2012].

Since now, clinical trial outcomes showed encouraging, although often moderate or short-termed, clinical benefits. These observations highlight the need of a rational optimization of therapeutic strategies with an adequate assessment of risk-benefit factors [Pacini, 2014].

1.5.4 Homing and engraftment

One of the most persistent paradoxes in MSC therapy is that systemically administered MSCs are able to exert their beneficial effects in the absence of stable engraftment in target tissues [Parekkadan & Milwid, 2010].

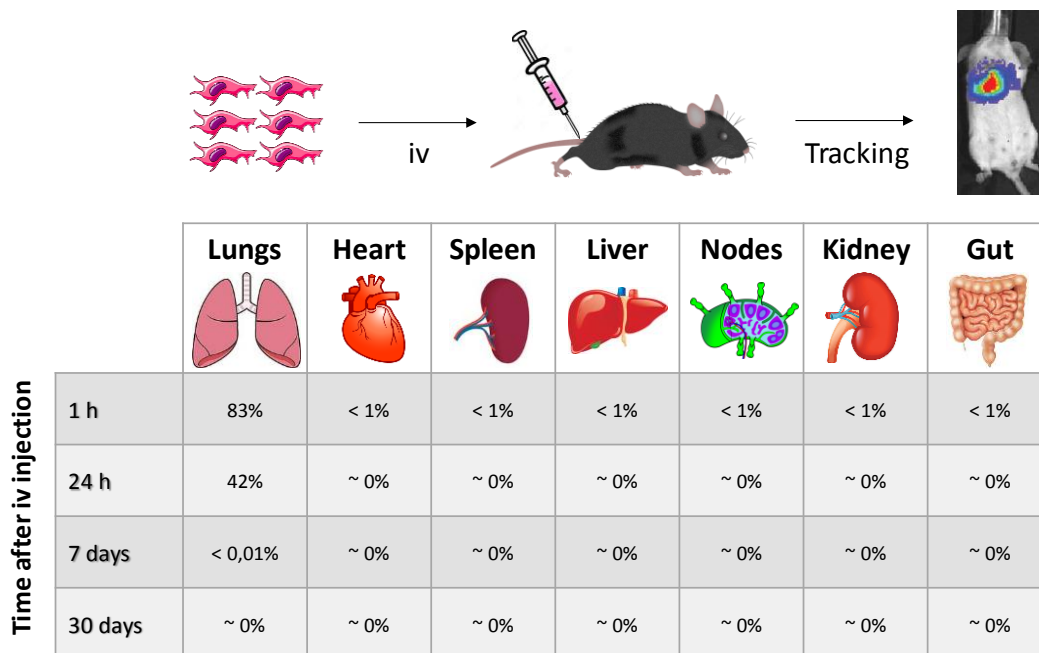


Image 9 | In vivo distribution of MSCs upon systemic administration. Whole-organism distribution of intravenously (i.v.) injected hMSCs in mice tissues.

[Image modified from Parekkadan et al., 2012]

Indeed, most homing and engraftment studies indicated that less than 1% of systemically administered MSCs persist for longer than a week after the injection [Image 9]. Immediately after administration, the majority of MSCs (>80%) accumulate in the lungs and are cleared with a half-life of 24 hours [Lee et al., 2009]. Trafficking studies have reported that systemically administered MSCs fail to engraft in most tissues and that engrafted cells may eventually be rejected, resulting in immunological memory to subsequent treatments [Zangi et al., 2009].

The most used tracking approach is the detection of MSCs expressing a reporter gene (mainly green fluorescent protein, GFP), even if it is unclear whether the transfection could modify cell behavior [Dominici et al., 2008]. A more powerful approach is magnetic resonance imaging (MRI) of MSCs labeled by traceable iron particles [Salamon et al., 2014]. However, the development of new and more accurate cell tracking techniques is a fundamental requisite to assess safety and efficacy of administered MSCs, in the light of their possible future clinical application.

One of the major characteristics of MSCs is that they have migratory abilities, [Nagyova et al., 2014] and after administration, they specifically migrate to the sites of inflammation and tissue damage which is typically associated with cytokine outburst [Eggenhofer et al., 2014; Sohni and Verfaillie, 2013], though the mechanism by which MSCs home to tissues is still not completely clear. However, it seems that injured tissues express specific receptors or ligands that allow MSC migration across endothelium, similarly to the way leukocytes are recruited to inflammation sites [Katsuda et al., 2013]. BMSCs, the most characterized MSCs, express on their surface different integrins (e.g. integrin $\alpha4/\beta1$), which mediate cell-cell and cell-extracellular matrix interactions by binding to the vascular cell adhesion molecule 1 (VCAM-1) and to the V-region of fibronectin. In damaged tissues there is an increase of fibronectin V-region exposure that allows MSCs to adhere and transmigrate into the extracellular matrix. Moreover, MSCs are able to secrete different metallo-proteinases (e.g. MMP-2 and MT1-MMP) which degrade the extracellular matrix barriers allowing extravasation [Ebrahim and Leach, 2014].

1.5.5 Paracrine action of MSCs

Although traditionally MSCs regenerative ability was associated to their stemness features, it is now clear that MSCs therapeutic effects are overall due to the secretion of a broad range of bioactive molecules, that mediate immunomodulation, chemo-attraction, angiogenesis, trophic support, anti-apoptosis and anti-scarring properties [Lavoie and Rosu-Myles, 2013]. In response to local micro-environmental cues, MSCs secrete variable concentrations of soluble factors that promote differentiation and survival of resident cells, remodeling of extracellular matrix and formation of new blood vessels [Meirelles et al., 2009]. The secretion of this wide variety of factors (growth factors,

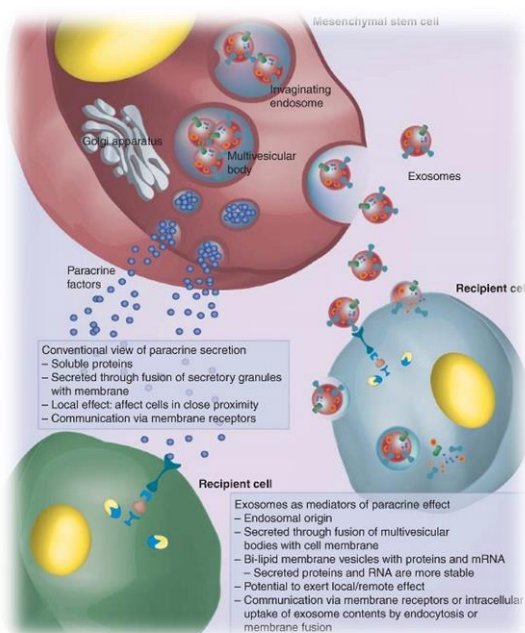


Image 10| Paracrine effects of MSCs.

[Lai et al., 2011]

cytokines, chemokines, metabolites and bioactive lipids) has the role to coordinate multiple interactions with the surrounding micro-environment. Beside known paracrine and autocrine activities, there is the possibility that MSCs could mediate endocrine effects on endogenous cells in a remote location.

The ability of MSCs to modulate the immune system seems to play a role in almost all MSCs treatment effects. The immunomodulatory action of MSCs is based mainly on cell-to-cell contact, production of inhibitory molecules and induction of regulatory T-cells. Interestingly, the release of soluble immunosuppressive factors seems to be specifically linked to the cause of inflammation. The modulation of the host immune

response mediated by MSCs plays also a role in tissue repair, by reducing tissue damage and scarring, during the regeneration of damaged organs. MSCs secreted factors promote tissue regeneration and repair of damaged organs, despite the absence of stable engraftment. Studies on many disease models (e.g. lung injury, chronic kidney disease and liver injury) demonstrated that MSC-derived conditioned medium (CM) alone is sufficient to mediate lasting therapeutic effects. Thus the beneficial effects observed after MSC administration can be completely recapitulated by MSC secreted factors alone. Moreover, many recent works have demonstrated that also the extracellular vesicles (EVs) produced by MSCs participate in the process of tissue repair [Image 10].

It is now evident that different paracrine actions of MSC secretome function in a co-operative manner to generate an advantageous microenvironment that allow tissue regeneration. Biological properties of MSCs and their ability to adapt to specific micro-environments define the new concept of “stem cell therapeutic plasticity” [Drago *et al.*, 2013].

The paracrine activity seems to be the main (even if not the only) mechanism by which MSCs exert their regenerative potential. Indeed, the often observed long lasting effects can't be explained by MSC short-lived engraftments after injection or transplantation. In this view, MSCs can be defined as drug delivery particles whose function follow a pharmacokinetic model: when administered they are subject to distribution and clearance similarly to other therapeutics.

1.5.5.1 Immunomodulatory properties of MSCs

Nowadays it is clear that MSCs action on tissue regeneration is only partially due to cell replacement, while the essential component is their ability to communicate with the inflammatory micro-environment by modulating inflammation. In response to inflammatory cues, MSCs produce a large amount of immune-regulatory factors that together with growth factors stimulate resident cells to repair the damaged tissue.

MSCs act on both the adaptive and innate immune systems and, depending on the type and intensity of inflammatory stimuli, these cells can have the ability to both suppress or enhance the immune response. For this reason, the type and the amount of cytokines released during the inflammatory process have a critical role in the influence and activation of immunoregulatory properties of MSCs [Wang *et al.*, 2014].

Many studies have demonstrated that MSCs can suppress the activation and function of various cells of the innate and adaptive immune systems, including macrophages, neutrophils, natural killer cells (NK), dendritic cells (DCs), T-lymphocytes and B-lymphocytes. MSCs act on T-cell inhibiting their differentiation into TH1 and TH17 types of helper T-cells and promoting the generation of regulatory T-cells (Treg cells) [Figure 4]. In addition, MSCs mediate the switch of macrophages from a pro-inflammatory type 1 to anti-inflammatory type 2 phenotype [Shi *et al.*, 2012].

Immunosuppressive factors produced by MSCs are mainly: interleukin 6 (IL-6), interleukin 10 (IL-10), transforming growth factor- β (TGF- β), prostaglandin E2 (PGE2), human leukocyte antigens (HLA-A, HLA-DR, HLA-G5), epidermal growth factor (EGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), insulin growth factor (IGF), COX-2 (cyclooxygenase 2), matrix metallo-proteinases (MMPs), tryptophan-catabolic enzyme IDO and nitric oxide (NO), a product of inducible nitric oxide synthase (iNOS) [Ma et al., 2014]. This great variety of mediators is probably due to the different tissue and micro-environment from which MSCs derive.

An important issue is that MSCs probably aren't able to elicit their immune-regulatory actions unless they are first in vivo activated by certain combinations of inflammatory stimuli, mainly interferon- γ (IFN- γ) together with other cytokines (e.g. TNF- α , IL-1 α or IL-1 β). Upon stimulation, MSCs secrete large amounts of chemokines that attract T-cells. In this way, MSCs influence the inflammatory process both recruiting T-cells and producing immunosuppressive factors. Moreover, MSCs are poorly immunogenic because they express low levels of major histocompatibility complex class I and II (MHC I – II), which can be up-regulated by IFN- γ and IL-1 β treatment [Schu et al., 2012].

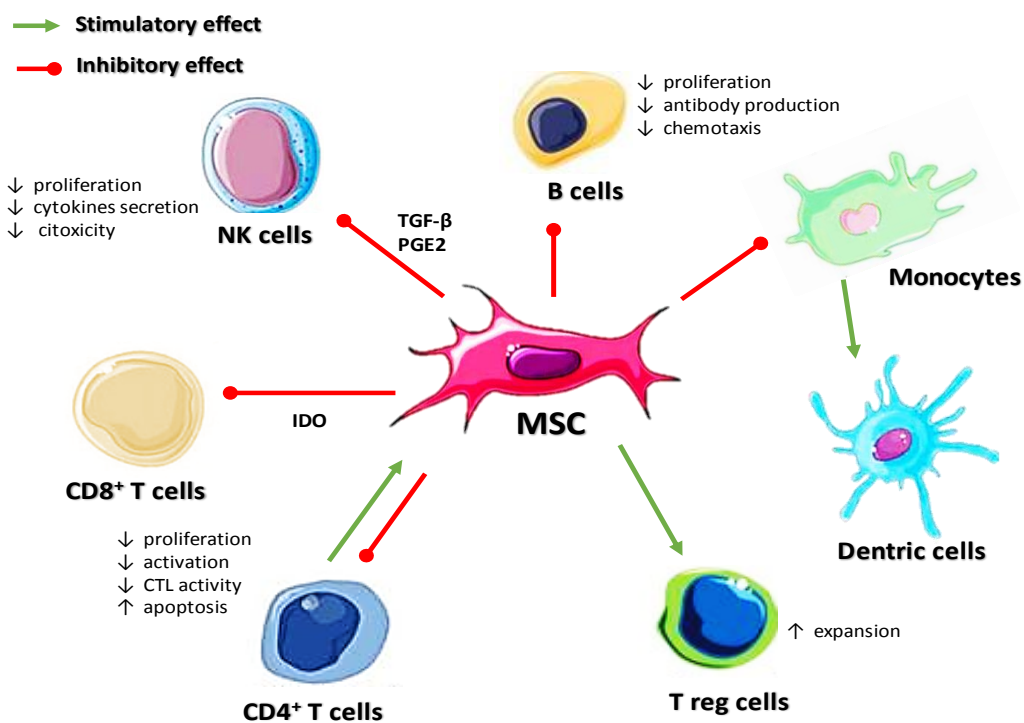


Image 11| Immunomodulatory effects of MSCs on innate and adaptive immune cells.

[Image modified from Yi et al., 2012]

However, MSC plasticity makes these cells able to inhibit but also promote the immune response, dependently on various inflammatory stimuli and on the state of activation of the immune system. Indeed MSCs activation depends on type and concentration of inflammatory mediators present in the surrounding micro-environment. Essentially, inflammation status determines the immune-regulatory fate of MSCs [Ren et al., 2008].

During the pathogenesis of inflammatory diseases, high concentrations of inflammatory cytokines in the acute phase promote disease progression, while low concentrations of these factors in the chronic phase prepare the microenvironment to facilitate tissue repair. It is thought that MSCs exert their immunosuppressive effect in the presence of strong inflammation, while weak inflammation paradoxically causes MSCs to enhance the immune response [Wang *et al.*, 2014].

For example, it has been reported that graft-versus-host disease (GvHD) treatment is more successful if MSCs are administered some days after bone marrow transfusion, when the inflammation is already established [Bernardo and Fibbe, 2013].

For this reason, a correct clinical design should consider the correct timing of MSC administration because the inflammatory status strongly influences the therapy outcome. Consistently, pre-treating MSCs with inflammatory cytokines before the administration could be a successful strategy to reinforce their immunosuppressive efficacy, while the concomitant application of immune-suppressants should be avoided.

It's still under debate the reason why endogenous tissue-resident MSCs aren't able to elicit these immunomodulatory actions. A possible explanation is that MSCs, before being administered, are expanded *in vitro* and could acquire part of these properties during culture. Furthermore, it should be considered that MSC administration is performed with a high number of cells (1-2x10⁶ cells/ kg).

1.5.5.2 Role and biological functions of microvesicles, exosomes and miRNA

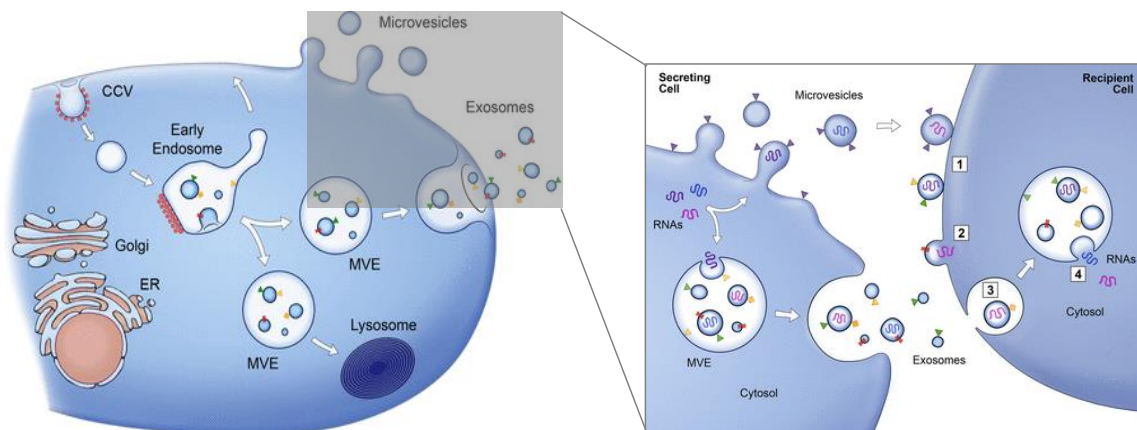


Image 12 | Microvesicles and exosomes. [Image modified from Raposo & Stoorvogel, 2013]

Cell-based therapy is associated to many ethical and practical problems (i.e. cell senescence, genetic instability, loss of function, limited cell survival and possibility of malignant transformation). Most of these issues might be eluded with the use of MSC conditioned medium (CM), microvesicles (MVs) and exosomes, which act as delivery vehicles containing a complex mixture of bioactive molecules. These particles have peculiar characteristics that make them a possible useful tool for cell-free therapies. For example, it has been shown that vesicles are able to cross the blood brain barrier, an interesting aspect for the treatment of neurodegenerative diseases (e.g. Alzheimer, Parkinson and prion diseases).

Moreover, many studies have shown that MSCs contain microRNAs, 20-22 nucleotide long RNA molecules that target messenger RNA for cleavage or translational repression, thus suppressing protein synthesis. It has been recently shown that miRNAs play critical functions in MSCs survival, proliferation, differentiation and immunomodulatory actions [Clark *et al.*, 2014]. Recent studies have also focused on the involvement of miRNAs in the paracrine action of MSCs. miRNAs can modulate the expression of MSC secreted proteins or alternatively they can be stored in microvesicles or exosomes and once secreted they exert their regulatory function in target cells.

1.5.5.3 Soluble factors identification in secretome

Recently, the conditioned medium (CM) of cultured MSCs has become the subject of intensive proteome profiling in the search for soluble factors which could be interesting for human regenerative medicine. Moreover, the proteomic study of extracellular vesicles (i.e. exosomes and microvesicles) is particularly attractive, since EVs are thought to be the real mediators of cell communication and could represent both an useful tool for regenerative medicine and markers for certain pathologies [Skalnikova, 2013].

Up to now a variety of growth factors, angiogenic factors, cytokines, chemokines and extracellular matrix proteins have been identified in the mammalian mesenchymal stem cell and other stem cell secretomes [Table 1 - Skalnikova *et al.*, 2011]. Other interesting studies are now focused on the secretome of conditioned medium obtained from cells cultured in hypoxic conditions, serum deprivation or inflammatory stimulation (e.g. TNF- α or lipopolysaccharide). Such conditions increase the amount of particular proteins and cytokines secreted into the culture media.

Two major approaches are employed to analyze secreted proteins: targeted proteomic approaches (immunological assays) and shotgun broad range scanning (protein separation followed by protein identification by mass spectrometry).

Target-based proteomic studies are based on the research of specific molecules with known biological roles (i.e. growth factors and hormones). This approach is based on immunological methods, mainly the Enzyme-Linked Immunosorbent Assay (ELISA).

Interestingly, proteomic studies do not always agree on the exact content of the MSCs secretome. This is probably due to differences in the analytic techniques used but it could also depend on the fact that MSCs are highly sensitive to their environment, therefore different culture conditions can result in changes of molecule secretion.

Table 1 | MSC secretome. [Table modified from Skalnikova et al., 2013]

<i>Extracellular matrix proteins and proteins involved in cell adhesion</i>	Biglycan Cadherin 2-11-13 Collagen α -1 and α -2 chains Decorin (Bone proteoglycan II) Dermatopontin Entactin (Nidogen 1) Extracellular matrix protein 1 Fibrillin 1-2 Fibronectin	Fibulin 1-2 Laminin γ 1 Lumican Osteoblast-specific factor 2 (Periostin) Serpin H1 (Collagen-binding protein) Tenascin Versican core protein Vitronectin	
<i>Growth and trophic factors</i>	Bone-derived growth factor (BDFG) Brain-derived neurotrophic factor (BDNF) Connective tissue growth factor Epidermal growth factor (EGF) Fibroblast growth factors (FGF) 4-7-9-17 Glial cell line-derived neurotrophic factor (GDNF)	Hepatocyte growth factor (HGF) Insulin like growth factor 1 (IGF-1) Insulin-like growth factor-binding protein (IGFBP) 2-3-4-5-6-7 Nerve growth factor (NGF) Placental growth factor (PIGF) Platelet-derived growth factor (PDGF)	
<i>Angiogenic factors</i>	Angiogenin Angiopoietin Vascular endothelial growth factor (VEGF)		
<i>Chemokines</i>	CCL1 (I309) CCL2 (MCP-1) CCL5 (RANTES) CCL7 (MCP-3) CCL8 (MCP-2) CCL11 (Eotaxin) CCL15 (MIP-1 δ) CCL16 (HCC-4) CCL18 (PARC)	CCL22 (MDC) CCL23 (Ckb8-1, MIP-3) CCL24 (Eotaxin-2) CCL26 (Eotaxin-3, MIP-4 α) CX3CL1 (Fractalkine) CXCL1 (GRO α) CXCL2 (GRO β , MIP-2 α) CXCL3 (GRO γ) CXCL5 (human ENA-78, mouse LIX)	CXCL6 (GCP-2) CXCL8 (IL-8) CXCL11 (I-TAC) CXCL12 (SDF-1 α) CXCL13 (BLC) XCL1 (Lymphotactin)
<i>Pro-inflammatory cytokines</i>	Interferon γ (IFN- γ) Interleukin-12 (IL-12) Interleukin-1 α (IL-1 α)	Interleukin-2 (IL-2) Tumor necrosis factor α (TNF- α)	
<i>Anti-inflammatory cytokines</i>	Interleukin-10 (IL-10) Interleukin-13 (IL-13)	Macrophage migration inhibitory factor (MIF) Transforming growth factor β (TGF- β)	
<i>Pleiotropic cytokines</i>	Interleukin-11 (IL-11) Interleukin-16 (IL-16) Interleukin-1 β (IL-1 β)	Interleukin-6 (IL-6) Leukemia inhibitory factor (LIF) Oncostatin M (OSM)	
<i>Hematopoietic cytokines</i>	Flt 3 ligand (FLT3LG) Granulocyte colony-stimulating factor (G-CSF) Granulocyte-macrophage colony-stimulating factor (GM-CSF) Interleukin-3 (IL-3)	Interleukin-7 (IL-7) Macrophage colony-stimulating factor (M-CSF) Stem cell factor (SCF, Kit ligand, steel factor) Thrombopoietin	
<i>Cytokine receptors</i>	CXCR3 (IP-10 receptor, CD183) Osteoprotegerin		
<i>Enzymes and enzyme inhibitors/enhancers</i>	72 kDa type IV Collagenase Chitinase-3-like protein 1 Glia-derived nexin Matrix metalloproteinase (MMP) 1-2-3 Metalloproteinase inhibitor (TIMP) 1-2-3	Plasminogen activator inhibitor (PAI) 1-2 Procollagen C-endopeptidase enhancer Serine protease HTRA1 Serine protease inhibitor J6	
<i>Other proteins</i>	Bone morphogenetic protein 1 (BMP-1) Clusterin Cystatin B-C Dickkopf related protein-3 Galectin 1-3 Galectin-3-binding protein Gelsolin Granulins Latent-TGF β -binding protein 1-2 Myocilin	Pentraxin-related protein 3 (PTX3) Pigment epithelium-derived factor (PEDF, serpin F1) Secreted protein acidic and rich in cysteine (SPARC, osteonectin) Stromal cell derived factor 4 Sulphated glycoprotein 1 (prosaponin) TGF β -induced protein ig-h3 Thrombospondin 1-2	

1.6 Stem cell therapy in experimental models of neuropathic pain

In recent years many preclinical studies have been carried out to assess the effects induced by several stem cells in different models of neuropathic pain. At the beginning, embryonic stem cells (ESCs) grabbed researchers' attention especially thanks to their wide differentiation potential, however they can easily induce tumor formation and are associated to ethical problems due to their collection, so today there are three main types of adult stem cells used for experimental neuropathic pain treatment: neural stem cells (NSCs), bone marrow mononuclear cells (BMMCs) and mesenchymal stem cells (MSCs). Marrow mononuclear cells containing mixed stem cell populations have been intravenously used in neuropathic rats showing recovery from pain [Klass *et al.*, 2007]; their implantation could be a possible solution for spinal cord injury; in fact BMMCs have the ability to be incorporated into spinal cord, differentiate, and to improve locomotor recovery [Schultz, 2005]. Klass *et al.* [Klass *et al.*, 2007] described that BMMCs infusion in CCI rat model was able to induce neuropathic pain recovery (both hyperalgesia and allodynia). The authors did not investigate into the mechanisms involved in such modulations. These cells also injected into the hind limb skeletal muscles in STZ-rats [Naruse *et al.*, 2011] were able to ameliorate mechanical hyperalgesia and cold allodynia in the BMMCs-injected side. Furthermore, the slowed sciatic nerve conduction velocities and decreased sciatic nerve blood flow in diabetic rats were improved in the BM-MNC-injected side.

It has been demonstrated that human NSCs can promote functional corticospinal axons regeneration and synapse reformation in the injured spinal cord of rats. The action is mainly through the nutritional effect of the stem cells on the spinal cord. Transplanted cells were found to migrate into the lesion, but not scatter along the route of axon growth. The cells differentiated into astrocytes or oligodendrocytes, but not into the neurons after transplantation [Liang *et al.*, 2006]. Moreover, spinal progenitor cells intrathecally transplanted in neuropathic rats are able to alleviate neuropathic pain [Lin *et al.*, 2002]. Murine neural stem cells (NSCs) grafted onto the injured spinal cord improved motor behaviour [Pallini *et al.*, 2005]. In CCI model, NSCs systemic injection when the pathology was already established, induced a significant reduction in allodynia and hyperalgesia; moreover it was also present a reparative process and an improvement of nerve morphology [Franchi *et al.*, 2014]. These positive results were observed also by Xu and colleagues [Xu *et al.*, 2013], that used another route for NSCs administration; the authors described that an intrathecal administration of neural stem cells, 3 days after CCI injury in rat, was able to significantly attenuate mechanical and thermal hyperalgesia.

Among stem cell population, mesenchymal stem cells (MSCs) rise probably best potential good results in pain-care research. One of the first groups to assess the effect of rat bone marrow stromal cells in an experimental CCI rat model was the group of Musolino [Musolino *et al.*, 2007]. They demonstrated that an ipsilateral intraganglionic injection of rat stromal cells was able to prevent the generation of mechanical allodynia and to reduce the number of allodynic responses to cold stimuli [Musolino *et al.*,

2007]. Rat bone marrow MSCs have also been used STZ diabetic neuropathy [Shibata *et al.*, 2008]. MSCs were able to ameliorate all the alterations induced by diabetes such as hypoalgesia, delayed nerve conduction velocity, and decreased sciatic nerve blood flow. Moreover, MSC transplantation was able to normalize sural nerve morphometry restoring the axonal circularity, decreased in diabetic rats. The same positive effect on nerve conduction velocity amelioration was also reported by Kim and Jin [Kim and Jin, 2011]. The Maione's group is the main user of human BMSCs for treating experimental neuropathic pain due to the spared nerve injury (SNI), the hBMSC therapeutic administration was performed either in the mouse lateral cerebral ventricle [Siniscalco *et al.*, 2010] or systemically into the caudal vein [Siniscalco *et al.*, 2011]. In both studies, hBMSC reduced pain-like behaviors, such as mechanical allodynia and thermal hyperalgesia, with an effect which was evident one week after cell transplantation and was long lasting. When cells were injected into the caudal vein, their effect on pain relief was still present three months after transplant while when hBMSCs were intravenously injected, cells were able to home into the spinal cord and prefrontal cortex of SNI neuropathic mice. The authors described the capacity of these cells to reduce glial [Siniscalco *et al.*, 2010] and macrophage activation [Siniscalco *et al.*, 2011] switching to an anti-inflammatory phenotype by decreasing the proinflammatory cytokines (IL-1 β and IL-17) and increasing the anti-inflammatory cytokine IL-10 [Siniscalco *et al.*, 2010; 2011]. The group of Waterman [Waterman *et al.*, 2012] developed a method to optimize the anti-inflammatory effects of hBMSC, skewing them *in vitro*, before their injections, towards a protective MSC2 phenotype. These BMSCs demonstrated a higher capacity to counteract mechanical allodynia and heat hypoalgesia induced in mice by STZ treatment. These cells were also able to decrease the serum level of pro-inflammatory cytokines and were described to be safe [Franchi *et al.*, 2014].

A recent study from our group evaluated the antinociceptive effect of hASC isolated from human adipose tissue intravenously injected in CCI model. Antihyperalgesic and antiallodynic effect was rapid, long lasting, and dose dependent, in fact 10⁶ hASCs were able to completely abolish thermal hyperalgesia [Sacerdote *et al.*, 2013a].

Aim of the research project

Diabetes mellitus is one of the most common and serious chronic disease in the world. Although the number of available agents to manage diabetes continues to rapidly expand, the treatment of diabetes complications, such as neuropathy, remains a substantial challenge and diabetic peripheral neuropathy is one of the most frequent complication of diabetes mellitus. Pathophysiology of diabetic neuropathy is complex and not fully elucidated; it has multipathogenic mechanisms that cause a diversity of physical symptoms: allodynia, hyperalgesia, numbness and cutaneous ulceration. Persistent Neuropathic Pain (NP) interferes significantly with quality of life, impairing sleep, and emotional well-being, and is a significant causative factor for anxiety, loss of sleep, and non-compliance with treatment. Recent advances in the mechanisms involved in NP have demonstrated that pro- and anti-inflammatory cytokines produced by immune cells as well as by glia and microglia in nerve, dorsal root ganglia and spinal cord are common denominators in neuropathic pain. These start a cascade of neuroinflammation-related events that may maintain and worsen the original injury, participating in pain generation and chronicization. By and large, activation of inflammatory cascade, proinflammatory cytokine upregulation, and neuroimmune communication pathways plays a vital role in structural and functional damage of the peripheral nerves leading to the diabetic peripheral neuropathy.

Unfortunately, most of the available analgesic drugs appear to be relatively ineffective in controlling diabetic neuropathic pain, both for insufficient efficacy and side effects. Thus, there is a clear need for new disease-modifying therapeutic approaches.

Mesenchymal stem/stromal cells may offer a novel therapeutic option to treat diabetic neuropathy; in fact in the last years the view of the biological action of Mesenchymal Stem Cells (MSCs) has been greatly changed. It has become evident that MSCs participate in tissue repair mostly by a paracrine mechanism through the release of soluble trophic and immunomodulatory factors. MSCs modulate the nervous system injured environment and promote repair as they secrete anti-inflammatory, anti-apoptotic molecules, and trophic factors to support axonal growth, immunomodulation, angiogenesis, remyelination, and protection from apoptotic cell death. Transplanted MSCs not only directly differentiate into endogenous cells on administration, but also secrete a broad range of biologically active factors, generally referred to as the MSCs secretome. Many studies provided pivotal support for the paracrine hypothesis such that MSC therapy is increasingly rationalized on MSCs secretion rather than its differentiation potential.

On these premises, the aim of my PhD project was to assess and compare in the type 1 Streptozotocin mouse model of diabetes the therapeutic effect of hASC (human adipose stem/stromal cells) and their secretome (CM-hASC) on painful diabetic neuropathy, evaluating mechanical and cold allodynia, and thermal hyperalgesia. In order to identify the mechanisms involved in the effect of hASC and CM-hASC we evaluated neuroinflammation, measuring pro- and anti-inflammatory cytokines expression in the main tissue stations involved in nociception transmission. Since hASC exert also an important

modulation of peripheral immunity, we considered also the activation and cytokine production of T-lymphocytes. We also evaluated the effect of both hASC and CM-hASC treatment on fiber loss and skin tickness, at the level of hind paws. Furthermore, to assess if treatments may be able to modify pathology development and progression, throughout the experiments blood glucose levels, body and organs weight were assessed. We also took into consideration the other important diabetes complication, i.e. nephropathy and measured kidney damage.

Type 1 diabetes is induced in mice by administration of Streptozotocin, a β -cytotoxine and when allodynia is established animals are intravenously injected in the tail vein with 10^6 hASC or CM-hASC from 2×10^6 cells. Behavioural measurements, biochemical and immune evaluation are performed at different time points after diabetes induction.

Moreover, in order to verify whether stemness is a fundamental prerequisite to obtain pain relief a group of STZ-mice is treated with CM obtained from 2×10^6 human fibroblasts (CM-hF). The present study will help to understand whether the hASC therapy may be effective for diabetes complication and whether eventually cells could be replaced by their conditioned medium, suggesting the chance to use a cell-free therapy.

Material & Methods

3.1 Animals

In all the experiments male mice of C57BL/6J strain, weighing 20-25g and 9 weeks old were used (Envigo, Italy). Animals were housed under controlled conditions with light/dark cycles of 12 hours, temperature of 22 ± 2 °C, humidity of $55 \pm 10\%$, food and water *ad libitum* and they were acclimatized to the new environment for at least one week before being used. A total of 120 animals were used in the described experiments. Each experiment consisted of 6 mice per group.

All animal care and experimental procedures complied with the International Association for the Study of Pain and European Community (E.C.L358/118/12/86) guidelines and were approved by the Animal Care and Use Committee of the Italian Ministry of Health guidelines (DL 116/92 and DL111/94-B). All efforts were made to minimize animal suffering and to reduce the number of animals used.

3.2 Diabetic neuropathy induction

Type 1 diabetes was induced in mice through chemical pancreatectomy by a repeated intraperitoneal (i.p.) administration of moderate low doses of streptozotocin (STZ, Sigma Aldrich, Italy), 80 mg/kg daily for three consecutive days (10 μ L/g), freshly prepared in citrate buffer 0.1 M, pH 4.55. Control mice were i.p. injected with citrate buffer [Castelli *et al.*, 2016].

STZ-mouse model is characterized not only by the development of neuropathic pain and hyperglycemia but also by other diabetic complications such as: body weight loss, fibers loss and skin thinning of the paws and, nephropathy as described below.

3.3 Therapeutic treatments

Cells and substances used for therapeutic treatments were provided by the group of Dr. Brini (University of Milan, Department of Biomedical, Surgical and Dental Sciences; I.R.C.C.S Galeazzi Orthopedic Institute of Biotechnological Applications Laboratory) and are the following:

- ***Human adipose-derived stem/ stromal cells (hASCs);***
- ***Conditioned Medium from hASC (CM-hASC);***
- ***Conditioned Medium from human Fibroblasts (CM-hF).***

3.3.1 Isolation and primary culture of hASC

Cells were isolated from subcutaneous adipose tissue obtained as wasted material from plastic and cosmetic surgery of healthy donors (male and females, age range 26-53 y/o) after written consent and Institutional Review Board (IBR) authorization from IRCCS Galeazzi Orthopaedic Institute (procedure PQ 7.5.125, version 4, 22.01.2015).

Raw lipoaspirate (20-180mL) were mechanically dissected, washed 3-5 times in PBS (137mM NaCl, 2.7mM KCl, 8.1mM Na₂HPO₄*2H₂O, 1.7mM KH₂PO₄; pH 7.4) and centrifuged at 1300g for 2 minutes to remove precipitated erythrocytes and cell debris. Adipose tissues were then enzymatically digested in a PBS-collagenase solution with 0.75mg/mL type-I collagenase (250U/mg – Worthington Biochemical Corporation) in water bath at 37°C for 30 minutes, shaking manually every 5 minutes. After digestion, collagenase was neutralized by adding an equal volume of culture medium. Sample were centrifuged at 1300g for 10 minutes to isolate the stromal vascular fraction (SVF) from cellular debris and undigested tissue. SVF was then filtered using cell strainers (pore size: 100µm) to remove remaining undigested tissue residues. After centrifugation at 350g for 4 minutes, pellets were resuspended in culture medium consisting of DMEM high glucose (D5671, Sigma Aldrich) supplemented with 10% FBS (Thermo Scientific), 2mM L-glutamine (G7513, Sigma Aldrich), 100U/mL penicillin, 100µg/mL streptomycin (P4458, Sigma Aldrich) and if necessary 2.5µg/mL amphotericin B (A9528, Sigma Aldrich). Cells were counted using a modified Burker chamber (Improved Neubauer) and cell viability was assessed by Trypan Blue (T8154, Sigma Aldrich) exclusion. Counted cells were plated at density of 10⁵ cells/cm² (passage 0) and maintained at 37°C in a humidified atmosphere with 5% CO₂. After 48-72 hours, non-adherent cells were discarded by washing with PBS.

During all the period of culture, medium was changed three times a week. After reaching 80-90% confluence, cells were detached by incubation with 0.5% trypsin – 0.2% EDTA (T4174, Sigma Aldrich) at 37°C for 3 minutes. hASCs were plated at density of 10⁴ cells/cm² for expansion. Cells were characterized as described below and they were administered between the third and seventh-passage.

3.3.1.1 hASC characterization and flow cytometric analysis

hASCs			
	%mean	SEM	+ / -
CD13	96.08	2.03	+
CD54	83.34	7.26	+
CD73	99.76	-	+
CD90	94.14	1.67	+
CD105	94.77	3.13	+
CD14	1.61	0.95	-
CD45	0.8	0.37	-

Table 2| hASC characterization.

Characterization of hASCs for their immunophenotypic markers was carried out by flow cytometry (FACSCalibur, Becton Dickinson) using CellQuest Pro software. Cells were analyzed for MSC surface antigen profile, evaluating CD13, CD54, CD73, CD90, CD105, CD14 and CD45 expression. Cells (passage 3) were deached washed twice in PBS to completely remove culture medium, pelleted and resuspended in ice-cold FACS buffer (2% FBS in PBS). For each sample, 4x10⁵ hASCs were aliquoted in polystyrene FACS tubes (352052, BD Falcon). Sample were incubated with monoclonal antibodies specific for CD13, CD73, CD90, CD14 or CD45 (Biolegend)

conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE) on ice for 30 minutes at 4°C in the dark. Conversely, other samples were incubated with biotinylated monoclonal antibody specific for

CD54 or CD105 (Ancell) and then incubated with SA-PE (streptavidin, a biotin-binding protein covalently attached to phycoerythrin fluorescent label) secondary antibody on ice for 20 minutes at 4°C in the dark. After washing with ice-cold FACS buffer, samples were analyzed by flow cytometry. Cells were first discriminated for cell size and granularity by forward and side scatter, vital cells were gated and further analyzed.

From flow cytometric analysis, hASC populations showed positivity for surface antigen CD13, CD54, CD73, CD90 and CD105, while negativity for CD14 and CD45 [Table 2].

3.3.2 CM-hASC collection and concentration

Upon reaching 80-90% confluence, hASCs were washed once and then incubated at 37°C with 5% CO₂ for 72 hours in serum-free medium containing DMEM high glucose (w/o phenol red) supplemented with 2mM L-glutamine, 50U/mL penicillin and 50µg/mL streptomycin. Starving medium was phenol red-free because the presence of this pH indicator could interfere with concentration step and because great part of collected CM had *in vivo* applications.

Conditioned medium was collected, centrifuged once at 350g for 5 minutes to remove cell debris and then concentrated using Ultra-15 Centrifugal Filter Units with 3 kDa cut-off (Meck Millipore) by centrifugation at 4000g for 90 minutes [Cantinieaux *et al.*, 2013]. After collecting the medium, cells were detached and counted to obtain a correspondence between CM/secretome volume and number of hASCs. hASC secretome was stored at -80°C or lyophilized.

3.3.2.1 Lyophilization protocol

Freeze-drying of concentrated conditioned medium was performed by using Lio5P freeze dryer (5 Pascal) and RV8 vacuum oil pump/Oil Mist Filter (Edwards). Following lyophilization, samples were stored at -80°C, until use. Before *in vivo* administration samples were reconstituted in PBS + 2.5% heparin.

3.3.3 Isolation of human Fibroblast and CM-hF collection and concentration

Human dermal fibroblasts were collected from de-epidermized dermis obtained as wasted material from plastic and cosmetic surgery of healthy donors (male/females, age range 26-53 y/o). Tissue was mechanically minced and then fragments were enzymatically digested with 0.1% type I Collagenase at 37°C for 6 hours. After collagenase digestion, samples were filtered, centrifuged and the pellet was resuspended in DMEM. Cells were cultered at the plate density of 5×10^3 cells/cm² as reported above for hASC. Procedure for obtain CM-hF was the same used ti prepare CM-hASC.

3.4 Experimental design

A sets of experiments were performed. Schematic experimental designs used throughout study are depicted in **Scheme 1**.

For the treatments, 10^6 hASC and CM derived from 2×10^6 hASC or hF were resuspended in 200 μ L of PBS supplemented with 2.5% heparin. Animals were intravenously injected through the caudal vein, CTR mice were administered with the same volume of vehicle (PBS + 2.5% heparin).

Moreover, to localize hASC both in diabetic and naïve mice 10^6 hASC were systemically administrated and Alu-sequences were identified.

SINGLE TREATMENT

Two weeks after STZ-injection (W2), mice were randomly allocated to different groups of treatment:

- Control mice treated with vehicle (CTR)
- Control mice treated with 10^6 hASC (CTR-hASC)
- Diabetic mice treated with vehicle (STZ)
- Diabetic mice treated with 10^6 hASC (hASC)
- Diabetic mice treated with Conditioned Medium derived from 2×10^6 hASC (CM-hASC)
- Diabetic mice treated with CM derived from 2×10^6 hASC lyophilized (Lyo CM-hASC)
- Diabetic mice treated with Conditioned Medium derived from 2×10^6 hF (CM-hF)

REPEATED TREATMENT

Two weeks after STZ-injection (W2), mice were randomly separated to different groups of treatment:

- Control mice treated with vehicle (CTR)
- Diabetic mice treated with vehicle (STZ)
- Diabetic mice treated with 10^6 hASC (hASC)
- Diabetic mice treated with conditioned medium derived from 2×10^6 hASC (CM-hASC)

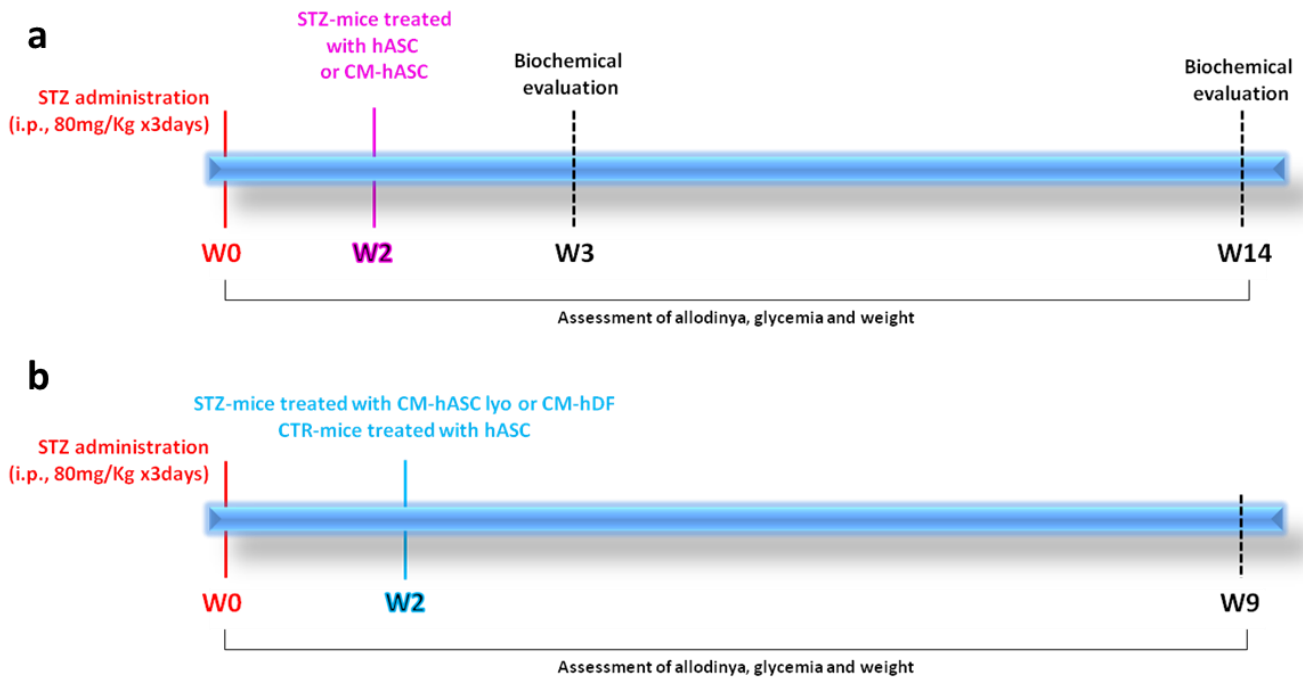
Moreover, 6 weeks after STZ (W6), corresponding to 4 weeks after the first injection two groups of mice received a second hASC or CM-hASC treatment using the previously mentioned doses.

SINGLE TREATMENT IN ADVANCED STAGE OF DISEASE

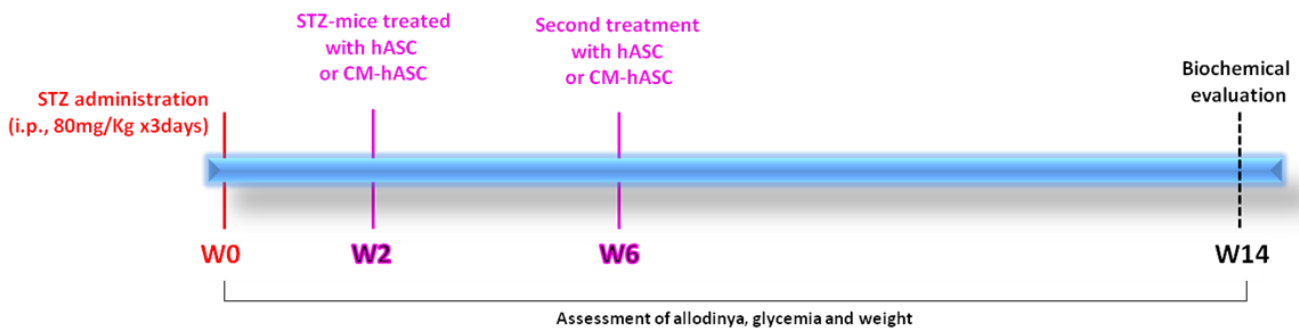
Six weeks after STZ (W6), mice were divided into the following groups:

- Control mice treated with vehicle (CTR)
- Diabetic mice treated with vehicle (STZ)
- Diabetic mice treated with 10^6 hASC (hASC)
- Diabetic mice treated with conditioned medium derived from 2×10^6 hASC (CM-hASC)

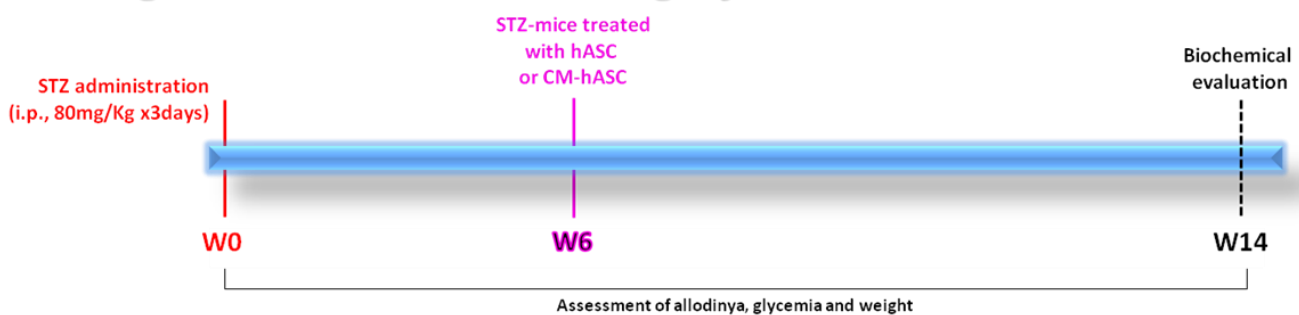
A. Single treatment



B. Repeated treatment



C. Single treatment in advanced stage of disease



Scheme 1 | Experimental protocol schema. (A-a) STZ-mice treated once (W2) with hASC or CM-hASC; (A-b) STZ-mice treated once (W2) with CM-hASC lyo or CM-hDF and CTR-mice treated with hASC. (B) Repeated treatment (W2+W6) with hASC or CM-hASC in STZ-mice. (C) Single hASC or CM-hASC treatment in advanced pathological state (W6).

3.5 Nociceptive behaviour test

In order to characterize the nociceptive behaviour of neuropathic mice, two of the most frequent symptoms encountered in neuropathic patients were evaluated, i.e. allodynia (mechanical and thermal) and thermal hyperalgesia, monitoring over time the threshold responses of the animals to thermal and mechanical stimuli. Behavioural testings were performed before neuropathic induction (T0), i.e. before chemical pancreatectomy with STZ, to establish a baseline for comparisons with post-diabetic induction values, and at different successive times after diabetes induction, weekly or every two weeks respectively, for whole period of experimental study on both hind paws of animals. Thermal allodynia was determined before and after STZ-injection and 1 week after treatments. The acute effect of both treatments was studied in STZ-mice, evaluating the responses to mechanical and thermal stimuli 3, 24 and 72 hours after hASC or secretome administration.

All behavioural tests were carried out in the morning in a stabulary-designed rooms after a habituation period of 30 minutes that allows an “appropriate behavioural immobility” of the animals. The behavioural evaluations were always performed by researchers who were blind to treatments.

3.5.1 Von frey test: mechanical allodynia evaluation

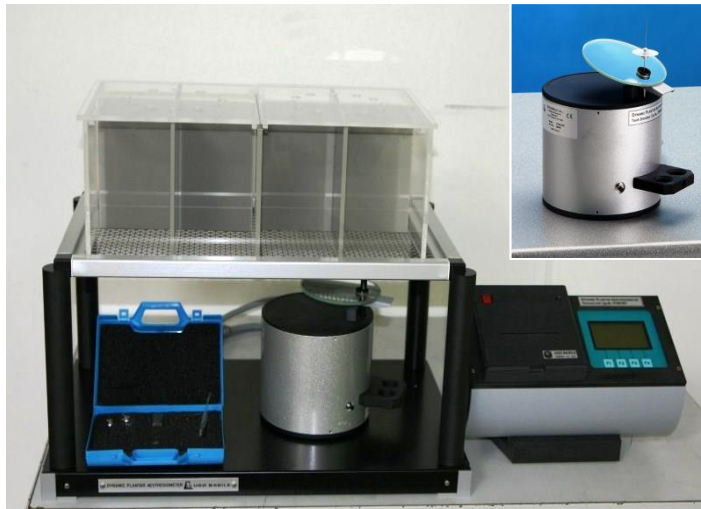


Image 13 | Dynamic plantar Aesthesiometer.
[www.ugobasile.com]

Mechanical allodynia, painful response to innocuous mechanical stimuli, was monitored evaluating the mechanical touch sensitivity through a blunt probe (Von Frey filament, 0.5 mm diameter) on the mid plantar surface of the animal hind paw, using the Dynamic Plantar Aesthesiometer (Ugo Basile, Italy). This instrument consists of a moveable force actuator containing the Von Frey filament, placed below a metallic perforated platform upon which the researchers deposit the animals, whose mobility is restrained by a Perspex cage; two compartments, further subdivided by wooden structures in order to test at the same time more animals, divide the Perspex box and delimit the space within which the animals are free to move, helping the operator to carry out a rapid “testing” work. The moveable force actuator is a cylindrical vessel equipped with an adjustable angled-mirror in order to position the touch probe below the target area of the paw and start keys to actuate a vertical movement of the filament. A controller allows the setting of the force exerted by the filament on the mouse paw and the reading (in grams) of the paw withdrawal threshold (PWT).

An increasing force (ranging up to 10 grams in 10 seconds) starting below the threshold detection is applied on the mid plantar surface of the hind paw; when animals feel pain they remove their paw and the PWT recorded. PWT was measured three times on both hind paws and the mean of the values was calculated.

3.5.2 Acetone drop test: thermal allodynia evaluation

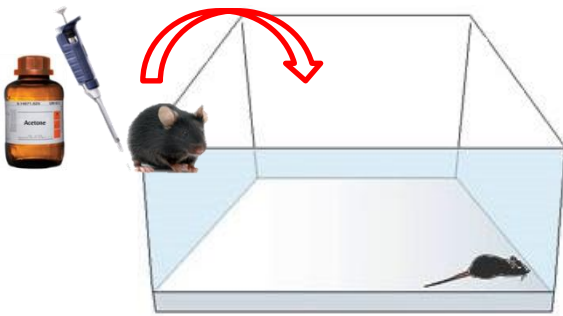


Image 14 | Experimental procedure of acetone drop test.

Cold-allodynia was assessed by Acetone drop test; a drop of acetone (50 μ L) was placed, without touching the skin, in the middle of the ventral side of the hindpaw. Mouse was immediately placed in a perspex box (size: 60cm \times 40 cm \times 15 cm) and allowed to move freely. A stopwatch was started. The response of the animal to acetone was monitored for 20s. If the mouse did not withdraw, flick or stamp its paw within

this 20s period then no response was recorded for that trial (0, see below). However, if within this 20s period the animal responded to the cooling effect of the acetone, then the animal's response was assessed for an additional 20s, for a total of 40s from the application. The reasons why a longer period of time was needed to assess the acetone-evoked behaviour was to measure only pain-related responses and not startle responses that can occur immediately after acetone application. Moreover, painful responses evoked by acetone application is often an interrupted series of behaviours, thus it is important to give the animals enough time to see all pain-related behaviours. Responses to acetone were graded to the following 4-point scale:

- **0**, no response;
- **1**, quick withdrawal, flick or stamp of the paw;
- **2**, prolonged withdrawal or repeated flicking (≥ 2) of the paw;
- **3**, repeated flicking of the paw with licking directed at the ventral side of the paw.

Acetone was applied alternately three times to each hindpaw, with a 5 minutes gap, between the applications. Mean values were then generated for each mouse [Flatters and Bennett, 2004].

3.5.3 Hot plate test: thermal hyperalgesia evaluation

Animals were placed individually into a perspex cylinder on a clean and dry aluminum hot plate maintained at $55 \pm 1^\circ\text{C}$ (Ugo Basile, Varese, Italy). This test measures the time that elapses before the mouse demonstrates hind paw licking/shaking and jumping, which indicate pain in response to the applied heat. Basically, each animal was placed on the heated surface, and the time (in seconds) between placement and pain response was measured. Exposure to heat continued until nociceptive reaction of either hind paws occurred. The latency of the withdrawal response was determined before induction of neuropathy and then every two weeks until the end of the experimental study (W14). The heat source was maintained at constant intensity. A maximal latency value (cut-off) of 20s was set to avoid tissue damage, and the withdrawal latency was approximately 10-13s in naive mice. Animals were tested once at every time points to minimize skin damage and adaptation. Heat hyperalgesia was defined as the average-group of these measurements [Romanovsky *et al.*, 2010; Alshahrani *et al.*, 2012].



Image 15 | Hot plate.
[www.coulbourn.com]

3.6 Blood glucose level and body weight reduction evaluations

The development of diabetes was monitored every week from STZ administrations by evaluating the blood glucose levels and the body weight of each animal, parameters typically altered also in diabetic patients. Blood samples were obtained from a small prick on the animals tail vein and glucose concentration was assessed using a glucometer (GLUCOCARD G+ meter, A. Menarini diagnostics, Italy).



Image 16 | Blood glucose levels evaluation.

Only the animals with blood glucose values above 250 mg/dl were considered diabetic; mice with blood glucose values inferior to this concentration were excluded from the study. Animal body weight was monitored at T0 and weekly after STZ-injection throughout the experimental study using a digital scale (PE1600, Metler Toledo, USA); the evaluations were always performed in the morning.

3.6.1 Glucose metabolism: glucose tolerance test

At W10 from STZ (8 weeks after treatments) 4 mice for each experimental group performed glucose tolerance test. This test was carried out in the morning after an overnight fast (14-18h) during which animals had access to drinking water. Basal blood glucose levels, before and after fasting, were recorded using a glucometer according to the previously mentioned protocol used for canonical glycemia measurement, subsequently animals were intraperitoneally injected with a D-glucose solution 2g/Kg . Blood was then sampled at 30, 60, 90, 120 and 180 minutes after the injection.

After the first measure, bleeding is started by removing the scab of the first incision, massaging the tail in case the blood flow was not sufficient. Further loss of blood from the incision was minimized by apply pressure directly to the incision after each measurement.

At the end of the experimental session, the mouse was placed in a clean cage with food and water ad libitum. Animals were monitored carefully to observe any abnormal behaviors.

3.7 Biochemical evaluations

Mice were killed by CO₂ inhalation at different times:

- ✘ 3 weeks from STZ, one week after treatments (**short time**)
- ✘ 14 weeks from STZ (**long time**):
 - ✘ mice treated 2 weeks from STZ (*W2, single treatment*);
 - ✘ mice treated after 2 and 6 weeks from STZ (*W6 + W2, repeated treatment*);
 - ✘ mice treated after 6 weeks from STZ (*W6, in advanced disease treatment*).

Other animals were also sacrificed in other time points (see Table 3)

3.7.1 hASC localization by Alu sequence detection

Lungs, livers, pancreas and sciatic nerves of STZ and CTR mice were taken and immediately frozen at day 1, 3, 7, 14 and 21 after 10⁶ hASC i.v. injection. Genomic DNA from organs was extracted following standard procedures. Briefly, 25 mg of each tissue (except for the sciatic nerve) were homogenized and lysed in 0.5 mL of lysis buffer (1% SDS, 400mM NaCl, 5mM EDTA [pH 8.0], 100mM Tris [pH 8.0]) containing 0.2 mg/mL of Proteinase-K (Sigma-Aldrich). Differently, both sciatic nerves from each mouse were lysed in 50µl of the same lysis buffer. Samples were incubated overnight at 56°C and following phenol/chloroform extraction, DNA was precipitated in 100% ethanol, washed with ice-cold 70% ethanol and finally resuspended in MilliQ water. Primate specific ALU sequences were amplified by PCR using appropriate primers: (forward, 5'-TGGGCGACAGAACGAGATTCTAT-3'; reverse, 5'-CTCACTACTTGGTGACAGGTTCA-3') that produce DNA amplicons of 224bp [Yu *et al.*, 2012]. Human DNA isolated from hASC was used as positive control. The PCR products were analysed by agarose gel electrophoresis and images were captured using SyngeneGBox-HR Gel Doc System.

Table 3 | In vitro analysis scheme.

Times of biochemical evaluations	Animals/ treatments	Tissue/ cells	Methods	Target	
3 hours after treatment	CTR STZ STZ + hASC STZ + CM- hASC	Splenocytes	ELISA	IFN- γ , IL-2, IL-4 and IL-10	
		Nervous tissues	ELISA	IL-1 β , IL-10 and CGRP	
3 weeks after STZ <i>(short time)</i>	CTR STZ STZ + hASC STZ + CM- hASC	Splenocytes	RT-PCR	IFN- γ and IL-10	
			ELISA	IFN- γ , IL-2, IL-4 and IL-10	
		Nervous tissues	ELISA	IL-1 β , IL-6, TNF α , IL-10 and CGRP	
		Pancreas	ELISA	IL-1 β and IL-10	
		Plasma	ELISA	insulin	
		Epidermal paws	Immunohistochemistry	PGP9.5 (% nerve fibers)	
			Morphology/ histology	Epidermis thickness	
		14 weeks after STZ <i>(longer time)</i>	W2	Splenocytes	ELISA
RT-PCR	IFN- γ and IL-10				
Spinal cord (L4-L6)	ELISA			IL-1 β , IL-6, TNF α , IL-10 and CGRP	
Plasma	ELISA			insulin	
Pancreas	ELISA			IL-1 β and IL-10	
Epidermal paws	Immunohistochemistry			PGP9.5 (% nerve fibers)	
	Morphology/ histology			Epidermis thickness	
Kidney	Morphology / histology			Bowman's space area	
W2 + W6	CTR STZ STZ+hASC STZ+CM-hASC		Splenocytes	ELISA	IFN- γ , IL-2, IL-4 and IL-10
			Spinal cord (L4-L6)	ELISA	IL-1 β , IL-6, TNF α , IL-10
			Plasma	ELISA	insulin
			Pancreas	ELISA	IL-1 β and IL-10
			Kidney	Morphology/histology	Bowman's space area
W6	CTR STZ STZ+hASC STZ+CM-hASC		Splenocytes	ELISA	IFN- γ , IL-2, IL-4 and IL-10
			Spinal cord (L4-L6)	ELISA	IL-1 β , IL-6, TNF α , IL-10
			Plasma	ELISA	insulin
			Pancreas	ELISA	IL-1 β and IL-10
			Kidney	Morphology/ histology	Bowman's space area
1 and 3 days and 1, 2, 3 weeks after treatments	CTR STZ+hASC CTR+hASC		Lungs, liver, pancreas and sciatic nerves	PCR	Alu-sequences

3.7.2 Tissues collection

Nervous tissues/ pancreas and serum collection for cytokines and insulin assay respectively

At different time points (reported in Table 3) Pancreas, DRG, spinal cord and sciatic nerves were rapidly dissected from diabetic neuropathic mice treated with hASC, CM-hASC, saline and their controls, immediately frozen in liquid nitrogen and conserved at -80°C until cytokine protein content assay. Nervous tissues were homogenized in 0.3 ml of ice-cold PBS containing a protease inhibitor cocktail (lysis buffer) (Roche Diagnostics, Italy). Pancreatic tissues were homogenized in 2 ml of the same lysis buffer. All samples were centrifuged at 13000 rpm for 15 minutes at 4 °C. Supernatants were collected and used to measure pro- and anti- inflammatory cytokines levels and total protein content (Lowry's method, see paragraph 3.7.3). In order to evaluate CGRP concentration spinal cord and DRG were homogenized in 2 N acetic acid (250µL), heated at 90°C for ten minutes, centrifuged, supernatant was dried, and then dissolved in EIA buffer. Before proceeding at tissues removal, a small aliquot of blood from all each mice was collected in tubes for plasma insulin dosage.

Spleen cells collection and stimulation for cytokine assay

Spleen cells collection was performed 2, 3 and 14 weeks from STZ. At these time points mice were killed and their spleens rapidly and aseptically removed. Splenocytes were spilled out from an incision on spleen cuticle made with 20-gauge needles, adjusted in 24-well plates at the final concentration of 4×10^6 cell/ml of culture medium (complete RPMI, i.e. RPMI 1640 supplemented with 10% FCS, 1% glutamine, 2% antibiotics and 0.1% 2-mercaptoethanol) and incubated at 37 °C in 5% CO₂ and 95% air with or without 10 µg/ml Concanavalin A (ConA) for Th1 and Th2 cytokines stimulation. The stimulus was added to the cell cultures in a final volume of 1 ml/well in complete RPMI. After 24 (for IFN-γ and IL-2) or 48 hours (for IL-4 and IL-10) of culture, times of maximum cytokines release [Sacerdote et al., 2000; Martucci et al., 2007], the supernatant was collected and stored frozen at -80 °C for cytokine assay. Moreover, 4×10^6 cells were pelleted for mRNA evaluation (see 3.7.4) and stored at -80 °C.

3.7.3 Enzyme-Linked Immune-Sorbent Assay (ELISA)

Cytokine concentration was determined by Enzyme-Linked Immuno-Sorbent Assay (ELISA) using ultra-sensitive ELISA kits according to the manufacturer's instruction. DuoSet® ELISA development system for mouse IL-2, IFN-γ and IL-4 was purchased from R&D Systems (Minneapolis, USA) while mouse IL-10, IL-1β, TNF-α and IL-6 ELISA Ready-SET-Go! from eBioscience (San Diego, CA). CGRP EIA Kit was purchased from Cayman. Serum insulin dosage was performed using a specific ELISA kit provided by Mercodia (Uppsala, Sweden). Sensitivity of the kits are reported in **Table 4**.

Table 4 | ELISA Kits

	Sensitivity	Kit
Interleukin 1 β (IL-1 β)	8 pg/mL	eBioscience
Interleukin 2 (IL-2)	15.6 pg/mL	R&D
Interleukin 4 (IL-4)	15.6 pg/ mL	R&D
Interleukin 10 (IL-10)	32 pg/ mL	eBioscience
Interferon γ (IFN- γ)	31.2 pg/mL	R&D
Tumor Necrosis Factor (TNF- α)	8 pg/mL	eBioscience
Interleukin 6 (IL-6)	4 pg/mL	eBioscience
Calcitonin Gene Related Peptide (CGRP)	3.91 pg/mL	Cayman
Insulin	0.2 ng/mL	Mercodia

Briefly, a first primary anti-cytokine monoclonal antibody (Ab-I) was seeded onto a polystyrene plate with U bottom 96 wells. Ab-I adhesion to the plastic polymer was favoured by basic pH and occurred during an over-night incubation at 4 °C (eBioscience kit) or at room temperature (R&D kit). In the insulin kit (Mercodia), wells were already Ab-I pre-coated. Several washes were then carried out to eliminate antibody excess that was not bound. The nonspecific sites of the plate were blocked a with blocking buffer solution; samples and standards were subsequently added. Plates were incubated to ensure that any cytokine present in the sample bound to the antibody already attached to the wells. After several washes, secondary anti-cytokine biotinylated monoclonal antibody (Ab-II) was added: this bound the immobilized cytokine already linked to Ab-I.

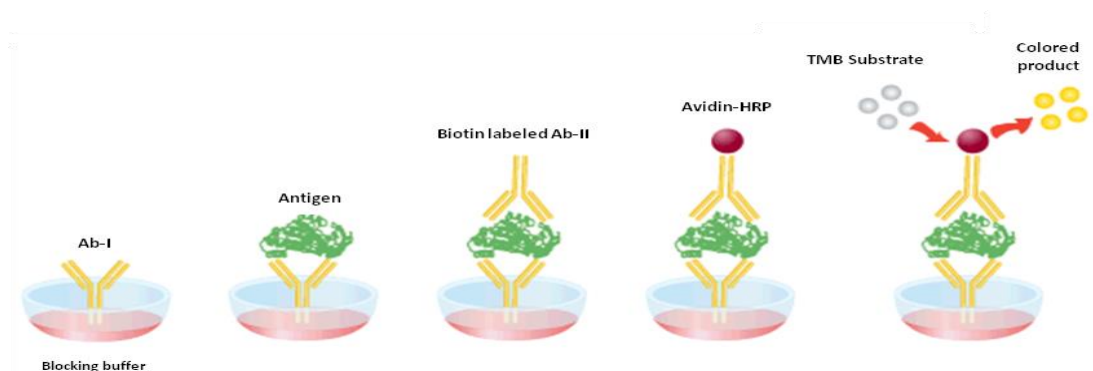


Image 17 | Illustration of sandwich ELISA method
[\[http://www.leinco.com/sandwich_elisa\]](http://www.leinco.com/sandwich_elisa)

Therefore a "sandwich" was formed with the substance in the center and on the sides the antibodies. After another series of washes, avidine peroxidase, which has the task of binding the biotinylated antibody, was added.

Finally, enzyme substrate was added: a colored complex was formed the intensity of which was proportional to the amount of cytokine present in the sample; color development was stopped with

H₂SO₄ [1M] addition. Results were obtained by reading the absorbance of each well in a spectrophotometer, using a specific wavelength to each cytokine studied. In cell cultures (splenocytes and macrophages) released cytokine concentrations were determined by interpolation with standard curve assayed the same plate (response pg/mL); for tissues (nervous tissues and pancreatic tissues) ELISA obtained values were then normalized considering the total protein content in of each sample.

3.7.4 Determination of total protein concentration

Total protein concentration is necessary to compare the quantity of cytokines obtained with ELISA to the total quantity of proteins present in the samples. It was evaluated according to the Lowry method which uses bovine serum albumin (BSA) as a standard.

A standard curve was prepared as follows: BSA powder was dissolved in distilled water and diluted to a concentration of 1 mg/mL. In each standard test tube: 5, 10, 20, 40, 60, 80, 100 µL of BSA (1mg/mL) were added and brought them to a final volume of 100 µL with distilled water. Blanks were obtained substituting albumin with 100µL of distilled water. All standards, and samples, were made in replicates of 2.

Samples were diluted, depending from the tissue of origin, such that they would fall within the BSA standard range (0-100 µg/µL) and a total volume of 100 µL was placed in each tube. Then 200µL of NaOH 1N and 2mL of Cooper Reagent¹ were added to each test tube. Samples were incubated without light for 10 minutes and then 100µL of Folin Ciocalteu reagent were added to each one of them. A second incubation without light for 45 minutes was needed to proceed to the reading of absorbance through a spectrophotometer (Uvicon 941 PLUS, Kontron Instruments), with a wavelength of 750nm.

Samples were obtained with analogous procedure of that used for the standard straight line using a fixed quantity of sample, instead of albumin, by adding distilled water we reached the final volume of 100µL.

¹ composed of 2% Na₂CO₃, 1% CuSO₄ and 2% Na K-tartrate dissolved in distilled water in a proportion of 100:1:1

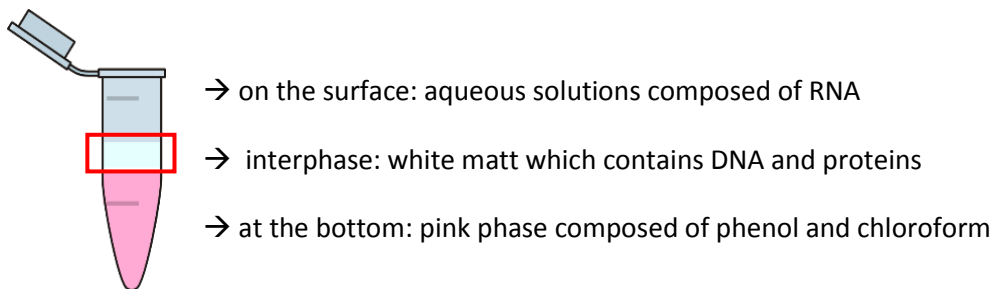
3.7.5 Evaluation of mRNA levels of IL-10 and IFN- γ in spleen cells

Every manipulations was done with the use of RNase-free material and sterile solutions in order to avoid any contaminations of samples.

3.7.5.1 Homogenization of samples and total RNA extraction

To evaluate levels of mRNA of Th1 (IFN- γ) and Th2 (IL-10) cytokines, a part of isolated spleen cells were homogenized with turrax in 1 mL of Trizol[®] (Trifast Eurogold[®], Euroclone, Italy), which is a monophasic solution of phenol and guanidine isothiocyanate that destroys cell membrane allowing leak from cells of RNA. Using insulin syringes (one for each sample to avoid contamination) they were transferred in a new series of tubes and 200 μ L of chloroform (Serva) were added to each one. Samples were manually shaken for 30 seconds and then they were incubated for 5 minutes at room temperature.

After this pause they were centrifuged at 13000 rpm for 15 minutes at 4°C. At this point there is a separation of the solution in three different phases:



The superficial phase was transferred in a new series of tubes and then 500 μ L of Isopropanol (Sigma Aldrich) were added to each sample to allow the precipitation of the RNA. Test tubes are then manually shaken for 30 seconds, incubated at room temperature for 10 minutes and centrifuged at 13000rpm for 15 minutes at 4°. At the end of this centrifugation step, the bottom of the tube contained a pellet of RNA. Supernatant was eliminated and pellet was washed with 1mL of ethanol 75%. Samples were subjected to another centrifugation step at 13000rpm for 10 minutes at 4°C, ethanol was eliminated and the pellet dried in the air. At the end dry RNA pellet was resuspended in 20 μ L of DEPC water (Fluka) and stored at -80°C to avoid degradation of the sample.

3.7.5.2 Digestion of genomic DNA

Before proceeding to the quantification of total mRNA contained in samples and to the retrotranscription to cDNA which is the final substrate for the PCR reaction, samples were incubated in a thermostate bath for 10 minutes at 55-60°C. To avoid any contamination of genomic DNA, samples

underwent to DNase treatment² (DNA-free™ DNase kit Treatment and Removal Reagents, Ambion, Applied Biosystem, Italy) to avoid false-positive results. At the end of this treatment, they were incubated in a thermocycler (T100™, BioRad) for 40 minutes at 37°C. Subsequently, a quantity of 0,1 of the final volume (in our case 2 µL) of DNase Inhibitor Reagent, which inactivates DNase, was added to each sample. Samples were left for 3 minutes at room temperature. They were finally centrifuged at 12000 rpm for 3 minutes and then the supernatant (which contained total RNA) was transferred to another series of test tubes.

Samples could now be used for quantification of the total mRNA or can be conserved at -80°C until use.

3.7.5.3 Quantification of total mRNA

Total RNA concentration was determined using spectrophotometer U.V. (Biophotometer, Eppendorf). From each sample a rate of 1µL was taken and diluted with 49µL of DEPC water. The total volume was transferred in uvette for the reading at the spectrophotometer set to a wavelength of 260 nm. Total concentration of RNA for each sample was calculated whereas OD260=1 is equal to 40 ng/µL, using the following formula:

$$[] \text{ total RNA} = \text{OD} \times 40 \text{ ng}/\mu\text{L} \times \text{FD}$$

To test the purity of extracted mRNA the ratio between absorbance of RNA (OD260) and absorbance of proteins (OD280) is evaluated. This ratio, in absence of contamination, has to be between 1,8 and 2.

3.7.5.4 Retrotranscription of mRNA

Extracted RNA is converted in cDNA. This is done thanks to the use of random primers, commercial exanucleotide that pair to the isolated RNA giving the docking (represented by an initial oxidrile group) for the activity of the reverse transcriptase enzyme. After the quantification of the total RNA, 1000ng were taken from each sample and a fixed quantity of 4µL of Mix for retrotranscription, Iscript RT supermix 5X (BioRad)³ was added to the RNA. Final volume of reaction was reached through the add of a quantity of DEPC water of: 20µL - (4µL RT Supermix + µL sample)

Samples are then incubated in thermocycler for 40 minutes with the following procedure: 5 minutes at 25°C (priming), 30 minutes at 42°C (Reverse Transcription) and the last 5 minutes at 85°C (RT inactivation).

Samples of cDNA obtained were preserved at -80°C for further analysis, RT-PCR.

² DNA-free™ kit contains Dnase-I Buffer (10X) and rDNase. In our case $V_f = 20\mu\text{L}$, for each sample 2µL of DNase-I buffer (10X) and 1µL of rDNase were added (the latter quantity is independent from V_f).

³ 5x RT supermix with iScript MMLV-RT (RNase H⁺), RNase Inhibitor, dNTPs, oligo (dT), random primers, Buffer, MgCl₂ and stabilizer

3.7.5.5 Real Time-PCR

Real Time-PCR is a semi-quantitative technique that allows the study of gene expression. Amplification of cDNA is monitored during all the exponential phase of growth.

The amplification reaction, through Real Time-PCR, was performed using ABI PRISM 7000 system (Applied Biosystem, Foster City, CA) and carried out in a final volume of 25µL constituted by:

- 2 µL of cDNA obtained from previously retrotranscription of mRNA
- 1,25µL of TaqMan primers/probe
- 10 µL of Real Master Mix Probe Rox (Eppendorf) containing Taq-polymerase enzyme
- 11,75 µL of RNase-free water

Specific TaqMan primers/probe for cytokines of interest, IL-10 (Mm00439616_m1) and IFN-γ (Mm01168134_m1), were used. As endogenous control gene GAPDH (Mm99999915_g1) (Taqman[®] Assay-on-Demand[™] Gene Expression Products, Applied Biosystem) was used.

All Real Time PCR assays were performed in duplicate. Before using the $\Delta\Delta C_t$ method for relative quantification, a validation experiment was performed in order to demonstrate that the efficiencies of targets and reference were approximately equal. As controls reaction mix without cDNA was used.

The reaction conditions were as follows: 95°C for 2 minutes (Initial Denaturation), followed by 45 cycles at 95°C for 15 seconds (Cycled Template Denaturation) and at 60°C for 60 seconds (Annealing and Extension).

Relative quantification was performed using the comparative threshold method. Threshold cycle numbers (C_T) of the specific gene of interest and the endogenous control gene GAPDH were determined by ABI PRISM 7000 Sequence Detection System.

Gene expression values, of each gene, were standardized on correspondent values of the endogenous control gene GAPDH and relate as relative values of calibrator sample using the following formula:

$$2^{-\Delta\Delta C_T}$$

Briefly, ΔC_T value was calculated subtracting GAPDH C_T to target gene of interest C_T (ΔC_T); while $\Delta\Delta C_T$ was calculated subtracting the GAPDH C_T average of saline treated healthy control group (non-diabetic mice) to ΔC_T value of each sample.

3.8 Skin immunohistochemistry and morphometry

Mice plantar skin was collected and fixed in 10% buffered formalin for 24 hours. Skin was embedded in paraffin according to standard procedures and cut at 8 µm by a microtome (Microm HM 325). Alternate paraffin sections from the middle part of plantar skin were processed by immunohistochemistry. The sections were deparaffinised, rehydrated and subjected to antigen retrieval in 0.05M sodium citrate buffer (pH 6.0) in hot water bath (98°C for 20'). Endogenous peroxidase activity was blocked by incubation with a solution of 3% hydrogen peroxide. Sections were

immunostained with the monoclonal antibody anti-mouse PGP9.5 (protein gene product 9.5, dilution 1:250, EMD Millipore, Darmstadt, Germany), a marker of nerve fibers. All sections were processed using UltraVision Quanto Detection System horseradish Peroxidase (HRP; ThermoScientific, Bio-Optica, Milan, Italy), followed by development with diaminobenzidine (Amresco, Prodotti Gianni, Milan, Italy). Finally, the sections were dehydrated and mounted and some of them were previously counterstained with hematoxylin. Immunohistochemical control was performed by omitting the primary antibody, in presence of isotype matched IgGs and performing pre-adsorption assay using the related peptide and gave negative results.

3.8.1 Quantification of epidermal innervation

Digitally fixed images of slices were analyzed using an image analyzer (Image Pro-Premier, Immagini e Computer, Milan, Italy). The density of nerve fibers was evaluated measuring the percent area occupied by positive PGP9.5 nerve fibers in epidermal area including the sub-basal one. The data of all animals were analyzed at a final 400X magnification measuring three randomly collected fields for each section.

3.8.2 Morphological evaluation of epidermal thickness

Digitally fixed images of slices were analyzed using an image analyzer (Image Pro-Premier, Immagini e Computer, Milan, Italy). Epidermal thickness was evaluated by means of the distance (in μm) between basal membrane and stratum granulosum at a final 400X magnification measuring three randomly collected fields for each section by researcher unaware of the animal group assignment.

3.9 Kidney histological analysis

Kidneys were fixed in 10% buffered formalin (Sigma-Aldrich) for 24 hours at RT and then processed for paraffin embedding through a graded ethanol series through STP 120 Spin Tissue Processor (Bio Optica). Morphological analysis was performed on 2-3 μm Harris' hematoxylin and eosin (Bio Optica) stained sections (images acquired by Olympus BX51, Japan). Bowman's space area (73,74) in at least five randomly collected fields were quantified by ImageJ software at a final 200X magnification, by researchers unaware of the animal group assignment. At least 30 glomeruli/ group were analyzed.

3.10 Data analysis

Statistical analysis were performed using GraphPad Prism 5 Software (San Diego, CA, U.S.A). Data were tested for equal variance before choosing statistical analysis.

Data from mechanical allodynia measurements were analyzed by mean of *Two-Way ANOVA* considering the type of treatment and the time as factors. Baseline values, i.e. responses before STZ injection, were not included in the analysis. If an overall test comparing group was significant, Bonferroni's test was used for between-group comparisons in the post hoc analysis. Cold allodynia scores were compared with *Mann–Whitney U-test*.

Cytokines results were analyzed using *One-Way ANOVA* or *Two-Way ANOVA*, followed by Bonferroni's post hoc test for multiple comparison. Body weight and blood glucose levels were statistically evaluated by *Two-way ANOVA* considering the type of treatment and the time as factors. If an overall test comparing group was significant, Bonferroni's test was used for between-group comparisons in the post hoc analysis.

Data from histological experiments (skin thickness and kidney damage) and of immunohistochemistry (PGP9.5) were analyzed and compared by analysis of variance (*One-Way ANOVA*) and by a Bonferroni's multiple comparison test.

The overall significance level was 0.05 for each hypothesis. Each group consisted of 6 animals. The group size was chosen on the basis of the results obtained in our previous studies. For antiallodynic response, considering an expected difference in means of 40%, SD of 10%, number of treatments 4, power 0.95 and α 0.05, a number of 3 animals for group could have been enough. However, considering also cytokines as important outcome, where the SEM can be estimated in 30%, the number must be increased to 6 animals. Results are expressed as mean \pm SEM.

Results

4.1 Effect of therapeutic treatments on allodynia, hyperalgesia and hyperglycemia

In order to study whether hASC and CM-hASC treatments were effective in alleviating diabetic neuropathic pain we performed a series of experiments using a mouse model of diabetes induced by chemical destruction of pancreatic insulin-secreting β -cells; animals were injected with multiple low doses of β -cell toxin, streptozotocin (STZ, 80 mg/kg, i.p.; daily for three consecutive days).

STZ administration induced in animals a rapid establishment of hyperglycaemic state associated to the development of painful symptoms. As depicted in **Figure 1 (panel A)**, blood glucose levels of STZ mice strongly increased after 2 weeks from diabetes induction and were still significantly higher on weeks 14 compared to normo-glycaemic values of controls; no treatments modified blood glucose levels that were always elevated in STZ mice over time.

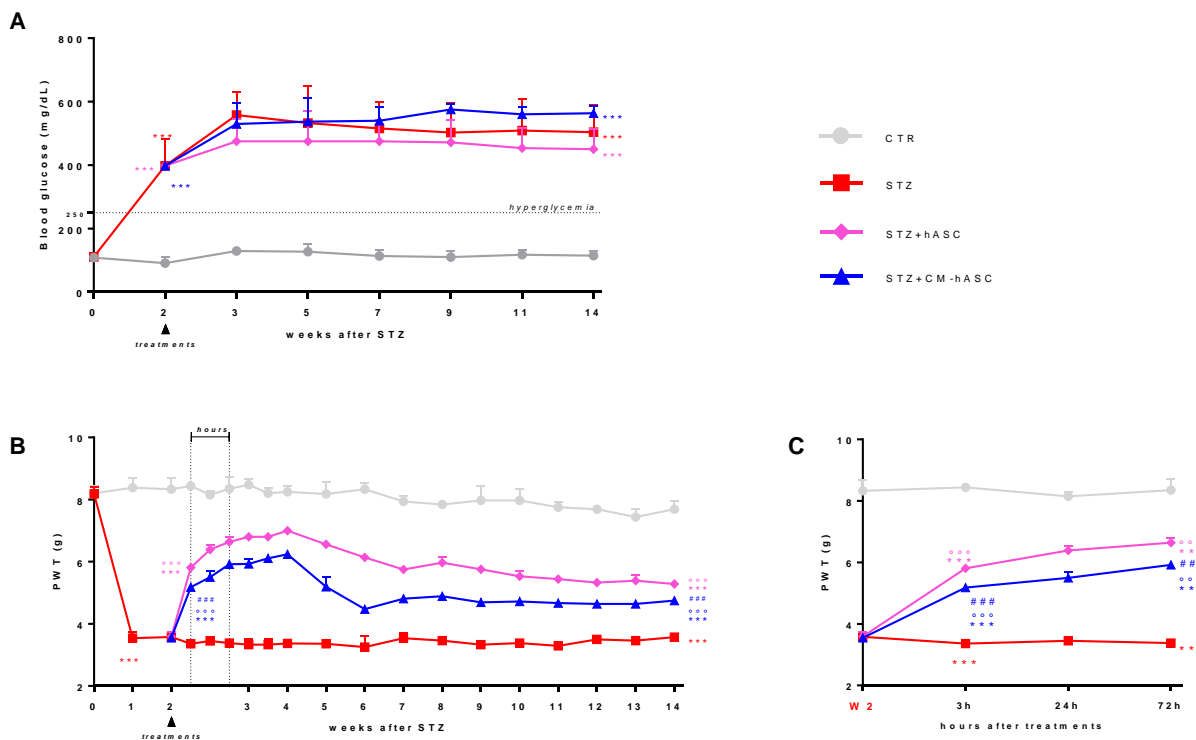


Figure 1 | Effects of a single hASC and CM-hASC treatment on hyperglycemia and mechanical allodynia. Mice received a single hASC (10^6) or CM-hASC (obtained from 2×10^6 cells) treatment, 2 weeks after STZ, and the effects on hyperglycemia (A) and mechanical allodynia (B, C) were monitored up to 14 weeks after STZ (*long-lasting effects*, B) and few hours (3 to 72 h) after the injection (*short-term effects*, C). Data represent mean \pm SEM of 6 mice per group. Two-way ANOVA was used for statistical evaluation, followed by Bonferroni's post hoc test for multiple comparisons. *** $p < 0.001$ vs CTR; ** $p < 0.001$ vs STZ; ### $p < 0.001$ vs STZ + hASC.

A marked reduction of paw withdrawal thresholds (PWT) of mice is observed one week after STZ (*panel B*). Pain thresholds to mechanical stimuli of STZ mice remained significantly lower than those of controls, i.e. non-diabetic mice, for the entire period of observation, i.e. up to 14 weeks.

Two weeks after STZ, when mechanical allodynia was fully developed, hASC or CM-hASC were i.v. injected. As reported in **Figure 1 - panel B**, both treatments were able to significantly reduce mechanical

allodynia, although the effect of hASC was significantly stronger than that elicited by CM-hASC. A significant reduction of mechanical allodynia was evident already 3 hours after treatments (**Figure 1, panel C**). Their anti-allodynic effect was maximal between 1 and 2 weeks, and was extremely long lasting since allodynia was maintained significantly reduced up to 12 weeks after a single hASC and CM-hASC injection (14 weeks after STZ, W14).

Since the sensory alterations associated to diabetic neuropathy in patients are often diverse and associated to modified response to several stimuli, we also decided to test the action of hASC and CM-hASC treatments on thermal hyperalgesia for the whole period (up to 14 weeks after STZ) and on cold allodynia during the first week after injection (3 weeks after STZ). As shown in **Figure 2 - panel A**, STZ-mice are characterized by a hyperalgesic condition between 1 and 3 weeks after STZ; 4 weeks after diabetic-induction, STZ-mice showed a clear shift toward an hypoalgesic state, which was stabilized 6 weeks after STZ and that remains up to 14 weeks.

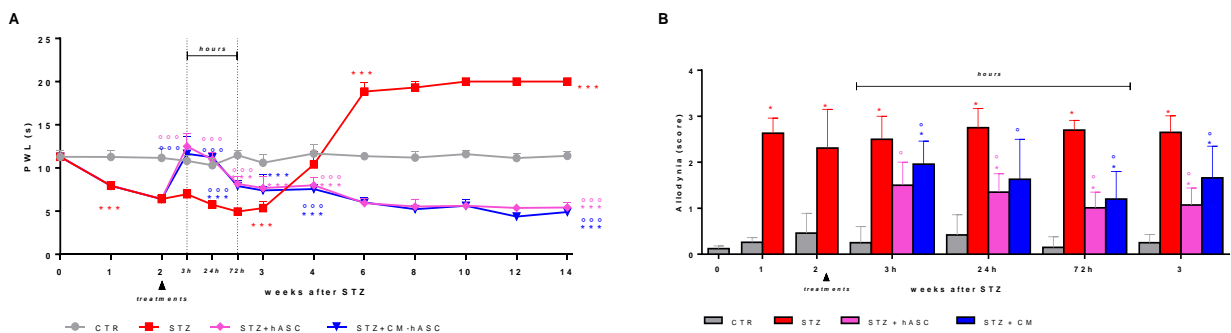


Figure 2 | Effect of a single hASC and CM-hASC treatment on thermal hyperalgesia and cold allodynia. Mice received a single hASC (10^6) or CM-hASC (obtained from 2×10^6 cells) treatments, 2 weeks after STZ, and the effects on hyperalgesia (A) and cold allodynia (B) were monitored up to 14 or 3 weeks after STZ, respectively. Values are mean \pm SEM of 6 mice per group, and were compared with Two-way ANOVA followed by Bonferroni's test for multiple comparisons (thermal hyperalgesia, A) and Mann-Whitney U-test (cold allodynia, B) * $p < 0.05$, *** $p < 0.001$ vs CTR; ° $p < 0.05$, °°° $p < 0.001$ vs STZ.

In the first hours after hASC and CM-hASC injection (3 and 24h) thermal hyperalgesia in STZ-diabetic animals was completely reduced by both treatments, but 72 hours after treatments mice return to a hyperalgesic state and this condition remains until the end of observation (W14); it is clear that both treatments were able to counteract the transition toward the hypoalgesic state. In addition, as reported in **Figure 2 - panel B**, administration of both hASC and CM-hASC rapidly reduces cold allodynia displayed by STZ-diabetic mice and their effect was still present 1 week later. These data demonstrate the ability of hASC and their conditioned media to relieve hypersensitivity due to different stimuli that are peculiar of diabetic pain.

Moreover, 4 weeks after the first treatment (6 weeks after STZ) a group of diabetic animals was administered a second time with either hASC or CM-hASC. As shown in **Figure 3 - panel A**, the second CM-hASC injection increased the PWT restoring sensitivity to the level reached after the first treatment and prolonging its effect. The anti-allodynic effect was potentiated already few hours after the second CM-hASC injection (**Figure 3, panel B**) and its trend completely mimicked both the rapid effect evoked by the early treatment (compare **Figure 1 - panel C** to **Figure 3 - panel B**) and the hASC effect up to 10 weeks after diabetes-induction (**Figure 3, panel A**). Repeated hASC treatment did not further ameliorate allodynia.

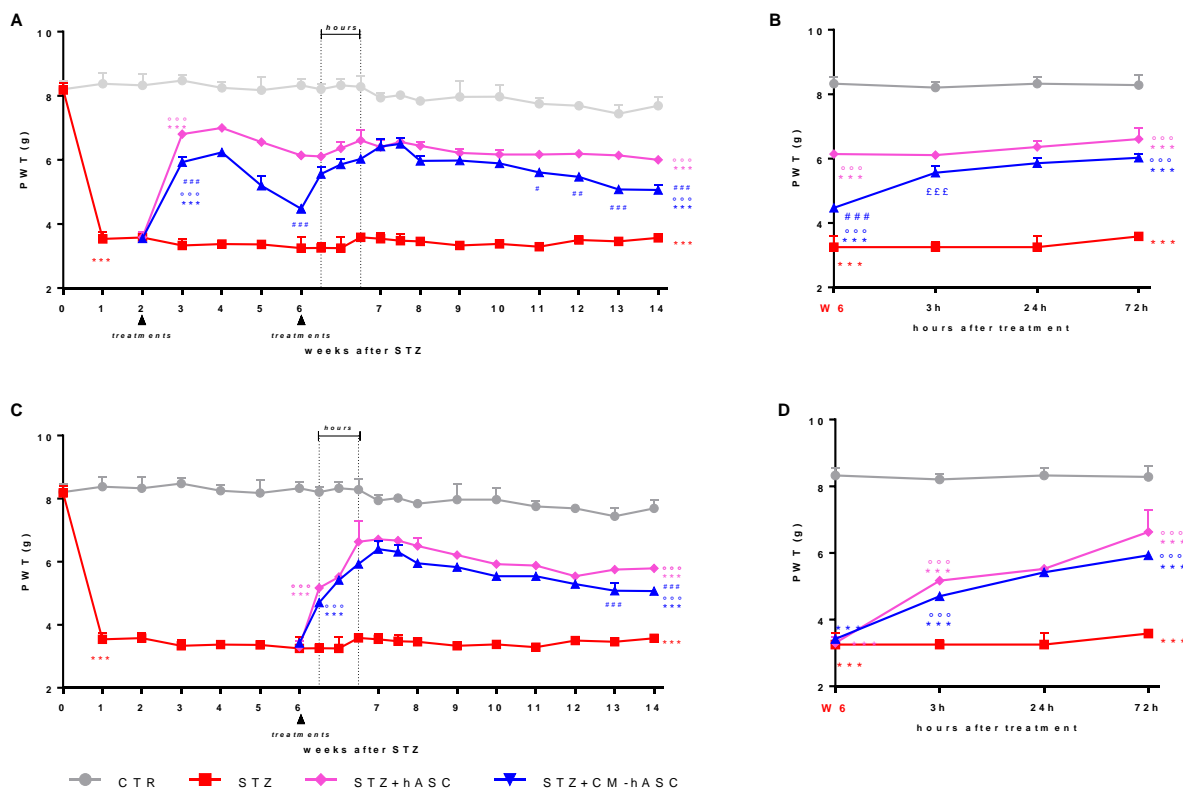


Figure 3 | Effect of repeated or advanced hASC and CM-hASC treatment on mechanical allodynia. (A and B) Mice received two repeated hASC or CM-hASC administrations 2 and 6 weeks after STZ; long-lasting effects (A) and short-term effects after the second administration (B) are shown. (C and D) Mice received a single hASC or CM-hASC injection, 6 weeks after STZ; long-lasting (C) and short-term effects (D). Data represent mean \pm SEM of 6 mice per group. Two-way ANOVA was used for statistical evaluation, followed by Bonferroni's post hoc test for multiple comparisons. *** $p < 0.001$ vs CTR; °°° $p < 0.001$ vs STZ; # $p < 0.05$, ### $p < 0.01$, #### $p < 0.001$ vs STZ + hASC; £££ $p < 0.001$ vs W6

In order to elucidate whether hASC and CM-hASC treatments were also therapeutically effective at a later time point, animals were treated for the first time 6 weeks after diabetes induction. Also this late treatment was able to provide a fast and irreversible antiallodynic effect (**Figure 3, panels C and D**). In addition, 13 weeks after STZ, the effect of hASC appeared significantly more pronounced than the one exerted by CM-hASC. Moreover, 6 weeks after diabetic-induction neither second nor first hASC/CM-hASC treatment were able to modulate blood glucose level: hyperglycemia of all treated mice remained similar to diabetic mice (data not shown).

We also treated STZ-mice with lyophilized CM obtained from 2×10^6 hASC, to evaluate whether the secretome had the same effects on mechanical allodynia once lyophilized. The possibility to store secretome in a lyophilized form would be useful for a potential clinical application. **Figure 4 – panel A** shows that lyophilized CM-hASC had lost its anti-allodynic effect, in fact it exerts a very weak antiallodynic effect that is significant in comparison to STZ-mice only in the first week after treatment; this result suggests that during the lyophilization process bioactive factors essential for pain relief are lost.

Concurrently, to prove evidence that CM-hASC effect was due to the specificity of the cell source, 2 weeks after diabetic induction STZ-mice were treated with conditioned media derived from human fibroblasts (CM-hF). As shown in **Figure 4 - panel B**, CM-hF does not counteract mechanical allodynia, indicating that specific factors contained in the secretome of adipose-derived mesenchymal stem/stromal cells are responsible of the effect observed *in vivo*.

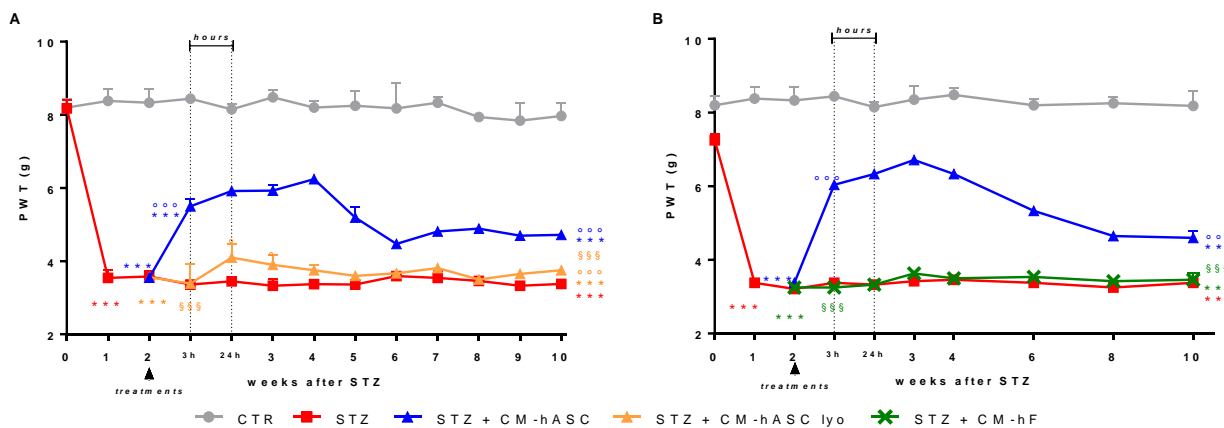


Figure 4 | Effect of other CM on mechanical allodynia. Two weeks after diabetic induction, STZ-mice received lyophilized CM obtained from 2×10^6 hASC (A) or CM obtained from 2×10^6 human fibroblasts (B). Data represent mean \pm SEM of 6 mice per group. Two-way ANOVA was used for statistical evaluation, followed by Bonferroni's post hoc test for multiple comparisons. *** $p < 0.001$ vs CTR; * $p < 0.05$, *** $p < 0.001$ vs STZ; §§§ $p < 0.001$ vs STZ + CM-hASC.

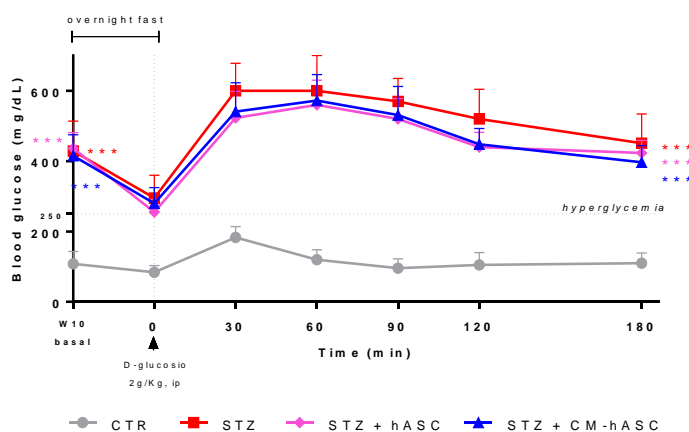


Figure 5 | Glucose tolerance test.

10 weeks after diabetic induction, intraperitoneal (i.p.) glucose tolerance tests were performed following an overnight fast (~16 hours) and a of 2 g/kg glucose injection. Blood glucose concentration was assessed at the indicated time points in mice. Data represent mean \pm SEM of 4 mice per group. Two-way ANOVA was used for statistical evaluation, followed by Bonferroni's post hoc test for multiple comparisons. *** $p < 0.001$ vs CTR.

Since we observed therapeutic effects of treatments on hypersensitivity but no change in basal blood glucose levels, we decided to investigate whether the treatments were able to modify the way glucose was metabolized and mice were subjected to the glucose tolerance test. Ten weeks after STZ diabetic, hASC or CM-hASC treated mice (1 single treatment) and naïve CTR, were intraperitoneally injected with 2 g/kg D-glucose, after an overnight fast. Blood glucose levels were monitored every 30 minutes after the injection for the following 3 hours (**Figure 5**). hASC/CM-hASC treated mice, showed a glycaemic peak similar to that of STZ- mice, thus indicating that in both cases mice were unable to metabolize glucose.

4.2 Localization of hASC

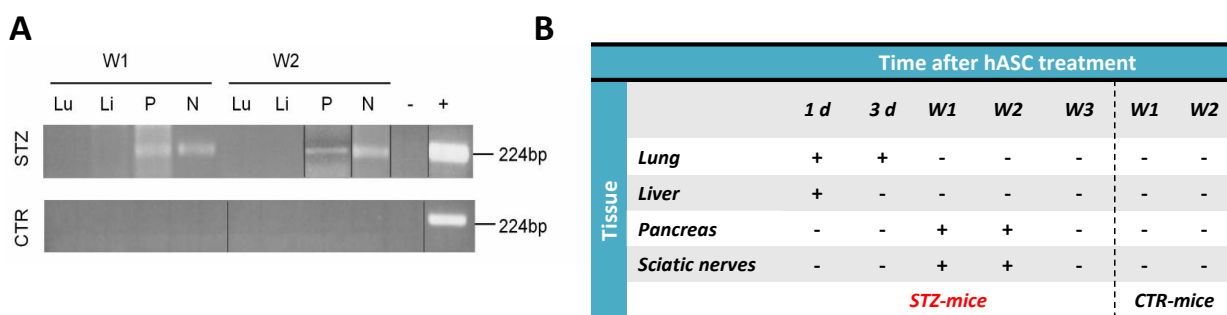


Figure 6 | hASC tracking by human ALU sequences detection. Representative gels showing human ALU sequences detection in STZ and naïve (CTR) mice tissues collected 1 week (W1) and 2 weeks (W2) after treatment (**A**). Lu: lungs; Li: liver; P: pancreas; N: sciatic nerves; + : positive control; - : negative control. Summary of the Alu PCR products observed in lung, liver, pancreas and sciatic nerve after 1 and 3 days and 1, 2 and 3 weeks from hASC administration (**B**).

We monitored the fate of the injected hASC and investigated localization of infused hASC up to 3 weeks after the injection by using ALU sequences, dimeric primate's specific sequences considered human DNA's molecular markers. The presence of human DNA was assessed in lungs, liver, pancreas and sciatic nerves of both diabetic (STZ) and healthy (CTR) mice (**Figure 6 - panels A and B**). As expected, at day 1 after treatment, human DNA was present in filter organs such as lungs and liver, at 3 days was identified only in lungs and it was not detectable in the damaged tissues such as pancreas and sciatic nerves (**Figure 6 - panel A and data not shown**).

However, when the antiallodynamic effect was well established (week 1 and week 2 after injection), human DNA was noticeable only in pancreas and sciatic nerve of STZ-mice, confirming the tropism and recruitment of hASC to the injured tissues, and not to the CTR-undamaged ones (**Figure 6, panels A and B**). At later time, 21 days after injection, human DNA was undetectable in all the tested tissues (**Figure 6, panel A and data not shown**).

4.3 Effects of hASC and CM-hASC on cytokine levels and CGRP in the main stations involved in pain transmission

It is well known that in diabetic neuropathy a neuroinflammatory cascade, characterized by altered levels of pro- and anti-inflammatory cytokines, is present in the main sites involved in nociception transmission. To verify an anti-inflammatory or immunomodulatory mechanism for hASC and CM-hASC mediated anti-hypersensitivity action, we checked IL-1 β , IL-6, TNF- α and IL-10 levels in the sciatic nerves, DRG and spinal cord of STZ mice. *Panels A-D of Figure 7* illustrate cytokine levels 3 weeks from neuropathy induction and 1 week after hASC and CM-hASC injection. Proinflammatory cytokines IL-1 β , TNF- α and IL-6 (*Figure 7, panel A-C*) were overexpressed in peripheral (sciatic nerve and DRG) and central (spinal cord) nervous system of diabetic mice.

Both hASC and CM-hASC treatments were able to restore IL-1 β , TNF- α and IL-6 basal levels, 1 week after treatment. In addition, IL-10 levels (*Figure 7 - panel D*) appeared significantly reduced in sciatic nerve, DRG and spinal cord of diabetic animals. Both hASC and CM-hASC significantly increased IL-10 concentrations in DRG and spinal cord. In the sciatic nerve of hASC/CM-hASC treated animals the anti-inflammatory cytokine was significantly elevated in comparison to STZ animals. Indeed after hASC, IL-10 increased over basal levels, indicating a rapid switch towards an anti-inflammatory environment in all these areas involved in pain transmission.

Fourteen weeks after STZ, *Figure 8*, spinal cord IL-1 β , TNF- α and IL-6 levels were still significantly elevated and IL-10 levels were reduced in diabetic mice, indicating the persistence of neuroinflammation. As previously described for the antiallodynic effect (*Figure 1 - panel B*), cytokines modulation induced by hASC and CM-hASC was long lasting. In *Figure 8*, 12 weeks after treatments (W2), the levels of IL-1 β (*panel A*), TNF- α (*panel B*) and IL-6 (*panel C*) were still significantly reduced by hASC and CM-hASC, while only in animals treated with hASC we observed a significant normalization of IL-10 (*panel D*). As reported in *Figure 8*, a second hASC and CM-hASC treatment (W2+W6) did not further modify pro-inflammatory cytokine levels (IL-1 β , TNF- α and IL-6, *panel A-C*) in comparison to single (W2) treatment; but the second CM-hASC treatment was able to normalize IL-10 levels (*panel D*). Moreover, both treatments were still effective when administered at a later stage of the pathology (W6); as reported in *Figure 8, panels A-D*, 8 weeks after the administration of both hASC and their CM there is a significant modulation of cytokine levels, although the effect on IL-1 β was more evident with hASC than with CM-hASC treatment

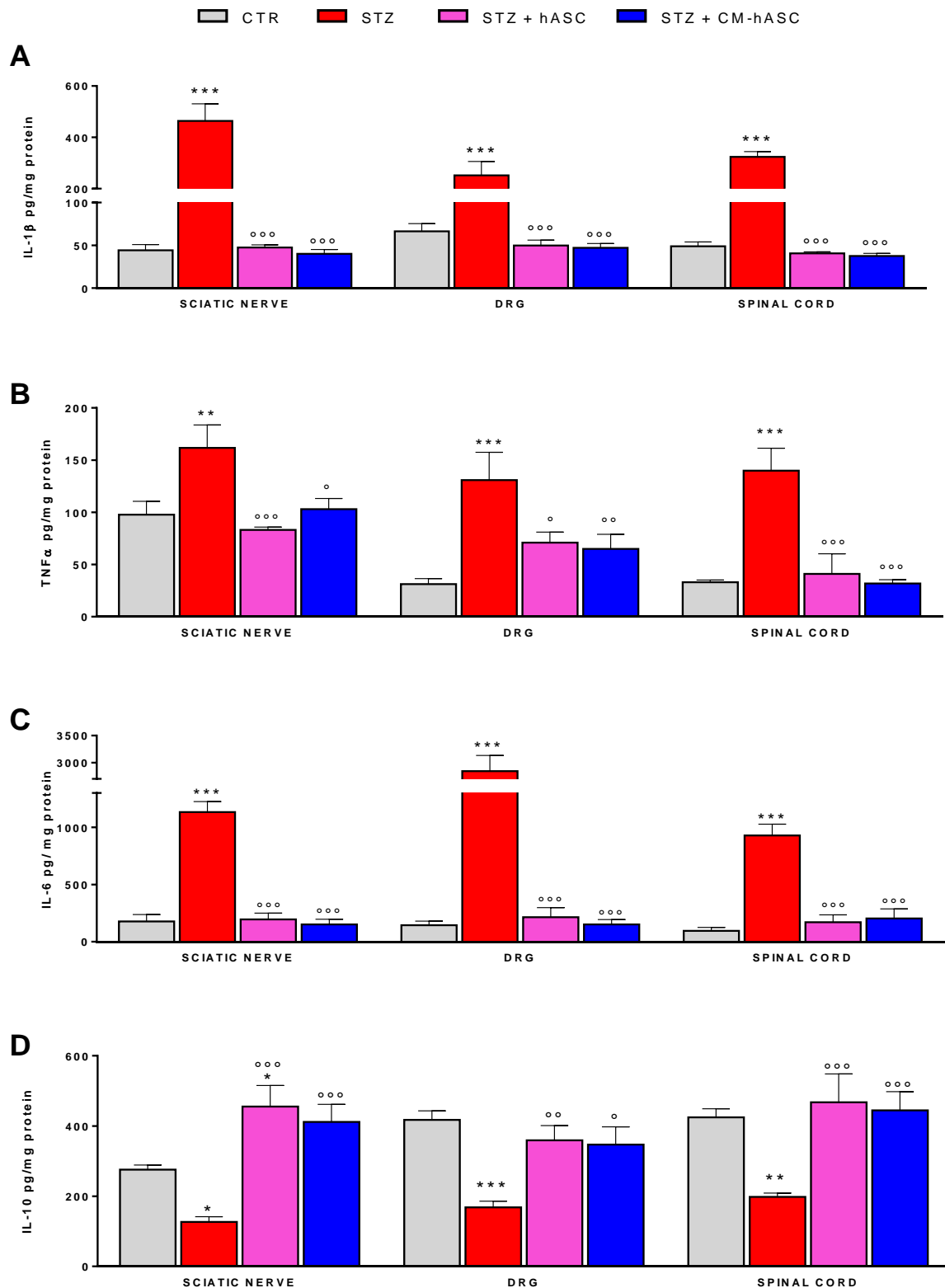


Figure 7 | hASC and CM-hASC maintain a correct cytokine balance in sciatic nerves, DRG and spinal cord of STZ mice. IL-1 β (A), TNF- α (B), IL-6 (C) and IL-10 (D) in sciatic nerve, DRG and spinal cord of STZ mice treated 2 weeks after STZ with hASC or hASC-CM; cytokines were evaluated after 1 week from treatments. Protein content in nervous tissues was evaluated by ELISA and reported as pg cytokine/mg total protein. Data represent mean \pm SEM of 6 mice per group. One-way ANOVA was used for statistical evaluation, followed by Bonferroni's post hoc test for multiple comparisons. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs CTR; ° $p < 0.05$, °° $p < 0.01$, °°° $p < 0.001$ vs STZ.

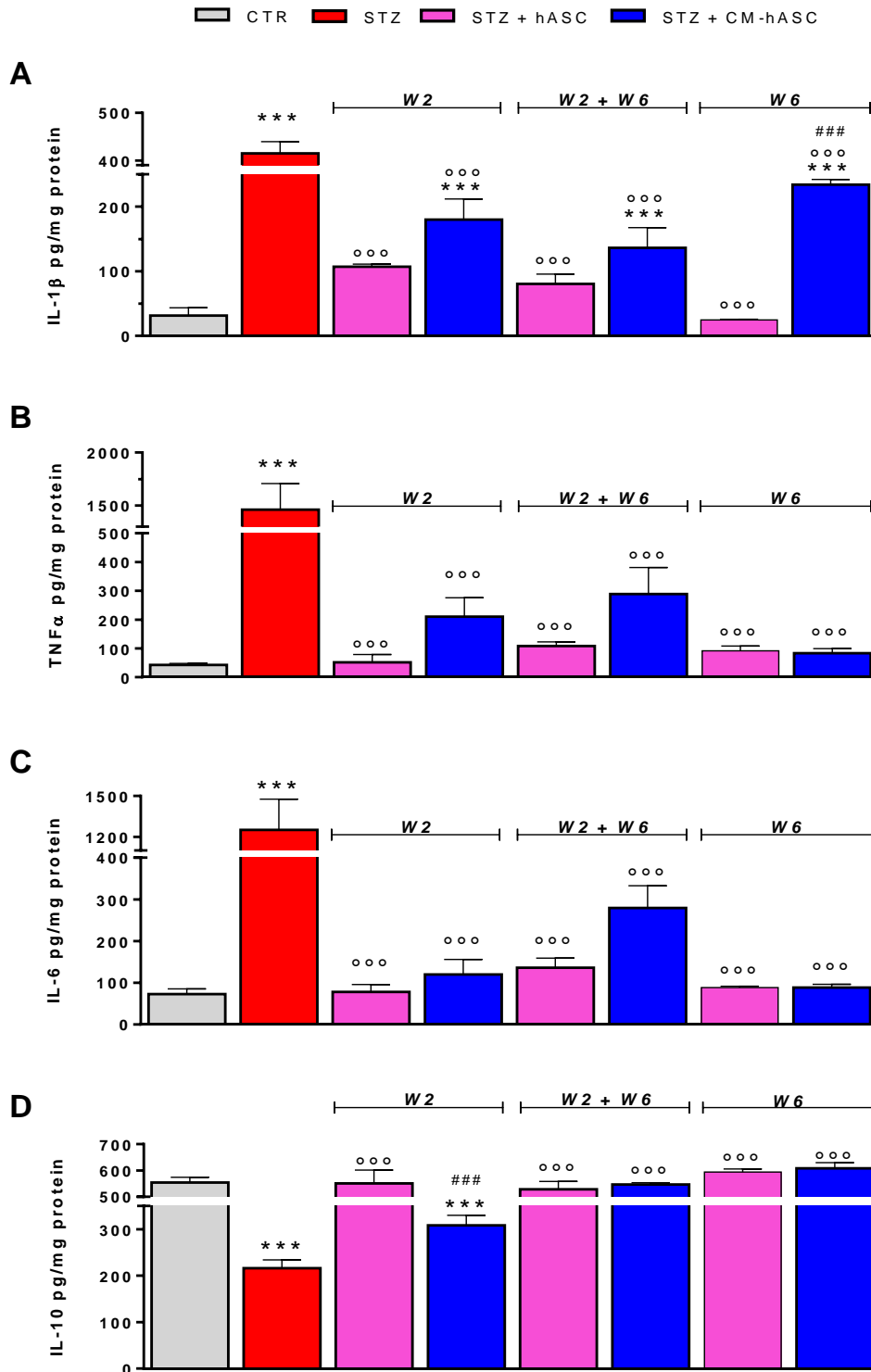


Figure 8] hASC and CM-hASC maintain a correct cytokine balance in spinal cord at longer time. IL-1 β (A) TNF- α (B), IL-6 (C) and IL-10 (D) in spinal cord, measured 14 weeks after STZ in animals treated with hASC or CM-hASC at 2 weeks (W2) or 2 and 6 weeks (W2+W6) or 6 weeks (W6) after STZ. Protein content in spinal cord was evaluated by ELISA and reported as pg cytokine/mg total protein. Data represent mean \pm SEM of 6 mice per group. One-way ANOVA was used for statistical evaluation, followed by Bonferroni's post hoc test for multiple comparisons. *** p < 0.001 vs CTR; °°° p < 0.001 vs STZ; ### p < 0.001 vs STZ + hASC.

Finally, to investigate the timing of cytokines modulation exerted by hASC and CM-hASC treatment, pro- and anti- cytokine levels in sciatic nerve, DRG and spinal cord 2 weeks after STZ, 3h from treatments, were measured. At this time, IL-1 β levels were significantly elevated and IL-10 levels decreased in all analyzed tissues, as shown in *panels A and B of Figure 9*. Three hours after injection, both treatments were already able to positively modulate cytokine levels, that were not any more different from CTR, although a complete restoration was evident only for IL-1 β in sciatic nerve and for IL-10 in DRG.

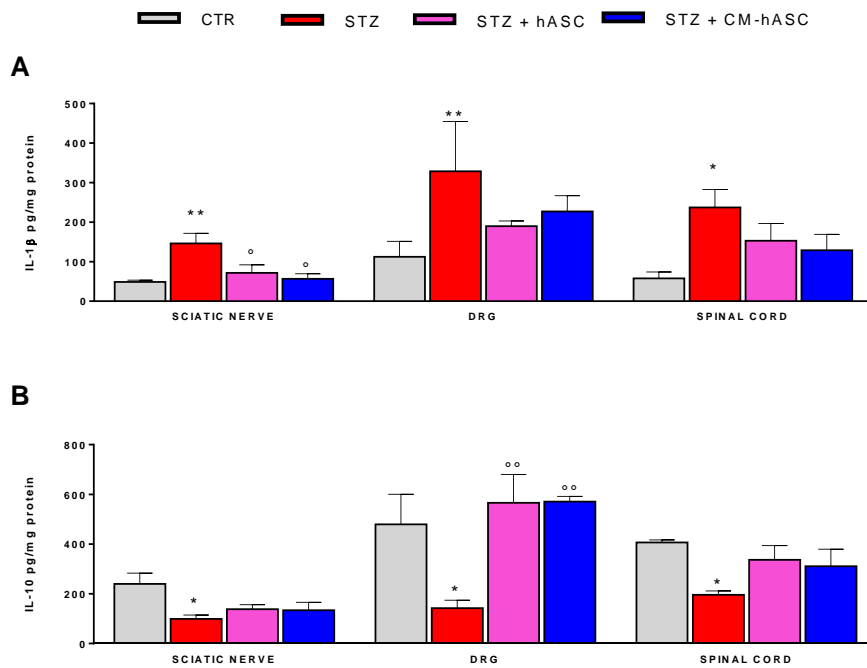


Figure 9| Acute effect of hASC and hASC-CM on IL-1 β and IL-10 in nervous tissues. IL-1 β (A) and IL-10 (B) protein content in nervous tissues was evaluated by ELISA and reported as pg cytokine/mg total protein. Cytokines were evaluated in sciatic nerve, DRG and spinal cord 3 hours after hASC or CM-hASC treatments, two weeks after STZ. Data represent mean \pm SEM of 6 mice per group. One-way ANOVA was used for statistical evaluation, followed by Bonferroni's post hoc test for multiple comparisons. * p <0.05, ** p <0.01 vs CTR;

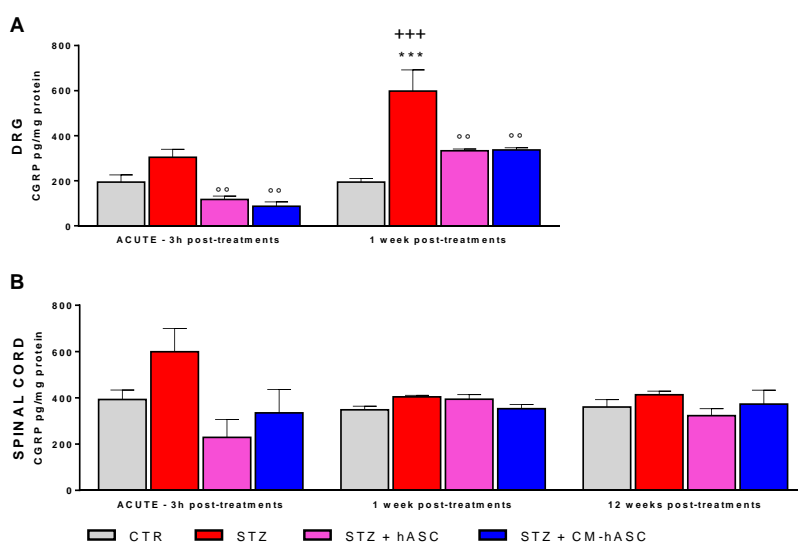


Figure 10| hASC and CM-hASC prevent alteration of CGRP levels in DRG. Levels of CGRP measured 3h and 1 week after hASC and CM-hASC treatment (2 and 3 weeks after STZ, respectively; panel A) in DRG. Spinal cord levels of CGRP detected 3h, 1 and 12 weeks after treatments, that correspond to 2, 3 and 14 weeks after diabetic-induction. Protein content in nervous tissues was evaluated by ELISA and reported as pg cytokine/mg total protein. Data represent mean \pm SEM of 6 mice per group. Two-way ANOVA was used for statistical evaluation, followed by Bonferroni's post hoc test for multiple comparisons. *** p <0.001 vs CTR; ^{oo} p <0.01 vs STZ; +++ p <0.001 vs STZ - 3h after treatment (W2)).

In order to further confirm the effect of treatments on hypersensitivity, we measured the effect of hASC and CM-hASC on Calcitonin Gene Related Peptide (CGRP) protein levels in DRG and spinal cord of STZ mice. As shown in **Figure 10 - panel A**, 2 weeks after STZ-injection, diabetic mice showed an increase (even if it is not significant) in CGRP levels in DRG. Both hASC and CM-hASC 3 hours after administration were able to significantly decrease CGRP levels respect to diabetic-mice. Three weeks after STZ-injection, CGRP levels were significantly increased in STZ mice and both treatments, 1 week after administration, were able to prevent CGRP increase (**Figure 10 - panel A**). On the contrary, we did not find any CGRP modification in spinal cord of diabetic mice at any time points (**Figure 10 - panel B**).

4.4 hASC and CM-hASC normalize skin thickness and PGP9.5+nervous fibers

Neuropathic pain is associated with epidermal thinning and reduced innervation, measured as expression of Protein Gene Product 9.5 (PGP 9.5) in axons of the epidermis and dermis [Van Acker *et al.*, 2016]. Both skin thickness and cutaneous innervation of the plantar skin were evaluated 3 and 14 weeks after STZ diabetes induction, corresponding to 1 and 12 weeks after hASC and CM-hASC treatment (**Figure 11, panels A-D**). Three weeks after STZ, skin thickness was not altered yet in STZ mice and neither treatments had effect (**Figure 11, panels A and C-3W**). At fourteen weeks, STZ animals showed a significant decrease in skin thickness (**Figure 11, panels B and C-14W**) and both hASC and CM-hASC prevented this decrease, in fact the skin thickness of treated mice was significantly higher than diabetic mice.

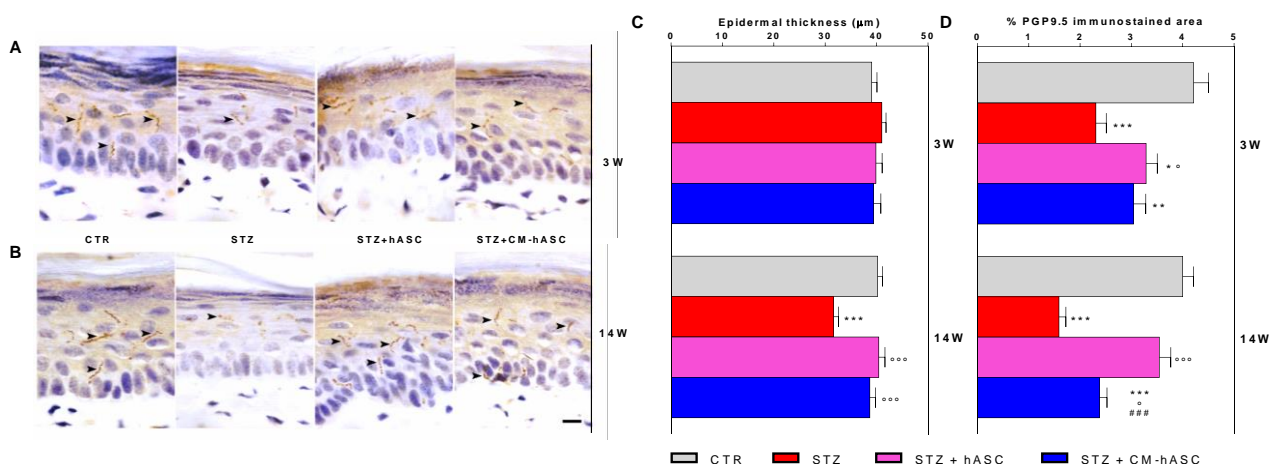


Figure 11| hASC and CM-hASC prevent thickness reduction and nerve fiber loss in paw skin of STZ mice. Microphotographs of plantar skin after PGP9.5 + immunohistochemistry and counterstaining with haematoxylin at 3 (A) and 14 weeks (B) after STZ, in CTR, STZ, STZ + hASC and STZ + CM-hASC groups. Animals were treated 2 weeks after STZ. Nerve fibers are stained in brown; arrow heads indicate PGP9.5+ fibers. Quantitative evaluation of epidermal thickness (C) and of PGP9.5 immunopositivity as percentage of immunopositive area in epidermal and subepidermal area (D) at 3 and 14 weeks after STZ. Data represent mean \pm SEM and were compared by One-way ANOVA followed by a Bonferroni's multiple comparison test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs CTR; * $p < 0.05$, ** $p < 0.001$ vs STZ; ### $p < 0.001$ vs STZ + hASC.

The high density of PGP9.5+ nervous fibers distributed both in epidermis and in subepidermal layer in CTR mice is progressively decreased in STZ mice between 3 and 14 weeks after diabetic induction (**Figure 11, panels A, B and D**). One week after hASC treatment, neuropathic animals showed a PGP9.5+ density fibers significantly higher than STZ group but still lower respect to the CTR (**Figure 11, panel D-3W**). No significant effect of CM-hASC on fibers' density was observed. Fourteen weeks after STZ (12 weeks from treatments, panel D-14W) hASC-treated animals completely recovered nerve fibers density. In CM-hASC-treated animals, a significantly increase in PGP9.5+ nervous fibers was present, especially at subepithelial levels, although it never reached the CTR values (**Figure 11, panels B and D-14W**).

4.5 hASC and CM-hASC modulate splenocytes cytokine levels

The STZ multiple low-doses protocol used in our study is known to induce an autoimmune response against pancreatic tissue which is sustained by a Th1 pattern of activation [Van Belle *et al.*, 2009; Rahavi *et al.*, 2015]. For this reason we decided to investigate whether a T-helper polarization was present in splenocytes from diabetic mice and whether hASC or CM-hASC did exert any immunomodulatory activity. As reported in *panels A and D* of **Figure 12**, 2 weeks after STZ, ConA-stimulated splenocytes released higher levels of IFN- γ (*panel A*), while IL-10 release (*panel D*) was reduced; 3 hours after hASC and CM-hASC administration, both treatments were able to significantly increase IL-10 levels (**Figure 12, panel D**). Three weeks after STZ, 1 weeks after treatments, Th1/Th2 unbalance was more evident and hASC and CM-hASC treatments were able to re-establish both IFN- γ and IL-10 physiological levels (**Figure 12, panels E and H**, respectively). When cytokine levels were measured 14 weeks after diabetes induction, a clear shift toward a Th1 pattern was present, characterized by higher IFN- γ and IL-2 secretion (**Figure 12, panels I and L**, respectively) and lower IL-4 (*panels M*) and IL-10 levels (*panel N*). hASC and CM-hASC treatments were able to normalize cytokine levels, as demonstrated by the IFN- γ and IL-2 decrease (*panels I and L*) and IL-10 and IL-4 increase 8 weeks after hASC/CM-hASC injection (**Figure 12, panels M and N, W6**). When the effect was evaluated 12 weeks after administration (W2), both hASC and CM-hASC were able to restore IFN- γ and IL-2 (**Figure 12, panels I and L**). IL-4 and IL-10 reached basal levels only in hASC-treated mice while in CM-hASC-treated animals the recovery was not complete (**Figure 7, panels M and N**), suggesting that the effects exerted by hASC is longer than the hASC-CM. Altogether, our data indicate that both hASC and CM-hASC treatments are able to counteract the Th1 polarization developed in this experimental diabetes model.

In addition, to better understand whether hASC/CM-hASC act at transcriptional or post-transcriptional level, IFN- γ and IL-10 mRNA (**Figure 13 - panels A and B**, respectively), were evaluated. These cytokines were selected because they were altered by the pathology at the all time observed and their mRNA, were evaluated at 3 (1 week after treatment) and 14 weeks (12 weeks after treatment) after STZ.

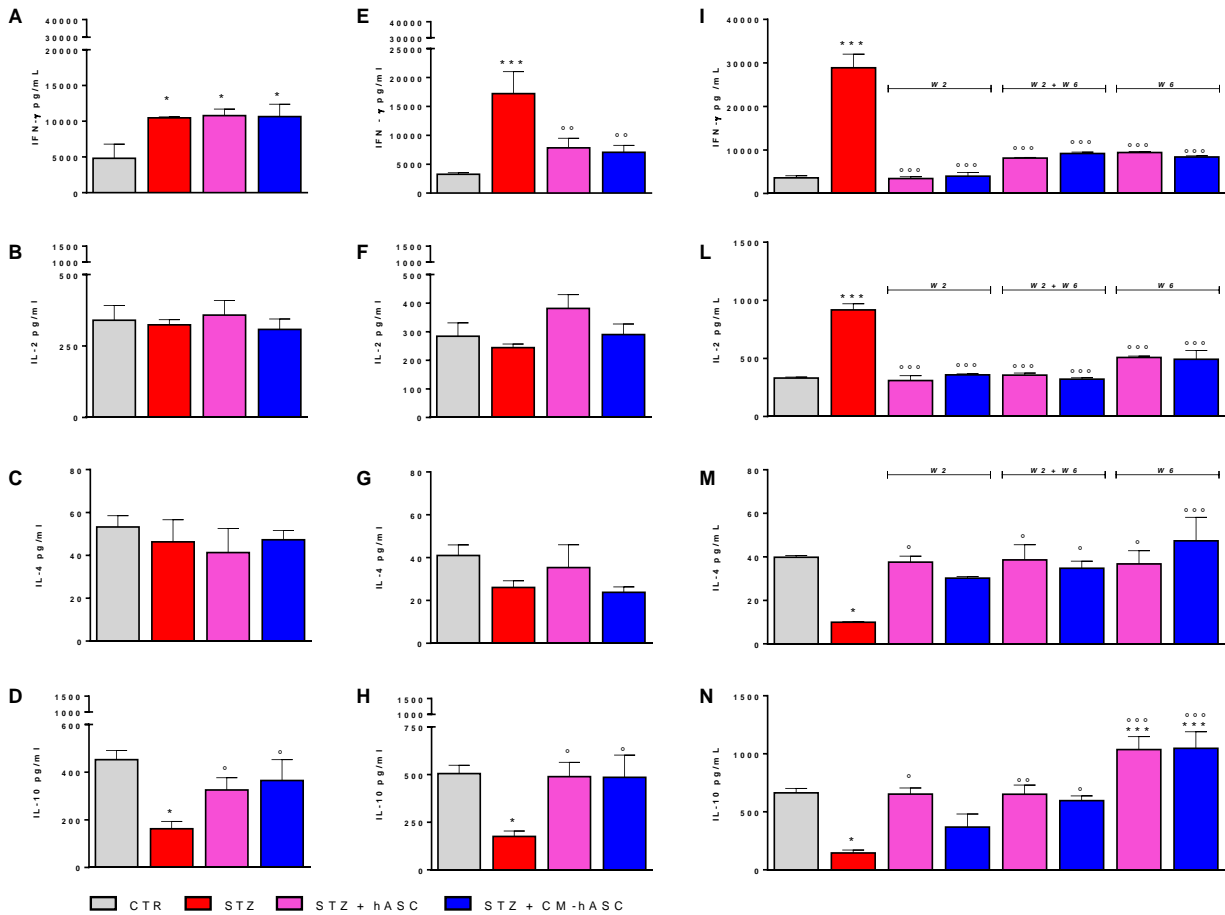


Figure 12 | hASC and CM-hASC treatments modulate cytokine release from splenocytes. IFN- γ (A, E and I), IL-2 (B, F and L), IL-4 (C, G and M) and IL-10 (D, H, N) were evaluated by ELISA, and reported as protein concentrations in culture media. Cytokine levels were evaluated 2 weeks after STZ, 3 hours after hASC and CM-hASC treatments (A-D); 3 weeks after STZ, 1 week after treatments (E-H). (I-N) Report cytokines levels measured 14 weeks after STZ in animals treated with hASC or CM-hASC either 2 weeks (W2), 2 and 6 weeks (W2+W6) or 6 weeks (W6) after STZ. Data represent mean \pm SEM of 6 mice per group, and have been statistically analyzed with One-way ANOVA, followed by Bonferroni's test for multiple comparisons. * $p < 0.05$, ** $p < 0.001$ vs CTR; $^{\circ}p < 0.05$, $^{\circ\circ}p < 0.01$, $^{\circ\circ\circ}p < 0.001$ vs STZ.

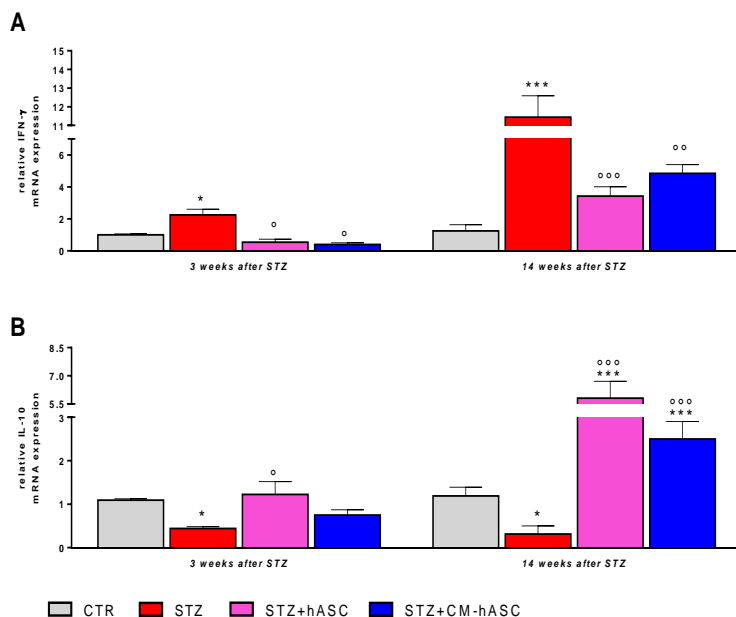


Figure 13 | hASC and CM-hASC treatments modulate mRNA expression of IFN- γ and IL-10 in splenocytes. IFN- γ (A) and IL-10 (B) mRNA levels, determined by Real Time PCR, were expressed in relation to glyceraldehydes 3-phosphate dehydrogenase (GAPDH) and presented as fold-increases over the levels in CTR animals. mRNA levels were evaluated 3 and 14 weeks after STZ (1 and 12 week after hASC and CM-hASC treatments, respectively). Data represent mean \pm SEM of 6 mice per group, and have been statistically analyzed with Two-way ANOVA, followed by Bonferroni's test for multiple comparisons. * $p < 0.05$, ** $p < 0.001$ vs CTR; $^{\circ}p < 0.05$, $^{\circ\circ\circ}p < 0.001$ vs STZ.

Similarly to cytokine release, 3 and 14 weeks after STZ, diabetic mice were characterized by a significant increase of IFN- γ and a decrease of IL-10 expression levels.

Three and 14 weeks after STZ, both hASC and CM-hASC treatments were able to reduce IFN- γ mRNA to baseline. 3 weeks after STZ, IL-10 mRNA levels were normalized only by hASC treatment while 14 weeks after STZ both treatments were able to increase IL-10 mRNA beyond physiological values (*panels A and B*). These data indicate that both treatments act upstream of mRNA translation, showing a correlation between mRNA and protein for both cytokines (see *Figure 12 panels E, I, H and N*, and *Figure 13 panel A and B*).

4.6 Effect of hASC and CM-hASC treatments on other diabetes associated alterations

Considering the strong effect of hASC and CM-hASC treatments on diabetic neuropathy, we decided to investigate whether these treatments positively influenced also other conditions related to diabetes.

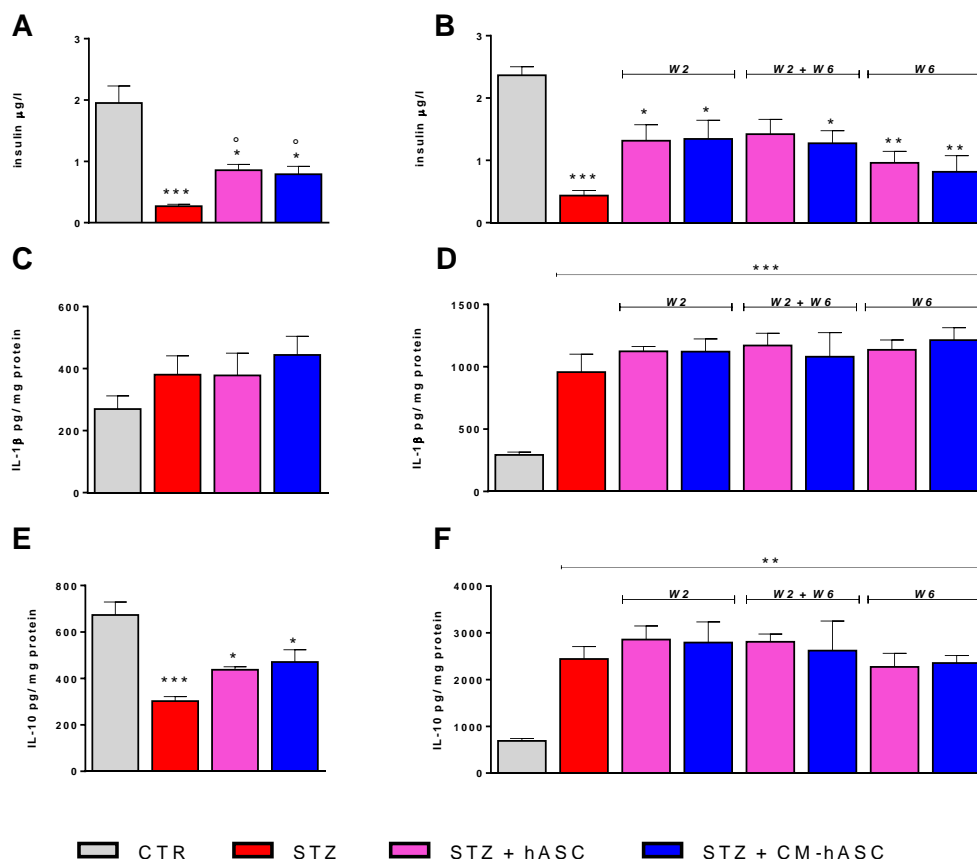


Figure 14 | Effect of hASC and CM-hASC administrations on insulin plasma levels and pancreatic cytokines. Plasma insulin levels were evaluated 3 (**panel A**) and 14 weeks (**panel B**) after. IL-1 β and IL-10 protein content in pancreatic tissue were evaluated 3 weeks (**panels C and D**) and 14 weeks (**panels E and F**) after diabetes induction.

Plasma insulin levels and pancreas protein content, evaluated by ELISA, were reported as protein concentrations in plasma and pg of cytokine/mg of total protein, respectively. Data represent mean \pm SEM of 6 mice per group. One way ANOVA was used for statistical evaluation, followed by Bonferroni's test for multiple comparisons. *p < 0.05, **p < 0.01, ***p < 0.001 vs

We measured plasmatic insulin levels and pancreatic cytokines levels, since it is known that in the STZ diabetes model the destruction of β -pancreatic cells induces an altered immune-inflammatory response characterized by a dysregulation of cytokine expression pattern in pancreas [Cnop *et al.*, 2005; Amirshahrokhi and Ghazi-Khansari, 2012], 3 and 14 weeks after STZ.

As reported in **Figure 14**, 3 and 14 weeks after STZ administration, plasmatic insulin levels were drastically reduced in STZ mice respect to the ones measured in CTR animals (*panel A and B*). Both hASC and CM-hASC treatments were able to counteract insulin decrease in the first phases of the pathology, that is 3 weeks after STZ. Moreover at longer time after diabetic induction, 14 weeks after STZ, single and double treatments (W2 and W2+W6, respectively) with hASC or CM-hASC were not able to re-establish physiological plasmatic insulin levels even if a positive trend was present after both hASC and CM-hASC treatments. When administered in an advanced stage of disease (W6), hASC and CM-hASC were not effective in modulating insulin levels.

Figure 14 - *panels C-F* report IL-1 β and IL-10 protein levels in pancreas that 3 and 14 weeks after diabetic induction, show a marked dysregulation of cytokine balance. In particular 3 weeks after STZ we observed a significant decrease of IL-10 levels (*panel E*). 14 weeks after STZ, both IL-1 β (*D*) and IL-10 (*F*) cytokines were significantly increased in diabetic mice, but we could not evidence any treatment effect. As shown in **Figure 15** - *panel A*, 3 weeks after diabetic induction body weight significantly decreased in STZ mice, but a single hASC or CM-hASC administration significantly prevented body weight loss.

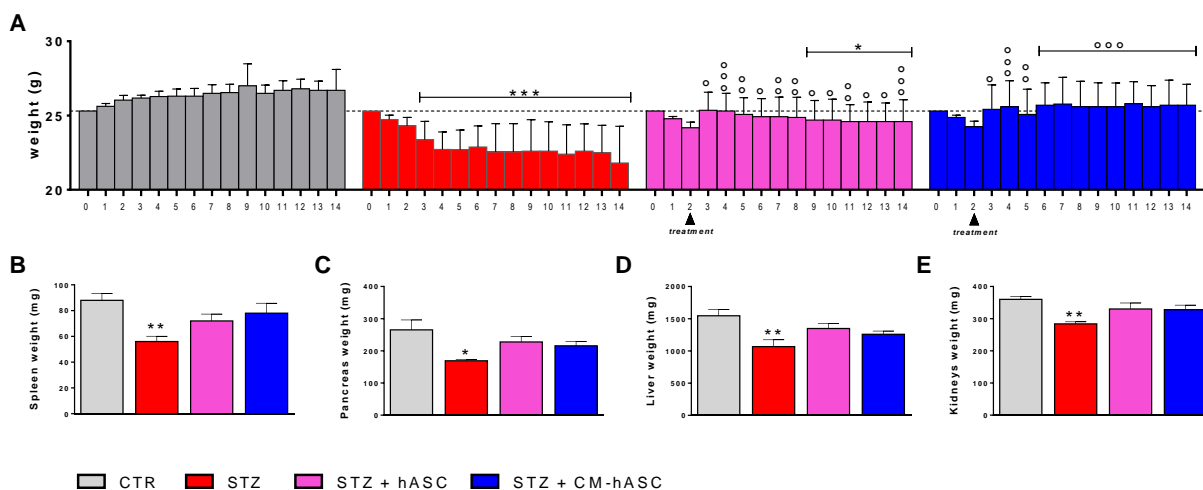


Figure 15| Effect of hASC and CM-hASC administrations on body and organ weight. Body weight time course of STZ mice treated with hASC or CM-hASC 2 weeks after STZ (A). Data are means \pm SEM of 6 animals. Two-way ANOVA was used for statistical evaluation, followed by Bonferroni's test for multiple comparisons. * $p < 0.05$, *** $p < 0.001$ vs CTR; $^{\circ}p < 0.05$, $^{\circ\circ}p < 0.01$, $^{\circ\circ\circ}p < 0.001$ vs STZ. Spleen (B), pancreas (C), liver (D) and kidneys (E) weight 3 weeks after diabetic induction. Data are means \pm SD of 6 animals. One-way ANOVA was used for statistical evaluation, followed by Bonferroni's test for multiple comparisons. * $p < 0.05$, ** $p < 0.01$ vs CTR.

Body weight of mice treated twice with hASC or CM-hASC (W2+W6) were comparable to those that were treated only once; on the contrary neither hASC nor CM-hASC were able to revert the body weight loss if administered 6 weeks after diabetes induction (data not shown).

Moreover, 3 weeks after STZ injection, diabetic mice showed a significant decrease in the weight of kidneys, liver, spleen and pancreas (**Figure 15, panels B-E**). Both hASC and CM-hASC were able to significantly contrast organ weight reduction in accordance with what was observed on total body weight (**Figure 15 – panel A**).

Since renal injury is a well known diabetic complication, we focused our attention also on glomerulopathy, which is a primary evidence of type I diabetes nephropathy. Analysing hematoxylin/eosin kidney sections, an evident expansion of Bowman's space of about 114% was

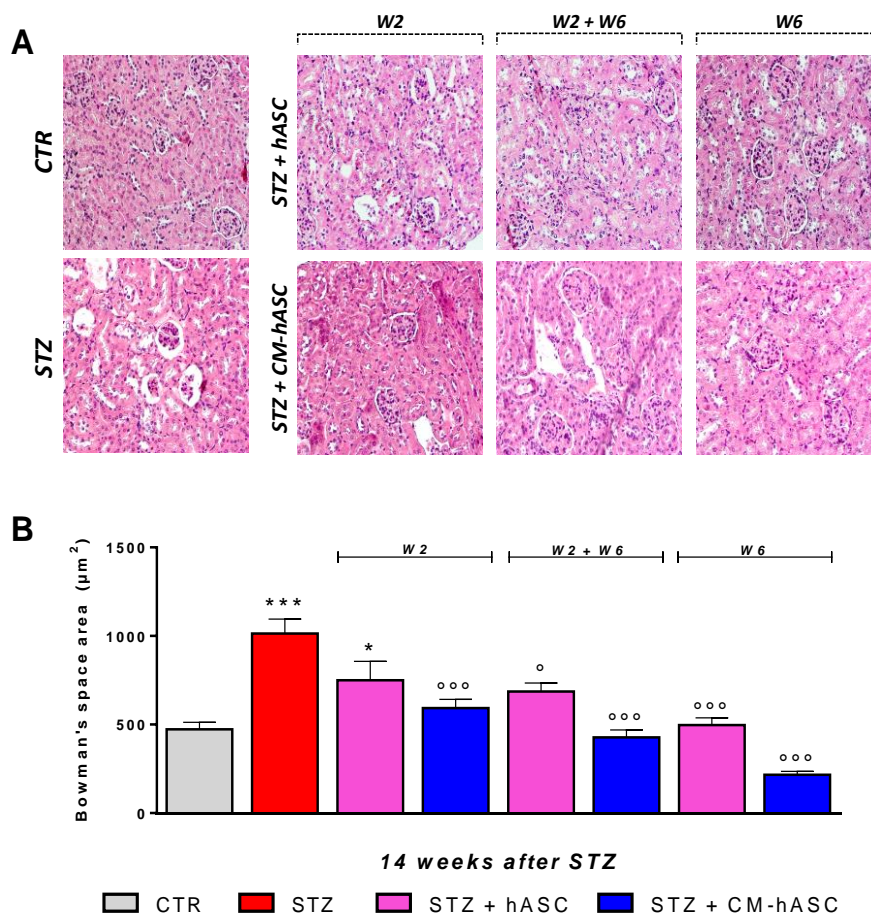


Figure 16 | Effect of hASC and CM-hASC on glomerular damage in STZ mice. A: Hematoxylin/eosin staining of kidney sections (magnification: 200X) from CTR, STZ and STZ treated with hASC or CM-hASC, 2 weeks (W2) or 2 and 6 weeks (W2+W6), or 6 weeks (W6) after diabetes induction. B: quantification of Bowman's space area for all the animal groups. More than 30 glomeruli were measured for each group. Data were analyzed and compared by One-way ANOVA and by a Bonferroni's multiple comparison test and are expressed as mean \pm SEM. * $p < 0.05$, *** $p < 0.001$ vs CTR; ° $p < 0.05$, °°° $p < 0.001$ vs STZ.

observed in diabetic mice compared to CTR (**Figure 16 - panels A and B**).

Interestingly, at all time observed CM-hASC injection significantly reduced Bowman's space area in respect to STZ mice; while only double or late hASC treatment was able to significantly reduce Bowman's space area. In more detail, effect of CM-hASC single (W2) or double (W2+W6) administration, appeared more pronounced than hASC, since the early cellular treatment was able to just mildly revert the alteration (*panel B*). In contrast, late treatments were more beneficial, since Bowman's space area was restored to physiological value by both treatments (**Figure 16 - panels A and B**). In all groups, tubules morphology appeared normal without necrosis or loss of brush border and neither vascular fibrosis nor sclerosis was observed..

Discussion and Conclusions

Neuropathic pain resulting from a damage or disease within the somatosensory system is a chronic pain largely resistant to treatment mainly because the underlying mechanisms are still poorly understood.

Much of the initial research on the neuropathic pain pathogenesis focused on the properties of neurons following a nerve injury, leading to proposal of both peripheral and central sensitization as important disease mechanisms [Sacerdote *et al.*, 2013; Costigan *et al.*, 2009]. However, in the last years it has emerged that the development and maintenance of neuropathic pain is not confined to the altered activity of sensory neurons and the reorganization of central nociceptive circuits, but also involves pathological interactions between neurons, glia and inflammatory immune cells, as well as a wide cascade of pro- and anti-inflammatory cytokines [Austin and Moalem-Taylor, 2010; Calvo *et al.*, 2012].

In the present work we provide evidences about the role of bioactive factors released from hASC or present in their secretome in modulating inflammatory events which occur in the peripheral and central nervous system following neuronal injury related to neuropathic pain development.

Considering that one of the most common causes of neuropathic pain in patients is the presence of diabetes, we used a diabetic painful neuropathy model induced by chemical pancreatectomy (STZ) to perform this study.

Diabetic neuropathic pain is characterized by sensory alterations, including hyperalgesia, tactile and thermal allodynia, and current treatments are often active only on some of them [Vincent *et al.*, 2011]. Our results demonstrate that hASC and their secretome can control diabetic complications such as neuropathic hypersensitivity, acting on several peripheral and central mechanisms involved in the development and maintenance of this condition, such as neural and immune elements. In particular, allodynia is known to be a cardinal symptom of diabetic painful neuropathy and in STZ-preclinical model it appeared within few days after diabetes induction, is fully developed in 1 week and persisted until 14 weeks after STZ administrations.

Systemic treatments with either hASC or CM-hASC are able to significantly relieve tactile and cold allodynia as well as heat hyperalgesia, although the effect of cells remains significantly higher than that of CM. Interestingly treatments seems to be effective also in modulating thermal hypoalgesia. While there is a general agreement on the development of allodynia in the STZ model, other studies have reported, as a result of diabetic neuropathy, the presence of thermal hyperalgesia, hypoalgesia or no changes in thermal latency tests [Ohsawa and Kamei, 1999; Pabbidi *et al.*, 2008a]. This discrepancy might be explained by the fact that there are many variables in these behavioural tests such as methodology, animal species, response criteria, duration of diabetic conditions, etc. [Calcutt, 2004]. In our experiments, STZ-mice showed thermal hyperalgesia from 1 to 3 weeks after STZ-injection, subsequently there was a switch from hyperalgesia to hypoalgesia that lasted until the 14th week. This change may depend on pathological alterations in the sensory system that result in reduced neurotrophic support of peripheral sensory nerves and loss of nerve fibers [Calcutt, 2004; Kakinoki *et al.*,

2006]. Moreover previous studies showed that insulin has a role in activating TRPV1 receptors of cultured primary sensory neurons [Sathianathan *et al.*, 2003], therefore hypoalgesia might be related to the reduction of insulin levels and diminished TRPV1 activity of primary sensory neurons in diabetic mice. Both hASC and CM-hASC treatments were able to contrast the shift from hyper- to hypo- algesia and this effect may be related to the ability of both treatments to contrast insulin levels decreased in diabetic mice up to 14 weeks after STZ and to the ability of treatments to protect nerve fibers from degeneration (see below).

The concept that MSC's secretome may be responsible for the beneficial effects of stem cell therapy is now a prevalent theory [Kapur and Katz, 2013; Blaber *et al.*, 2012; Sabin and Kikyo, 2014]. However, this is the first report demonstrating that the conditioned medium of hASC also results in quite similar therapeutic effects in diabetic neuropathic condition and comparing simultaneously the effect of CM and of the stem cells from which it has been produced.

One of the most striking result of our study is related to the time course of the antiallodynic effect exerted by both hASC and CM-hASC. In fact, their antiallodynic effect was not only rapid but also extremely long lasting, an effect that is hardly reached by any analgesic clinically used [The Diabetes Control and Complications Trial Research Group, 1993; Martin *et al.*, 2006].

Furthermore, the long lasting antiallodynic effect of CM-hASC, up to 14 weeks, may lead to speculate that a direct engraftment of stem cells in the nervous tissue has to be excluded, favouring the hypothesis of a precocious reprogramming of the immune and neuronal environments that, once activated, changes the course of the neuropathy. In a juxtacrine or paracrine fashion hASC can crosstalk and modulate endogenous stem cells that may also induce nerve regeneration, as previously described in a different model of neuropathy [Marconi *et al.*, 2012].

Similarly, the biologically active substances contained in the CM-hASC can activate/deactivate specific signaling pathways with a final protective outcome. It must be underlined that CM derived from human dermal fibroblasts are completely inactive on allodynia, confirming that the mesenchymal stem feature is a fundamental pre-requisite in this novel approach. Moreover lyophilized CM-hASC was not able to increase mechanical stimuli thresholds in diabetic mice, indicating that the process by which the secretome is obtained is crucial. The differences observed could provide a starting point to identify bioactive factors present in the CM through a comparison between the 3 secretomes with the final aim to create a mix bioactive ad hoc factors.

The ability of hASC and their secretome to modulate the host cell response is demonstrated by the effect on the cytokine levels in the nervous tissues. IL-1 β , IL-6, TNF- α and IL-10 in the peripheral (nerves, DRG) and central (spinal cord) nervous system are now recognized as a pivotal signal for maintenance of neuropathic pain, regardless of its origin [Sacerdote *et al.*, 2013; Calvo *et al.*, 2012; Yagihashi *et al.*, 2011]. Here we demonstrated that one week after hASC and CM-hASC treatment, neuroinflammation is

significantly blunted and a correct balance between IL-1 β , IL-6, TNF- α and IL-10 is re-established. Interestingly, the levels of IL-10 after treatments in the sciatic nerves are even more elevated than those measured in normal animals, confirming the relevant role for this cytokine in controlling sensory hypersensitivity.

In parallel with the long lasting modulation of allodynia, also the effect of hASC on IL-1 β and IL-10 in spinal cord is fully maintained up to 12 weeks after treatment, suggesting that once the switch toward an anti-inflammatory program has been started, it is permanently sustained. Significant similar effect of both treatments on TNF- α and IL-6 levels were observed. Interestingly, at later times following administration, the effect of CM-hASC on IL-10 is not as evident as with stem cells. When the antiallodynic effect was decreased, one month after the 1st CM-hASC treatment, a 2nd treatment was able to restore IL-10 physiological levels and increase mechanical stimuli thresholds. In addition, it was noted that second hASC/CM-hASC treatment did not modify IL-1 β , TNF- α and IL-6 levels compared to single treatment; this may depend on the fact that a single treatment with hASC or CM-hASC has already been able to restore cytokines basal levels. Since clinical diabetic neuropathy is a progressive disorder, and its manifestation may need several years to develop [Yagihashi *et al.*, 2011], we thought it could be interesting to evaluate whether hASC and CM-hASC could be able to modify allodynia and neuroinflammation also when administered at a later stage of the disease. In our model, hyperglycemia and abnormal sensitivity were developed soon after STZ injection while for structural nerve modifications it is necessary a longer time [Goyal *et al.*, 2016]. Interestingly, both hASC and CM-hASC were fully effective in reverting allodynia and restoring all analyzed cytokines levels (IL-1 β , IL-6, TNF- α and IL-10) in spinal cord also when injected 6 weeks after diabetes induction.

Moreover, to try to understand the rapid effect of both treatments on painful symptoms, we checked if already 3 hours after administration, hASC and CM-hASC were able to modulate cytokine levels; we demonstrate that the cytokine levels modulation begins almost immediately after hASC or CM-hASC. Already 3 hours after treatment we observed a trend in the restoration of IL-1 β /10 balance in nervous tissues (both central, spinal cord; and peripheral, DRG and sciatic nerve). In addition, since calcitonin gene-related peptide (CGRP) is widely distributed in nociceptive pathways in peripheral and central nervous system and its receptors are also expressed in pain pathways, we evaluated CGRP levels immediately after therapeutic treatments (3 hours) and 1 and 12 weeks after.

Although it is well known that CGRP peptide is involved in migraine pathophysiology its role in neuropathic pain has not been clarified [Schou *et al.*, 2017] and recent studies are discordant [Schou *et al.*, 2017; Russell *et al.*, 2014]. In our analysis we observed that in the early stages of the diabetic neuropathy, 2 weeks after STZ, diabetic mice showed a trend toward increased CGRP levels both at the central and peripheral nervous system level (spinal cord and DRG, respectively). Three weeks after STZ, CGRP peripheral levels were significantly increased. Both hASC and CM-hASC treatments at the two

observed time points (3 hours and 1 week after administration) were able to significantly decrease CGRP levels in comparison to STZ mice, demonstrating that both hASC and CM-hASC are able to modulate several important mediators or neurotransmitters involved in pain sensitivity.

The quantification of the density of intraepidermal nerve fiber to assess cutaneous innervation is considered a reliable mean of both diagnosing and staging of diabetic neuropathy [Van Acker *et al.*, 2016; Beiswenger *et al.*, 2008; Lauria *et al.*, 2005]. We decided to measure PGP-9.5 expression, since as shown in previous studies [Albrecht *et al.*, 2006; Fundin *et al.*, 1997; Pare *et al.*, 2007; Hou *et al.*, 2011] anti-PGP 9.5 labels all known types of peripheral innervation. Here we showed that nerve fibers damage began in 3 weeks after STZ and at later time point, 14 weeks after STZ, it get worse in untreated diabetic group, in agreement with previous findings in diabetic animals [Boric *et al.*, 2013]. We observed that both treatments were able to prevent loss of nerve fibers detected by PGP9.5+, supporting a neuroprotective activity of our treatments in diabetes. We are however aware that further immunohistochemical analysis with specific fibers' markers would be important in order to assess which fibers are lost and eventually recovered by treatments. Since it has been proven that in neuropathic pain partial denervation induces differences in epidermal thinning, we also evaluated this parameter and we observed that 14 weeks after STZ, when denervation of the paw skin is more evident, diabetic mice showed also epidermal thinning, which was completely prevented by both therapeutic treatments.

MSCs carry out pleiotropic effects on the immune system by both secreting bioactive molecules and by cell-cell contact involving dendritic cells, B and T cells [Fierabracci *et al.*, 2016; de Witte *et al.*, 2015]. Inflammation and immune activation have been recognized as fundamental mechanisms in the pathophysiology of diabetes and its complications [Agrawal and Kant, 2014]. An autoimmune reactivity characterized by a T helper 1 profile is consistently present in clinical diabetes and 2 weeks after diabetic induction also STZ-mice were characterized by a clear shift toward Th1 profile, with high IFN- γ and low IL-10 levels, which becomes even more evident in the following weeks. A progressive polarization of peripheral immune response towards a Th1 profile is present in our STZ model and we demonstrate that hASC are involved in modulating the peripheral immune response.

In fact hASC treatment, regardless the time of its injection, reduced IL-2 and IFN- γ release by peripheral splenocytes and increased IL-10 and IL-4 secretion, maintaining an optimal Th1/Th2 balance. This effect is almost completely mimicked by CM-hASC, indicating that cell-cell contact is not essential to obtain immune modulation, that instead appears to be mainly due to a paracrine mode of action.

We found a parallel modification between IFN- γ and IL-10 protein and their mRNA levels, and as with protein both treatments were able to reestablish a correct balance. It is possible that hASC and CM-hASC may induce the activation of IL-10 synthetic machinery in order to supply the strong demand and consumption of this cytokine required for contrasting the pro-inflammatory cascade induced by the lesion [Sacerdote *et al.*, 2013].

Based on these results we can envisage that hASC, when i.v. injected in diabetic animals, may start to release bioactive factors that immediately modulate allodynia, immune responses and thereafter convert a pro-inflammatory/neuro-destructive environment to an anti-inflammatory/neuroprotective one. We showed that hASC are recruited at the site of lesioned tissue, such as pancreas and nerves of diabetic mice, while they are undetectable when the cells are i.v. injected in naïve animals. Furthermore, we suggest that hASC disappear after reprogramming the tissue cells, since cellular life span in the lesioned tissue (2 weeks) is shorter than the duration of their effects (12 weeks), as already reported [Uccelli *et al.*, 2008]. In the case of CM-hASC, the cocktail of bioactive mediators may exert a similar precocious modulation of host tissue, that is maintained over-time. However, at longer time after the single injection, the hASC positive effect on the anti-inflammatory cytokines appears stronger, suggesting that either the recruitment of cells at the injured sites or the cell to cell contact with endogenous immune cells may be useful. The positive long lasting effect of CM-hASC have been already described in different models of pathological conditions, such as liver disease, urological dysfunction and Alzheimer [Lee *et al.*, 2015; Yiou *et al.*, 2016; Mita *et al.*, 2015] and here we demonstrated that a repeated injection of CM-hASC promotes a higher antiallodynic response and further extends its duration, giving promising indications for a future clinical treatment.

At the moment, we cannot exclude that the neuroprotective effects of hASC and CM-hASC might also be mediated by an improvement of neural vascularity that is induced by the angiogenic factors contained in hASC secretome. Nonetheless, this aspect, previously suggested by others [Han *et al.*, 2016; Davey *et al.*, 2014], can be combined with the effects described here of the hASC and their CM.

The content and the relevance of CM-hASC factors is still under study. Our CM obtained from hASC maintained in similar culturing conditions as previously described by others [Makridakis *et al.*, 2013; Kapur and Katz, 2013; Blaber *et al.*, 2012; Konala *et al.*, 2016], should contain a wide range of cytokines, chemokines, and growth factors such as BDNF, VEGF, IGF. Recently a paper by Chen *et al.* [2015], suggested that the release of TGF- β by bone marrow stromal cells may be particularly involved in the modulation of neuropathic pain in the CCI (chronic constriction injury) and SNI (spare nerve injury) models. From preliminary analysis we measured high levels of TGF- β also in our CM-hASC, and this cytokine may play an important role also in diabetic neuropathic pain.

We also detected the presence of exosomes in CM-hASC (Brini, unpublished observations), and the secreted exosomes carry specific mRNA or miRNA, which could potentiate the reparative process and heal the injured tissues [Marote *et al.*, 2016; Sabin and Kikyo, 2014]. Although it is possible that for different pathological conditions, diverse mediators may be responsible for the beneficial effect, we think that what makes the secretome unique is just the simultaneous presence of all these multiple factors. To confirm our hypothesis, we have also shown the CM-hASC effect on another diabetes alteration such as renal injury. This diabetes complication is due to the progressive inflammation and

immune activation promoted by the formation of advanced glycation-end products, oxidative stress, and activation of renin-angiotensin-aldosterone system within the kidney [Rivero *et al.*, 2009; Forbes and Cooper, 2013]. Indeed, inflammation activated by the metabolic, biochemical and haemodynamic derangements plays a key role in the development and progression of diabetic nephropathy [Reidy *et al.*, 2014]. Since in STZ-mice kidney damage is ameliorated by both treatments, we hypothesize that hASC and CM-hASC may control several aspects of diabetes linked to inflammation and immune activation. Weight loss is also a classical symptom associated to the STZ diabetes models, and a general positive effect of hASC and CM-hASC treatments is demonstrated by the prevention of weight loss in treated mice [Goyal *et al.*, 2016; Agrawal and Kant, 2014]. Several factors may contribute to the prevention of weight loss that we observed in treated mice. In STZ mice, altered protein and lipid catabolism due either to hyperglycemia, to the inflammatory condition and oxidative stress are likely at the basis of weight loss [Goyal *et al.*, 2016]. Moreover, it has been suggested that the presence of allodynia and painful symptoms may affect the locomotor activity and modify energy expenditure [Atif *et al.*, 2007]. Interestingly, as in our work, a reduction of body weight loss has been observed also in the absence of modification of the hyperglycemic state in STZ mice [Old *et al.*, 2015]. It can be suggested that the reduction of systemic inflammation and hypersensitivity may have a role. Moreover, recent studies [Zafar and Naeem-ul-hassan Naqvi, 2010] demonstrated that body weight reduction in animal models was associated with decreased organs weight (kidney, liver, pancreas and spleen), event also observed by us. It can also be hypothesized that hASC and CM-hASC may contribute with hormones and mediators directly involved in the control of adipogenesis or body weight [Delle Monache *et al.*, 2016]. This hypothesis, however, deserves to be studied in future work.

No significant change in blood glucose levels was observed, suggesting that the efficacious relief of nociceptive hypersensitivity is independent from glycemia.

It is known that the use of mesenchymal stem cells of several origin, including hASC, was able to induce β -cells protection in the STZ models and to restore a correct glyceic state [Rahavi *et al.*, 2015; Kono *et al.*, 2014; Guimaraes *et al.*, 2013; Yaochite *et al.*, 2015; Monfrini *et al.*, 2017] and we indeed observed the presence of hASC in lesioned pancreas starting from day 7 after administration. However, this discrepancy can rely on the different administration route, systemically or intra-pancreatic resulting in different numbers of cells reaching the pancreas, and, most of all, the timing of administration. In all our study cells and media were injected when hyperglycemia was fully developed. In fact, the data obtained with pancreatic cytokine study, suggest that the organ is already altered and immune responses modulated when we injected cells and media. Both here and in our previous study [Sacerdote *et al.*, 2013a] we never observed any significant variability among the *in vivo* effect of the different population of hASCs and their conditioned media. However to overcome the differences in cells' growth, in the

future we might consider to set a more standardized production of both cells and CM, maybe by pooling several hASC populations, as also suggested by *Bodle et al. [2014]*.

In conclusion, we confirm and explain the ability of i.v. hASC to exert a long lasting control of diabetic neuropathic hypersensitivity showing, for the first time, that the effect of the hASC is mimicked by their secretome, confirming the general view that stem cells act mainly throughout a paracrine action.

The safety of autologous MSCs has been documented by a number of clinical trials [*Nordberg and Lobo, 2015; Lalu et al., 2012; Pawitan et al., 2014*] and the use of secretome could be further safer. In addition, considering all the studies on the effects of MSCs on diabetes, and knowing that peripheral neuropathy affects up to 60% of diabetic patients [*Galer et al., 2000*], we believe that advanced diabetic neuropathy could become a first clinical target for this type of medicine cellular product. Thinking about the future, a novel therapeutic option with hASC secretome might be suggested for treating advanced peripheral painful neuropathy.



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*List of my publication
and other activities*

- Publications** Amodeo G, Bugada G, Franchi S, Moschetti G, Grimaldi S, Panerai AE, Allegri M, Sacerdote P. *Immune function after major surgical interventions: the effect of post-operative pain treatment. Journal of Pain Research* - Submitted
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- Book chapter** Sacerdote P and Amodeo G; (2016) *Basi farmacologiche della terapia antalgica in: IL DOLORE IN ORTOPEDIA E TRAUMATOLOGIA. Griffin editore*
- Abstract/ Congress** Therapeutic effect of systemic injection of human adipose stem cells or their secretome in an mouse model of diabetic neuropathy. Amodeo G, Moschetti G, Ferreira LM, Franchi S, Borsani E, Brini AT, Sacerdote P. 38° Congresso Nazionale della Società Italiana di Farmacologia, Rimini – October 24-28, 2017.

Role of Prokineticin system in an experimental model of chemotherapy-induced peripheral neuropathy. Moschetti G, Amodeo G, Lattanzi R, Sacerdote P, Franchi S. 38° Congresso Nazionale della Società Italiana di Farmacologia, Rimini – October 24-28, 2017.

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Best Poster Award - **2017**: *“Therapeutic effect of systemic injection of human adipose stem cells or their secretome in an mouse model of diabetic neuropathy - 38° Congresso SIF”* 500 euro

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- Training courses** *Corso di Formazione AiFOS, Corso di Formazione/Aggiornamento professionale, Prevenzione dei rischi Chimico e Biologico in Laboratorio; Università degli Studi di Milano; 19 Gennaio 2017, Milano*
- Corso Introduttivo alla Sperimentazione animale, Istituto di Ricerche Farmacologiche Mario Negri - Animal Care Unit; 16-18 Novembre 2016, Milano*
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- Modelli animali nella ricerca preclinica, aspetti scientifici, regolatori e tecnologie applicate; 9 Ottobre 2015; Tecniplast - Buguggiate, Varese*



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