Mesenchymal stem cells encapsulated into biomimetic hydrogel scaffold gradually release CCL2 chemokine in situ preserving cytoarchitecture and promoting functional recovery in spinal cord injury

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Spinal cord injury (SCI) is an acute neurodegenerative disorder caused by traumatic damage of the spinal cord. The neuropathological evolution of the primary trauma involves multifactorial processes that exacerbate the pathology, worsening the neurodegeneration and limiting neuroregeneration. This complexity suggests that multi-therapeutic approaches, rather than any single treatment, might be more effective. Encouraging preclinical results indicate that stem cell-based treatments may improve the disease outcome due to their multi-therapeutic ability. Mesenchymal Stem Cells (MSCs) are currently considered one of the most promising approaches. Significant improvement in the behavioral outcome after MSC treatment sustained by hydrogel has been de-monstrated. However, it is still not known how hydrogel contribute to the delivery of factors secreted from MSCs and what factors are released in situ.

Among different mediators secreted by MSCs after seeding into hydrogel, we have found CCL2 chemokine, which could account for the neuroprotective mechanisms of these cells. CCL2 secreted from human MSCs is delivered efficaciously in the lesioned spinal cord acting not only on recruitment of macrophages, but driving also their conversion to an M2 neuroprotective phenotype. Surprisingly, human CCL2 delivered also plays a key role in preventing motor neuron degeneration in vitro and after spinal cord trauma in vivo, with a significant improvement of the motor performance of the rodent SCI models.

Keywords: Hydrogel, Mesenchymal stem cells, Spinal cord injury, Human chemokine (C–C motif) ligand 2 CCL2, Microglia/macrophages, Motor neurons

1. Introduction

Spinal cord injury (SCI) is caused by a traumatic event that leads to neurological impairment with partial or total loss of sensory/motor function. The primary acute mechanical injury is followed by further damage to the spinal cord tissue (secondary injury) which worsens the clinical outcome. The secondary injury includes a wide range of subsequent events blood-brain barrier dysfunction, thrombosis, ischemia, free radical formation and glutamate release due to the acute damage; an uncontrolled inflammatory response, scar formation and cell death occur with time, limiting neuronal regeneration [1–3]. In the therapeutic scenario the multi-therapeutic ability of stem cells, able to release many potential beneficial factors at the damaged site [4], is evaluated as one of the most promising strategies for SCI. This promise depends on the fact that many stem cells act on the pathological outcome, combining trophic support, anti-inflammatory effect, immunomodulation, anti-apoptotic effect, neutralization of inhibitory factors and reduction of scar formation [4].

Alongside recent progress in studying the efficacy of stem cells for treating SCI, the effect of mesenchymal stem cells (MSCs) in CNS regeneration has emerged among the most promising.

However, different limitations are still to be overcome in using hMSCs as therapeutic opportunity in SCI [4–6]. Among them, a crucial aspect is the preservation of MSC viability and optimal delivery of regenerative factors in the injured site. Particularly, hydrogels offer the possibility to load and sustain hMSCs making an optimal niche close to

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the injured site and maintaining them preserved from the hostile tissue [4,5]. However, so far these scaffolds have been assessed only for their own capacity to preserve the cell viability [4,7] and very few attempts have been made for understanding how hydrogel contributes to the delivery of factors secreted from MSCs [4] and what factors are released.

Recent studies have changed our view of the MSC effect, indicating that transplanted stem cells do not differentiate into neuronal or glial cells but act through a by-stander effect releasing many modulating factors [4,8,9]. Among the potential mechanisms involved in the efficacy of these cells in SCI, the immunomodulatory properties seem to play a key role [4]. MSCs have a protective effect through anti-inflammatory action [4,6] suppressing lymphocyte proliferation and differentiation [4–6].

The pro-inflammatory response mediated by the immune system is a critical target for treating SCI [10,11], and better understanding of how MSCs and their factors released from hydrogel are involved in these mechanisms is essential to optimize the design of biopolymeric matrice and cell treatment. We know that MSCs play a protective anti-inflammatory role acting on infiltrated peripheral macrophages and promoting the transition from M1 pro-inflammatory to M2 pro-regenerative phenotype in SCI animal models [5,6,12], as also reported in our previous studies [5]. However, the mechanism of this efficacy after treatment of human MSCs (hMSC) loaded into hydrogel remains unknown. We postulated the existence of modulators secreted by MSCs and released by hydrogel that could act on macrophage recruitment and M2 conversion in the injury site, orchestrating the reconstitution and preservation of nervous tissue. The present study aimed at demonstrating the complete biocompatibility of the biomaterial and the efficacy of a delivery system optimized for a sustainable release of potential neuroreparative factors secreted by hMSCs. This further confirms the therapeutic action of hMSCs, identifying putative factors released by hydrogel.

2. Material and methods

2.1. Cord blood human mesenchymal stem cell (hMSCs) cultures

Human mesenchymal stem cells (hMSC) were obtained from umbilical cord blood (CB) and fully characterized for mesenchymal identity and optimal growth properties (long-living CB hMSCs) [13]. See Supplementary data.

2.2. Arginine-glycine-aspartic acid (RGD) – synthesis of extracellular matrix (ECM) hydrogels

The synthesis of the RGD ECM hydrogels used as scaffold for hMSC was previously described in a paper by Caron et al. [5]. See Supplementary data.

2.3. Loading hMSC in RGD-ECM lyophilized hydrogels

To obtain RGD-ECM hydrogels loaded with hMSC, cells were harvested and suspended at a density of 1.25×10^6 cells/mL in growth medium; 80 mL of cells were added directly onto the lyophilized hydrogel (40 mL for each side). Hydrogels were left to swell for 30 min in an incubator at 37 °C, and then 1 mL of growth medium was added. Each hydrogel contained approximately 100,000 cells [5].

2.4. Primary neuron cultures

Cultures were prepared from the spinal cord of 13-day-old C57 BL/6J mouse embryos, as previously reported [14]. See Supplementary data.

2.5. Number of motor neurons and neurite length in vitro

Stereological analysis was carried out to estimate the motor neuron count and length of their projections *in vitro*. See Supplementary data.

2.6. Peritoneal macrophage cultures

Peritoneal macrophages were prepared by adapting the protocol reported by Zhang and coworkers [15,16]. See Supplementary data.

2.7. Surgery

B6.129P-Cx3cr1tm1Litt/J mice (Charles River Laboratories International, Inc) were used for *in vivo* studies. Animals were placed on a Cunningham Spinal Cord Adaptor (Stoelting) mounted on a stereotaxic frame and laminectomy of T12 vertebra was done to uncover the lumbar spinal cord. Extradural compression of the spinal cord at T12 was achieved using an aneurysm clip with a closing force of 60 g, producing mechanical trauma. The clip was left in place for 1 min and then removed. After the surgery, the animals were kept on a warm pad for 30 min and then placed in separate cages for recovery. See Supplementary data.

2.8. Intraparenchymal injections of human ⌂hemokine (C−C motif) ligand 2 (hCCL2)

For the acute and chronic evaluation of hCCL2 treatment, 1 day or 20 days after SCI mice were injected intraparenchymally with hCCL2 (50 µg/mL). Six injections of 0.250 uL each were done to cover the injured area of spinal cord, with a glass capillary. The capillary was positioned ± 0.5 mm from the midline, and then it was deepened into the parenchyma to 0.6 mm below the pia mater. After treatment, dorsal muscles were juxtaposed using absorbable sutures and the skin was sutured and disinfected.

2.9. Behavioral evaluations

All treated mice were evaluated using the Basso Mouse Scale (BMS) [17] to test hind-limb locomotor performances, once a week from day 7 to 35 days post injury (DPI). The BMS is a 10-point scale (9 corresponds to normal locomotion, 0 complete hind limb paralysis). Videos of the locomotor performances (5 min) were taken with an ICD-49E camera (Ikegami) and assessments were made by two independent observers, blinded to the treatment. Individual hind limb scores were averaged for each animal at each time point.

2.10. Flow cytometric analysis

FACS analysis was carried out as previously reported [16]. See Supplementary data.

2.11. Total number of neurons in damaged spinal cord

Stereological analysis was used to obtain an unbiased estimate of the total number of motor neurons in a spinal cord area +1.5/−1.3 mm from the injury site [18–20]. See Supplementary data.

2.12. Spared myelin in spinal cord

Stereological analysis was done to obtain estimate the volume of the lesion measured as myelin spared in a spinal cord area +1.5/−1.3 mm from the injury site. See Supplementary data.

2.13. Statistical analyses

Statistical analyses were performed using Prism software

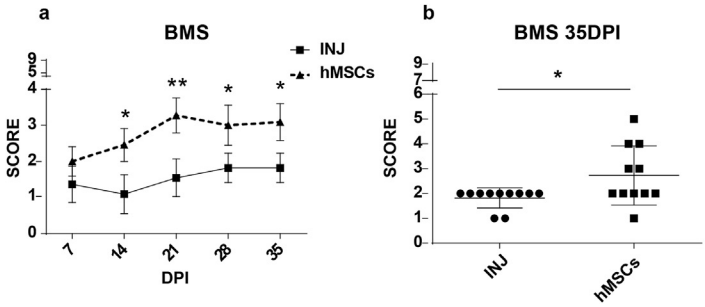

Fig. 1. *In vitro* evaluation of hCCL2 protein delivered after hMSCs loaded into hydrogel improved locomotor performance in SCI mice: a) untreated SCI mice (INJ) or treated (hMSCs) 1 DPI examined weekly starting 7 days post treatment, using the Basso Mouse Scale-BMS (score 0, complete paralysis, 9 complete mobility, referred to healthy mice). Locomotor performances in hMSC mice significantly improved starting from 14 DPI up to 35 DPI, compared to INJ. b) BMS scores of mice at 35 DPI. c) Positioning of the hydrogel + hMSCs in the SCI mouse model. Data are presented as mean ± SEM, (*) $p < 0.05$, (**) $p < 0.01$; 11 mice/group.

(Graphpad). ManneWhitney test, one-way ANOVA or two-way ANOVA followed by Bonferroni's *post hoc* test were applied, as reported in figure legends.

3. Results

3.1. Treatment with hMSCs loaded into hydrogel improve locomotor performance in SCI mice

We evaluated the effects of MSCs on the recovery of locomotor function in a mouse SCI model. *In vivo* mice were treated 1 day post injury (DPI). Mice were subjected to SCI and randomly divided into three groups: untreated (INJ), treated with empty hydrogel (HYDROGEL) and treated with hMSCs loaded into hydrogel (hMSCs). Behavioral tests were repeated weekly from 7 up to 35 DPI. Locomotor performance was assessed on the Basso Mouse Scale (BMS, score 0 indicates a complete paralysis and score 9 normal mobility) [17]. Spontaneous recovery was seen in INJ groups throughout the observation time after complete post injury paraplegia (Fig. 1; a,b). The positioning of an empty hydrogel *in situ* significantly improved the course of the SCI progression only at 21 DPI compared to the untreated injured group, with a partial healing effect of the scaffold on the injured site (Fig. S1). Hydrogel supplemented with hMSCs and applied into the epicenter of the lesion gave significant, lasting locomotor improvement compared to the untreated INJ group, with a better BMS score 14, 21, 28 and 35 DPI (Fig. 1; a,b). This experiment demonstrates the therapeutic effect of hMSC administered with a biocompatible hydrogel on the motor performance of SCI mice.

3.2. Human chemokine (C−C motif) ligand 2 (CCL2) is consistently released by hMSCs encapsulated into hydrogel in vitro and in vivo

To assess potential elements involved in the efficacy of hMSCs, we hypothesized that there might exist some factors involved in the recruitment of perypheral macrophages. Indeed, our previous study had found increased migration of macrophagic cells into the traumatized spinal cord after hMSC treatment [5]. So we focused on one of the cytokines most involved in macrophage recruitment into the damaged spinal cord [10], chemokine (C−C motif) ligand 2 (CCL2). We used RT-PCR (Fig. S2; a) and ELISA (Fig. S2; b) to analyze the expression of this cytokine from hMSCs. CCL2 was abundantly expressed by hMSCs *in vitro* (1.000 ± 1.142 relative mRNA expression and 2212 ± 1311 pg/mL protein, mean ± SD), with a potential contributing effect during the progression of SCI degeneration.

To evaluate the capability of hydrogel to release hCCL2 secreted from hMSCs, we assessed *in vitro* the release of this chemokine in the conditioned media, it is in, starting from 6 h up to 7 days after hMSC seeding (Fig. 2). We measured the levels of hCCL2 protein by ELISA assay. We observed an initial burst of hCCL2 release (20.86 ± 7.47 pg/mL, mean ± SD) at 6 h with a significant gradual reduction at 2 days (12.12 ± 2.03 pg/mL, mean ± SD); after the levels of hCCL2 remain constant up to 7 days (13.63 ± 3.2 pg/mL, mean ± SD) (Fig. 2).

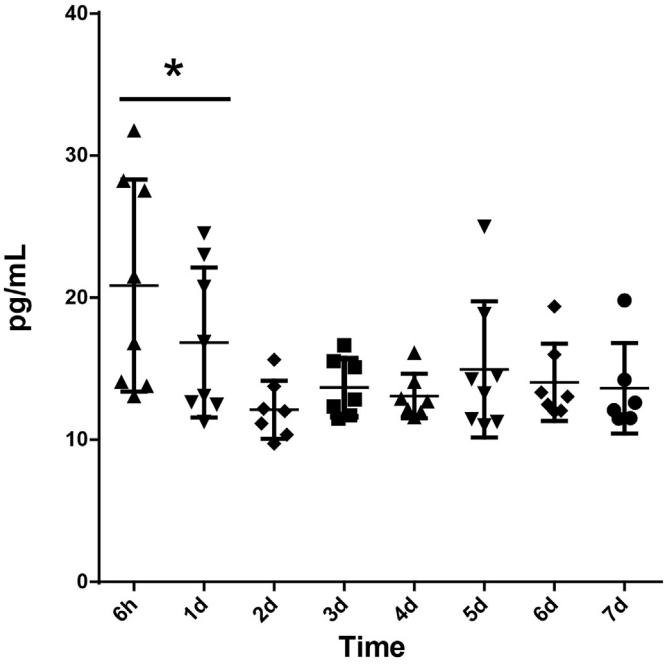

Fig. 2. *In vitro* evaluation of hCCL2 protein delivered after hMSC seeding into hydrogel. hCCL2 is delivered after 6 h (h), significantly reduced at 2 days, after the levels remain constant up to 7 days. Data are presented as mean ± SD; N = 6/8 replicates; (*) $p < 0.05$.

These data demonstrate the ability of hydrogel to release hCCL2 secreted form hMSCs for at least 7 days. In line with the *in vitro* finding, *in vivo* ELISA showed higher level of hCCL2 in the parenchyma of traumatized SCI animals (Fig. S2; c), confirming the ability of hMCS to secrete hCCL2 *in vivo* (203.3 ± 86.4 pg/mL, mean ± SD) and concomitantly the ability of this hydrogel to deliver this factor efficiently to the SCI site. hCCL2 was clearly distinguished from the murine CCL2 through a specific protein expression ELISA (see Methods). These results indicate that a high level of hCCL2 is potentially available to enhance the recruitment of peripheral macrophages *in situ* in the SCI mouse model.

3.3. Early, but not late, in situ hCCL2 treatment improves functional recovery after SCI in vivo

To assess the effect of hCCL2 in SCI, we ran *in vivo* experiments. Mice were subjected to SCI and randomly divided into two groups: untreated (INJ) and treated with hCCL2 recombinant protein 1 DPI. Six injections were made into the injury site with a glass capillary to deliver this chemokine (50 µg/mL). Behavior was assessed weekly from 7 up to 35 DPI. In the hCCL2 treated group (hCCL2), we found a significant and persistent behavioral improvement from 14 to 35 DPI compared to the INJ group (Fig. 3; a,b). However, later hCCL2 treatment in the SCI

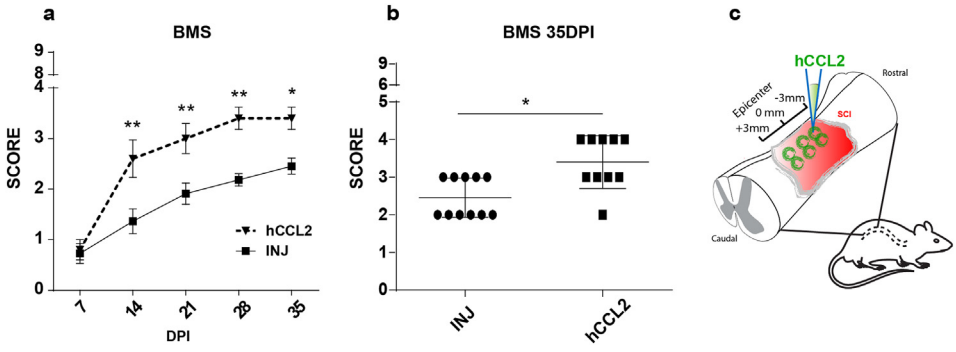

Fig. 3. Early treatment with hCCL2 improved locomotor performance in SCI mice: a) untreated SCI mice (INJ) or treated with hCCL2 1DPI (hCCL2), examined weekly starting 7 days post treatment by Basso Mouse Scale – BMS (score 0, complete paralysis, score 9 complete mobility, referred to healthy mice). Locomotor performances significantly improved in hCCL2 mice starting from 14 DPI up to 35 DPI compared to INJ. b) BMS scores of mice at 35 DPI. c) Injection of hCCL2 in SCI mice. Data are mean ± SEM, (*) $p < 0.05$,(**) $p < 0.01$; 10/11 mice/group.

chronic phase (20 DPI, corresponding to the recovery plateau in our own model) did not give any improvement in motor performance compared to the INJ group (data not shown). To validate the putative protective role of CCL2 after trauma, we examined the functional recovery of SCI mice immunodepleting the murine form of this chemokine in the injured site. The anti-mCCL2 treated group at 7 DPI showed a lack of recovery and progressive generalized malaise that made behavioral assessment impossible (data not shown). Microinjections of isotype IgG (Hamster) in the injury site, as control condition, did not modify the course of the SCI progression compared to the untreated group, up to 35 DPI (data not shown). This shows that CCL2 is essential for the functional recovery of the SCI rodent model and its supplementation is only protective in an initial acute-subacute phase, while chronic CCL2 seems not to be a vital player.

3.4. *In situ hCCL2 significantly enhanced macrophage recruitment and conversion to a M2 phenotype in vivo and in vitro*

Given the ability of CCL2 to recruit macrophages *in situ*, we examined the presence of M1/M2 in the injured site by FACS analysis, after chemokine treatment *in situ*. Microglia and peripheral macrophages in parenchyma were distinguished by double labeling with CD11b and CD45 antibodies, identifying microglia by a $CD11b^{+}/CD45^{low}$ expression and peripheral macrophages by a $CD11b^{+}/CD45^{high}$ expression [16]. The amount of microglia increased very early after the acute injury and then over time up to 7 DPI (data not shown). Peripheral macrophages were recruited only at 1 DPI and peaked 7 DPI (data not shown) [16]. The hCCL2 group showed significant increase in the recruitment of peripheral macrophages at 3 DPI compared to the untreated group (INJ) (Fig. S3A; a,b,d) without significantly affecting microglial cells (Fig. S3A; a,b,c). These results demonstrate the ability of human CCL2 in recruiting murine origin peripheral macrophages.

We also examined whether the proposed treatment could act on M1/M2 like macrophagic phenotypes in the injury site. We used FACS analysis to detect the expression of markers for M1 ($CD11b^{+}/CX3CR1^{low}/Ly6C^{high}$) or M2 ($CD11b^{+}/CX3CR1^{high}/Ly6C^{low}$) [16] polarized cells. Quantitative examination indicated an accumulation of both M1 and M2 macrophages starting from 1 DPI and increasing with time up to 3 DPI for M1 and 7 DPI for M2 (data not shown). Interestingly, hCCL2 increased M2 polarized cells at 3 DPI, but not M1, compared to untreated INJ mice (Fig. S3B; a,b,d). These results suggest that hCCL2 is mostly involved in establishing a neuroprotective environment during the recovery of SCI.

To validate the contribution of CCL2 in setting a beneficial milieu, we examined peripheral macrophage recruitment after immunodepletion of constitutive CCL2 in the injured site at 1 DPI, and found significantly reduced migration of peripheral macrophages (Fig. S4; a,b,d), but not of resident microglia, although with lower amount (Fig. S4; a,b,c), *in situ*. This confirms the importance of this chemokine in contributing for a reparative environment after the acute damage *via* macrophage recruitment –as demonstrated (Figs. 3 and S3) – and lack of recovery after anti-mCCL2 treatment.

We further explored the potential interplay between CCL2 and macrophage polarization *in vitro*. Peripheral macrophages were isolated from the mouse peritoneal cavity, cultured and treated with hCCL2 (1 μg/mL). The mRNA expression of tumor necrosis factor alpha (TNFα), interleukin 1 beta (IL-1β), Chitinase-like protein 3 (YM1), and Arginase I (Arg I), all accepted markers for M1 [2] or M2 macrophages [2], was measured to demonstrate the shift to the M2 anti-inflammatory phenotype. IL4, which converts macrophages to the M2 subset, was used as a control, demonstrating its ability to detect M2 polarization *in vitro* as evidenced by the significantly reduced transcript expression of TNFα (Fig. 4; a) and IL-1β (Fig. 4; b) and concomitant increase of YM1 (Fig. 4; c) and Arg1 (Fig. 4; d). These results showed that hCCL2 treatment induced a lower expression of M1 markers: TNFα (Fig. 4; a) and IL-1β (Fig. 4; b), and increase in M2 markers: YM1 (Fig. 4; c) and Arg1 (Fig. 4; d) compared with macrophages cultured alone; this suggests that this soluble factor has an important role in macrophage polarization.

To confirm that the M2 macrophage polarization is also induced by the soluble factors secreted by MSCs, we cultured macrophages for 24 h with conditioned medium from hMSCs. Again, there was a reproducible reduction of TNFα (Fig. 4; a) and IL-1β (Fig. 4; b) and induction of YM1 (Fig. 4; c), but not Arg1 (Fig. 4; d), compared to the untreated cells (Fig. 4; a,b,c,d). These findings provide the first evidence that hCCL2 can elicit M2 macrophage polarization, suggesting a key role for this chemokine in improving the SCI pathological outcome when secreted *in situ* by hMSCs. Arg I did not change after hMSC treatment; possibly some factors released from the hMSCs counteract Arg I over-expression mediated by CCL2, or else the lower concentration detected in conditioned medium of hMSCs (0,0022 ± 0,0013 μg/mL, data not shown), compared to the 1 μg/mL used for treating with recombinant hCCL2, was unable to act on Arg I expression.

3.5. *hMSCs and hCCL2 preserved motor neurons and myelin in vivo*

Structural recovery after SCI must be assessed to determine the therapeutic effect of hMSCs or hCCL2. We examined neuronal and myelin preservation by histological and quantitative stereological analysis (see Methods). We recorded the number of neurons (Fig. 5; A,B) and amount of spared myelin (Fig. S5) in untreated injured mice compared to hCCL2 or hMSC treated mice at 35 DPI. Based on the assumption that motor neurons are larger cells in the spinal cord, we stained neurons with NeuN or Fluorescent Nissl Stain (Neurotrace) and classified them in two categories: neurons measuring $< 400\,\mu m^2$ (mostly located in the dorsal horn) and neurons $> 400\mu m^2$ (motor neurons) [21]. We analyzed the distribution and frequency of all neurons in a spinal cord area $+1.5/-1.35$ mm from the injury site. Quantitative analysis indicated that neurons $< 400\,\mu m^2$, particularly those in the size range 100–150 μm^2, were significantly preserved after hMSC treatment compared to hCCL2 or untreated injured mice (Fig. 5; A), which had fewer small neurons. In contrast, we found a significantly

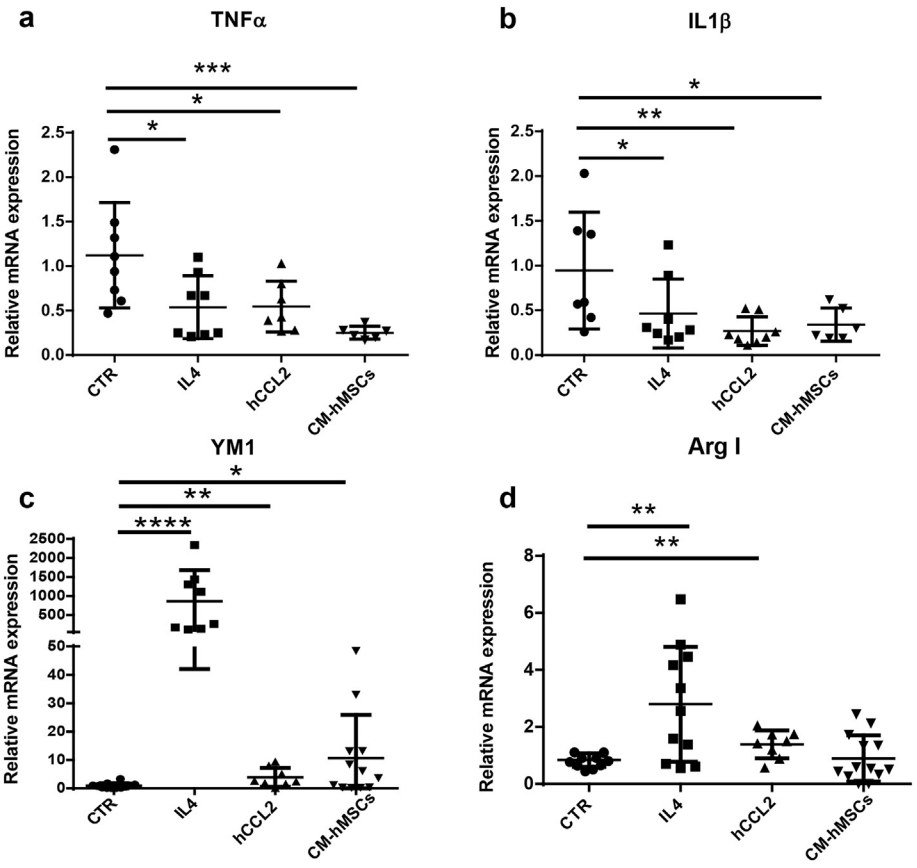

Fig. 4. hCCL2 and Conditioned Medium–MSCs (CM-MSCs) significantly skewed M1 toward the M2 phenotype *in vitro*.
Quantitative mRNA analysis of M1 (TNFα, a and IL1β, b) or M2 markers (YM1, c and Arg I, d) expressed by macrophage cultures after treatment with IL4, used as M2 positive control, hCCL2 or hMSCs conditioned media (CM-hMSCs). hCCL2 and CM-hMSCs significantly reduce the M1 markers (a,b) and significantly increase M2 markers (c,d), compared to untreated (CTR) cells. Data are mean ± SD, (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$, (****) $p < 0.0001$.

higher number of motor neurons in both hMSC and hCCL2 treated mice than untreated injured mice (Fig. 5; B). This suggests that the principal motor neurons were preserved across the spinal cord after MSC treatment and that CCL2 seems to have a key role in preserving these cells.

After injury there was atrophy of nervous tissue together with a loss of myelin in the spinal cord, impairing the conductive capacity of surviving axons and their functional activity [22]. Therefore, we analyzed the loss of myelin, stained by Fluormyelin, using a point count unbiased grid frame and Cavalieri method [20] in a spinal cord area encompassing $+1.5/-1.35$ mm from the injury site (Fig. S5) of untreated, hMSCs and hCCL2 treated mice. Significant myelin sparing was noted in hMSCs and hCCL2 treated mice compared to untreated mice in the injured site and in the distal peri-injury zone (Fig. S5). This neuron and myelin sparing strongly correlates with the improvement of motor function after hMSC and hCCL2 treatment in SCI mice.

3.6. *hCCL2 protect motor neurons against excitotoxic death in vitro*

Acute glutamate-mediated excitotoxicity due to the activation of the *N*-methyl-D-aspartate (NMDA) receptor is one of the documented events that follow SCI resulting in further loss of neuronal cells [1]. We sought to examine whether treatment with hCCL2 or conditioned medium collected from hMSCs improved the response to NMDA-mediated excitotoxic damage in cultured motor neurons. To this purpose, we used motor neuron/astrocytes cocultures (see Methods). The susceptibility of motor neurons to NMDA-induced cell death was determined over a range of concentrations, reaching significant cell death and neurite degeneration with dystrophic phenotype after 24 h from 10 μM NMDA treatment (Fig. 6; a,b,e,f). 2D Stereology cell count and neurite lengths were recorded after neurofilament (SMI 32) immunostaining (see Methods). Noteworthy, cotreatment with hCCL2 (1 μg/mL) significantly preserved motor neuron viability and neurite length decrease from NMDA-mediated excitotoxicity showing arborization patterns and morphologies similar to untreated cells (Fig. 6; c,e,f). Motor neurons also showed significant neurite outgrowth compared to control (CTR) when exposed only to hCCL2, suggesting a possible worthwhile trophic effect of the chemokine on neurite arborization of spinal motor neurons.

To demonstrate that CCL2 acted directly on the motor neuron protection after NMDA insults, we compared the survival and morphology of isolated motor neurons exposed to the excitotoxic challenge (10 μM NMDA) and isolated motor neurons treated with NMDA (10 μM) and conditioned medium of CCL2 pre-treated astrocytes. In this condition, no significant difference in motor neuron survival or neurite outgrowth compared to only NMDA treated cultures was detectable (data not shown).

4. Discussion

Engineered scaffolds are considered a promising approach to sustain a long lasting cell viability of stem cells in the injured site offering the opportunity to deliver factors appropriately for optimizing the therapeutic effect of the treatment [4,7]. However, it is still not known how hydrogel contribute to the delivery of factors secreted from MSCs and what factors are released *in situ* to exploit their full therapeutic potential and identify effective and reliable treatment for SCI.

There is mounting evidence that the therapeutic effect of hMSCs relies on the paracrine action of specific factors produced and delivered *in situ* [4,6,23]. In line with this, we reported previously that hMSCs administered in an optimized carbomer-agarose based scaffold showed a bystander effect when positioned very close to the injured site [5]. We also reported that this treatment recruited numerous anti-inflammatory macrophages in the epicenter of the lesion [5]. This extended our previous findings, demonstrating functional recovery in SCI animals treated with hMSCs encapsulated into hydrogel compared to untreated animals, persisting up to 35 DPI. This further confirms the complete

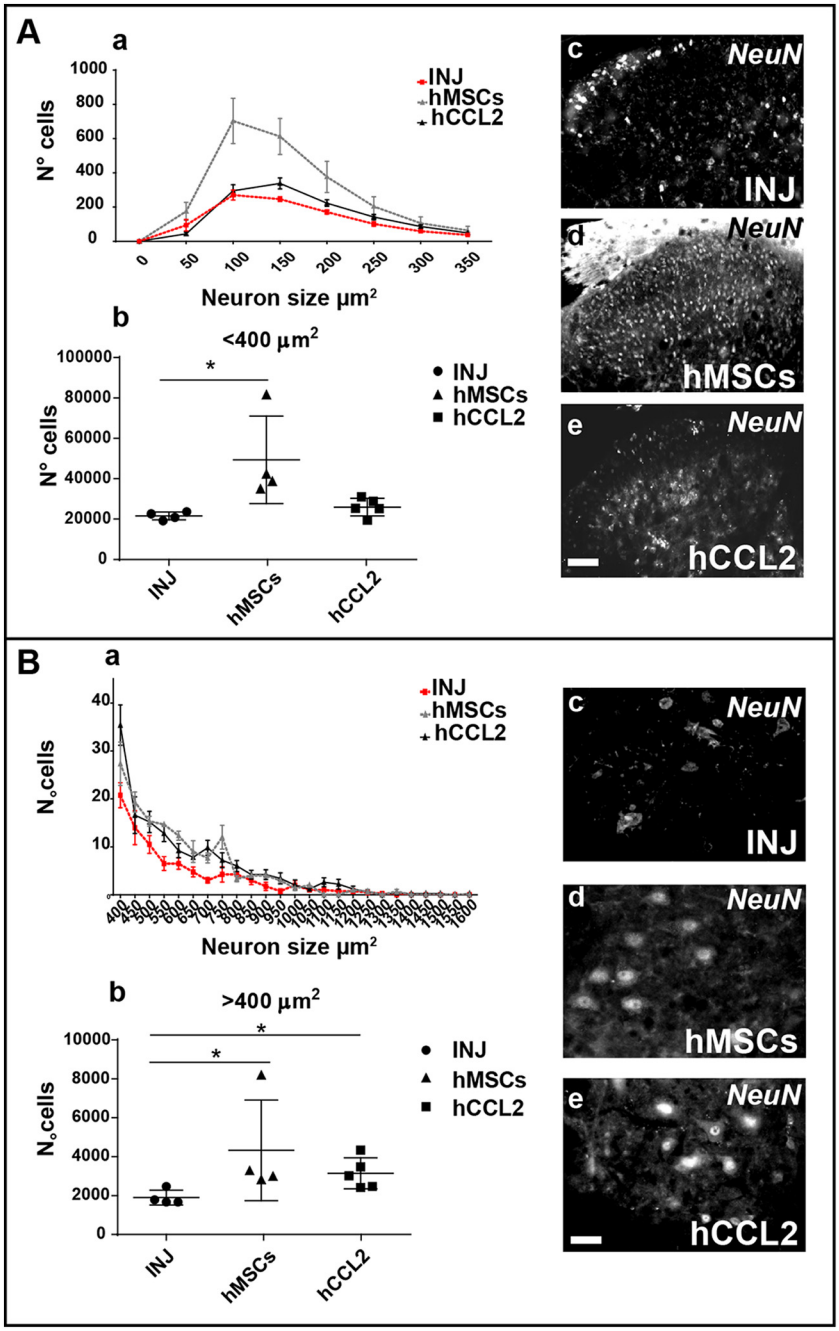

Fig. 5. hMSCs and hCCL2 skewed a significantly high number of motor neurons. hMSCs also preserved small neurons in the dorsal horn.
(A) (a,b) Quantification of sensory neurons (< 400 µm^2) expressed as number of cells for each size (from 0 to 350 µm^2) in untreated injured (red line), hMSCs (grey line) and hCCL2 (black line) treated mice. hMSC treated mice have more small neurons than untreated or hCCL2 treated SCI mice (data are mean ± SD, n = 4, *p < 0.05). (b). Representative insets of dorsal portions of spinal cord sections of untreated injured (c), hMSCs (b,) and hCCL2 (e) treated mice, stained with NeuN. (B) (a,b). Quantification of motor neurons (> 400 µm^2) expressed as number of cells for each size (from 400 to 1600 µm^2) in untreated injured (red line), hMSC (grey line) and hCCL2 (black line) treated mice. hMSC and hCCL2 treated mice have more motor neurons than to untreated injured mice (data are mean ± SD, n = 4, (*) p < 0.05). Representative insets of ventral portions of spinal cord sections of untreated injured (c), hMSCs (d) and hCCL2 (e) treated mice, stained with NeuN.

biocompatibility of the biomaterial and the efficacy of a delivery system optimized for a sustainable release of potential neuroreparative factors secreted by hMSCs. To dig deeper into the mechanism of this treatment, we postulated that a chemotactic factor might be involved, given the higher macrophage response in the injured site [5]. Among several factors secreted by hMSCs and released by hydrogel *in vitro* there is human CCL2, a chemokine that might play a key role in recruiting peripheral macrophages. We also found that the biomaterial used in this study allowed a sustainable human CCL2 delivery *in vivo,* secreted by hMSCs.

The therapeutic action of human CCL2 was examined further by administering isolated CCL2 recombinant protein of human origin early in the injured site of a SCI rodent model. This treatment recruits numerous peripheral macrophages with an anti-inflammatory M2 like phenotype in the damaged site. The substantial improvement seen with human CCL2 injected *in situ* 1 day after the lesion was confirmed in a behavioral test which showed significant motor recovery. CCL2 treatment is beneficial only in the acute phase of SCI, and a treatment in the chronic phase (20 DPI) did not affect the progression of the pathology. This point to the importance of early recruitment of pro-regenerative macrophages to boost reparative mechanisms *in situ*. These results suggest that human CCL2 from hMSCs might have chemotactic ability similar to the murine form, considering that the mouse or rat CCL2 gene has about 75% homology the human one [24].

The immune response after SCI has always been considered largely harmful because of the production of toxic mediators [25]. Various studies have reported a better neurological outcome after anti-inflammatory treatment in different SCI rodent models [26–28]. Although some aspects of the inflammation are clearly detrimental, some inflammatory responses are beneficial [10,28–30]. There appears to be a pro regenerative role for a subset of macrophages that remove debris and promote axonal regeneration and tissue remodeling [5,16,29,31],

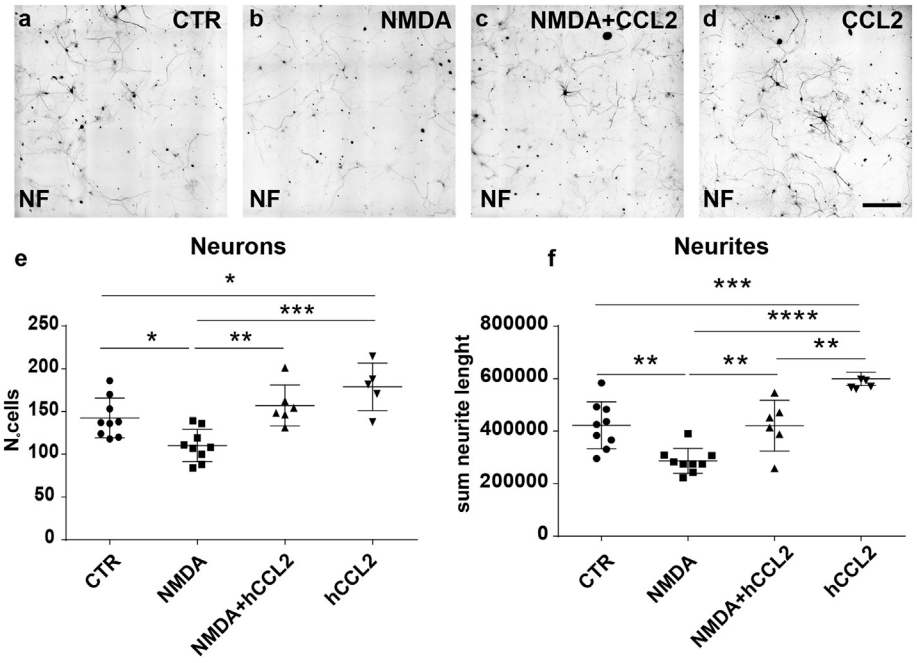

Fig. 6. hCCL2 treatment reverted NMDA motor neuron toxicity *in vitro*.
Primary neuron cultures (a, CTR) incubated with NMDA (b), NMDA and hCCL2 (c) or hCCL2 (d) and stained for SMI-32. Neuron count (e) and neuritis length quantification confirms the toxicity of NMDA treatment and shows that concomitant hCCL2 treatment is able to revert NMDA toxicity and to promote a protective effect (a,b,c,e,f). hCCL2 treated neurons shows an increased viability and neurite elongation compare to CTR (a,d,e,f). Data are presented as mean ± SD, (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$, (****) $p < 0,0001$. Scale bar = 100 µm.

whereas after delayed infiltration of activated macrophages myelin debris persisted after SCI, preventing remyelination [32]. This suggests that a pro-regenerative-like phenotype, when enhanced in the injury site, could help tissue healing and motor recovery [5,16,29,31]; time-dependent action on the inflammation may therefore be decisive for a destructive or constructive outcome [16].

CCL2 chemokine strongly promotes the pro-inflammatory response in SCI by recruiting mostly peripheral pro-inflammatory M1 monocyte-macrophages to the injured site [10]. However, some recent studies reported an anti-inflammatory effect of CCL2, responsible for a more regenerative macrophage-enriched milieu at the site of damaged [33]. CCL2 can recruit and stimulate macrophages toward the M2 phenotype, which is essential for motor function recovery [33]. We showed that *in vitro* peripheral macrophages were skewed toward the M2 phenotype after hCCL2 treatment (Fig. 7), as demonstrated by the expression of M2 markers. We also found a new, unexpected role of a human CCL2 released by MSC loaded hydrogel, with neuroprotective mechanisms *in vitro* and *in vivo*. Human CCL2 chemokine preserved motor neurons and increased neurite outgrowth (Fig. 7). This study demonstrates the role of this chemokine secreted by hMSC encapsulated into hydrogel in supporting motor neurons for the first time. It is quite likely linked to neuronal protection preserving neurons from excitotoxicity by reducing NMDA receptor 1 expression and extracellular glutamate levels [34] and/or contributing to the axogenic and pro-motility action of survival motor neuron protein [35,36]. These data therefore indicate that human CCL2 is a key player in the therapeutic effect of hMSCs. However, we cannot exclude that other factors are secreted and released by MSC loaded into hydrogel or other protective mechanisms involving different agent/or cells are mediated by CCL2. Secreted trophic factors of hMSCs also support neuronal cells in the dorsal part of the damaged spinal cord, protecting the tissue cytoarchitecture more.

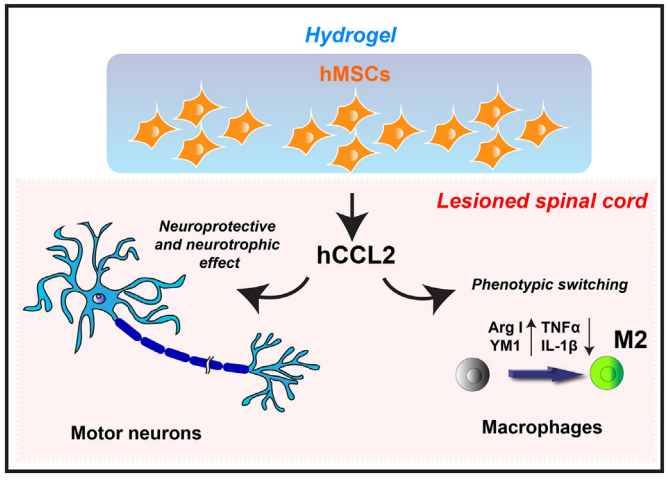

Fig. 7. Cartoon representation of protective effect of Hydrogel/hMSC treatment on SCI mouse model. hCCL2 is consistently release in the lesioned spinal cord and exerts a double protective effect, acting on macrophages phenotype switching and promoting motor neuron protection.

5. Conclusion

In conclusion, we identified a new factor secreted by hMSCs and released by carbomer-agarose based hydrogel involved in the efficacy of this cell therapy. These results also point to an important therapeutic opportunity for controlling the migration of beneficial macrophages in the lesioned site through a single factor for repairing the damaged spinal cord. CCL2 can be loaded in biomaterial for more controlled delivery. This latest approaches might offer several advantages over to cells: elimination of the variability of cell survival *in situ,* better reproducibility of delivery, and more controlled kinetics in response to specific therapeutic needs. These findings offer promising indications for future treatments with therapeutic factors that can be isolated and maximized for a successful clinical outcome.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jconrel.2018.03.034.

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