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Mesenchymal stromal cells for traumatic brain injury

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per essere stata il mio incentivo nei momenti di difficoltà

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Abbreviations

AKI	acute kidney injury
AMPA	α-amino-3-hydroxy-5-methyl-4-isoazole
BBB	blood brain barrier
BDNF	brain-derived neurotrophic factor
BM	bone marrow
BMMCs	BM-derived mononuclear cells
BrdU	5-bromo-2-deoxyuridine
CB	cord blood
CBF	cerebral blood flow
CC	corpus callosum
CCI	controlled cortical impact
CCl_4	carbon tetrachloride
CNS	central nervous system
CsA	cyclosporin-A
CSPGs	chondroitin sulfate proteoglycans
CTs	clinical trials
EAA	excitatory amino acid
CAD	caspase-activated deoxyribonuclease
DAG	dyacil-glicerol
DAMPs	damage-associated molecular pattern molecules
DCX	doublecortin
DISC	death inducing signaling complex
DMEM	Dulbecco's modified eagle's medium
GCS	Glasgow coma scale
GDNF	glial cell-derived neurotrophic factor
GFAP	glial fibrillary acidic protein
GMP	good manufacturing practices
ia	intra-arterial
ic	intracerebral
ICAM	intercellular adhesion molecule
ICU	intensive care units
icv	intracerebroventricular

IDO	indoleamine 2,3-dioxygenase
IL	interleukin
INF	interferon
IP_3	inositol trisphosphate
ISTC	international society for cellular therapy
iv	intravenous
M	microglia/macrophages
MAPC	multipotent adult progenitor cells
MCP-1	monocyte chemotactic protein-1
MIP-2	macrophage inflammatory protein-2
MMP	matrix metalloproteinase
MRI	magnetic resonance imaging
MSCs	mesenchymal stromal cells
NGF	nerve growth factor
NMDA	N-methyl-D-aspartate
NO	nitric oxide
NSCs	neural stem cells
NT-3	neurotrophin-3
PAMPs	pathogen-associated molecular patterns
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffer saline
PGE-2	prostaglandin E2
PL	platelet lysate
PRRs	pattern recognition receptors
ROS	reactive oxygen species
SGZ	subgranular zone
SVZ	subventricular zone
TBI	traumatic brain injury
TGF	transforming growth factor
TIMP-3	tissue inhibitor of matrix metalloproteinase-3
TNF	tumor necrosis factor
tPA	tissue plasminogen activator
Trk	tyrosine kinase receptor
TSG-6	tumor necrosis factor-stimulated gene-6
VCAM-1	vascular cell adhesion molecule-1

Chapter 1

General Introduction

1.1 Traumatic brain injury

Traumatic brain injury (TBI) is a critical public health and socio- economic problem in high income countries. It is a major cause of death for young people[1] and survivors suffer permanent disabilities and dysfunctions[2]. The main causes of TBI are related to car accidents[3], but brain trauma following falls in the domestic environment is an increasing problem for elderly people. Prevalence data indicate that in Europe about 7,7 million people live with TBI related disabilities[4] while in USA the prevalence is estimated around 5,3 million people[5]. The incidence in Europe is 235 case per 100.000 people[4] while higher values are reported for USA inhabitants with 506 new cases per 100.000 people[6]. When considering patients with severe TBI (with Glasgow Coma Scale [GCS][7] ≤ 8) the mortality rate is 36-40% and the survivors with unfavorable outcome are 52-60%[2,8]. In the last decades great improvement has been obtained in the management of TBI patients in the intensive care units (ICU), significantly reducing mortality rate[9]. TBI care in ICU is focused on the early identification and removal of mass lesions and on the detection, prevention and treatment of secondary cerebral insults (e.g. elevated intracranial pressure, hypoxia, seizures) exacerbating brain damage. However despite much researches and promising results in animal

models, there are still no pharmacological treatments available to reverse the pathological consequences of TBI[1,10]. For these reasons TBI remain an unsolved clinical problem that urgently needs new therapeutic strategies.

1.1.1 Pathophysiology of TBI

Brain trauma is the result of a "primary" and a "secondary" injury (figure 1).

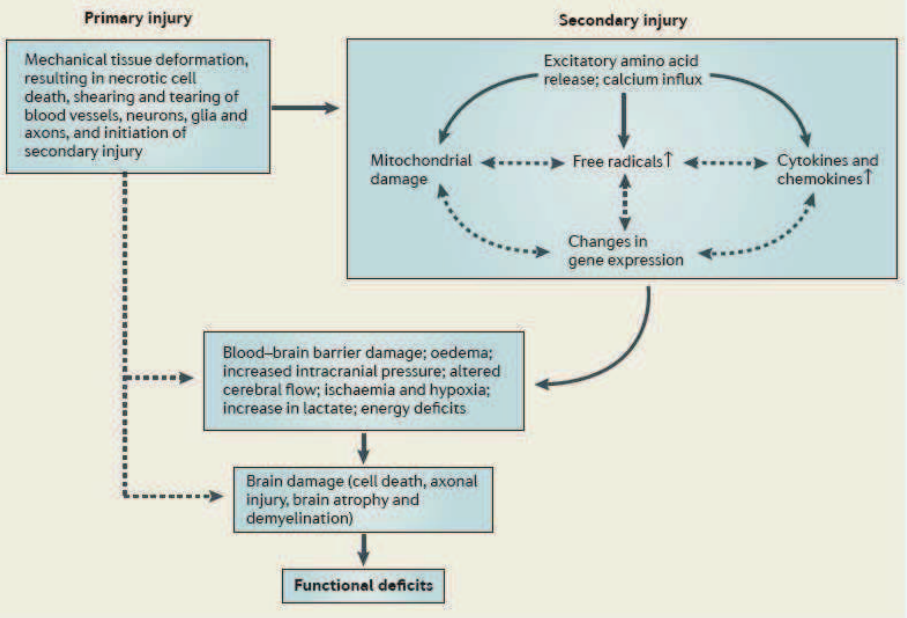

Figure 1. Schematic representation of TBI pathopysiology. *Both primary and secondary injury cascades are responsible for the final damage to brain tissue with consequent functional deficits. Figure from Xiong Y et al.*[11]

Primary injury is due to mechanical forces at the moment of the impact causing rapid tissue deformation and mechanical disruption resulting in physical damage responsible only for a part of the neurologic damage. Several "secondary" events

evolve over minutes to months after the primary injury, including neuroinflammation, toxic neurotransmitter release, production of free radicals, mitochondrial dysfunction, perturbation of calcium homeostasis, which have a crucial impact on the subsequent neurologic damage and the final outcome (figure 2A)[12].

As the primary injury occurs immediately after trauma, it can only be prevented (for example by the use of safety devices while driving). It induce irreversible brain damage causing neuronal death in the “core” of the lesion. The area adjacent to the core is called “penumbra” and represents potentially salvageable brain tissue in which neuronal activity is suppressed, but it is still potentially viable[13]. The viability of the penumbral area is threatened by the pathological cascades activated after brain trauma. The evolutionary nature of secondary injury development and the multiplicity of targets offer the possibility for therapeutic interventions. Recent preclinical studies have revealed that together with pathological cascades, a number of pro-regenerative events like neurogenesis, axonal sprouting, and angiogenesis are induced after TBI (figure 2B)[12,14]. However they are largely ineffective to counteract the lesion progression and tissue loss, suggesting that the promotion of neurorestorative processes may be an additional potential therapy for TBI. Today, preclinical promising results on single agent pharmacologic treatment have not been successfully translated into the clinical setting[1,10,15] underlining the need to focus on strategies that affect simultaneously multiple injury mechanisms. In this contest mesenchymal

stromal cells (MSCs) might provide an ideal candidate[16]. In the following paragraphs different pathologic/regenerative cascades activated after TBI will be analyzed while the potential of MSCs to counteract these cascades will be reviewed in chapter 1.2.

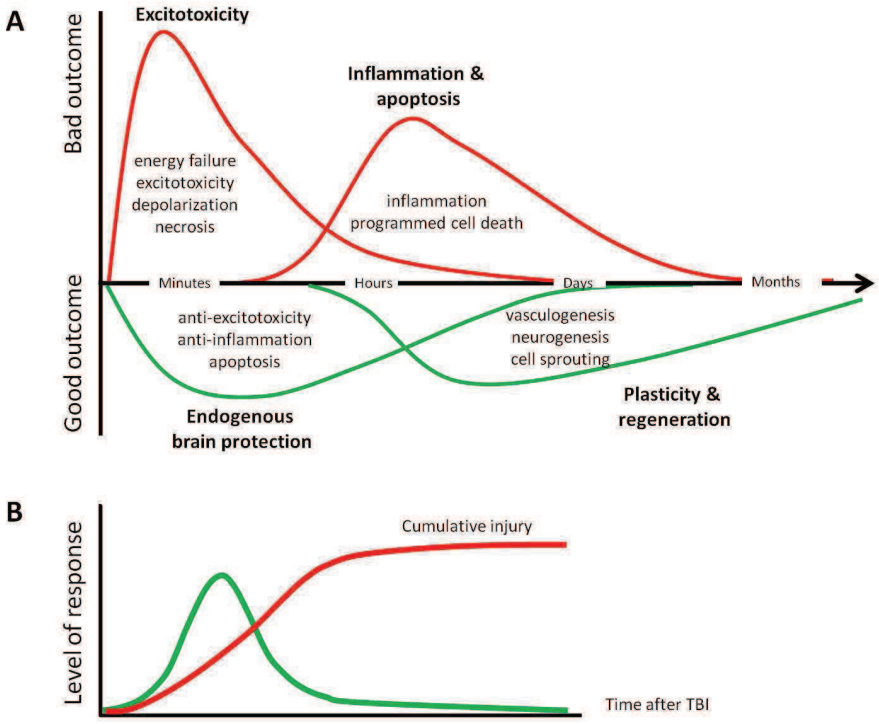

Figure 2. Injury progression after TBI. *A) Both toxic and regenerative cascades evolves after TBI with different kinetics. B) Protective pathways activated after acute brain injury are largely ineffective in antagonizing the progression of damage and cumulative injury increases over time. Graph A has been modified from Dirnagl et al.*[17]

1.1.2 Post-traumatic excitotoxicity

After TBI there is a massive increase of interstitial glutamate concentration[18,19]. This increase could belong to different sources: from traumatically damaged parenchymal cells in the injured brain, or from newly developed micropores in cell membranes[13], from the blood brain barrier (BBB) leakage[20] or following the reduced astrocytic reuptake due to the downregulation of the glutamate transporters GLAST and GLT-1[21]. The altered glutamate concentration in extracellular space lead to an over-stimulation of ionotropic N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoazole (AMPA) receptors with the consequent excessive calcium influx into neurons and astrocytes. Trying to compensate this disequilibrium, the cell increases the activity of Na^+/K^+-ATPase exchanger, however the lack of oxygen and metabolic supply prevents the restoration of the physiological ionic intra- and extra-cellular concentrations. As results, the exacerbated intracellular ionic overload leads to cytotoxic edema[22], with consequent cellular swelling and increase of intracranial pressure. Moreover the high Ca^{2+} influx due to overstimulation of glutamate receptors, induces the activation of second and third messenger triggering an array of downstream phospholipases and proteases that degrade membranes and proteins essential for cellular integrity[23,24]. Calcium activated downstream pathways include: 1) the activation of calpains[25], a family of cysteine proteases that proteolyze a wide range of cytoskeletal proteins such as spectrin, tubulin, microtubule-

associated proteins and neurofilamental proteins[26] as well as membrane associated proteins, including ion channels, excitatory amino acid (EAA) receptors and adhesion molecules; 2) the activation of the phospholipase A_2 [27] which hydrolyzes cellular phospholipids, liberating free fatty acids and lysophospholipids thereby providing the precursor substrates for the biosynthesis of eicosanoids and platelet activating factor[28], contributing to vascular damage increasing BBB permeability; 3) the activation of phospholipase C which hydrolyzes the membrane-associated phosphatidylinositols producing dyacil-glicerol (DAG) and inositol trisphosphate (IP_3)[29], giving rise to the second messenger cascades; 4) the perpetuation of mitochondrial dysfunction (Pharagraph 1.2.2); 5) activation of caspases and triggering of apoptosis (Pharagraph 1.2.3).

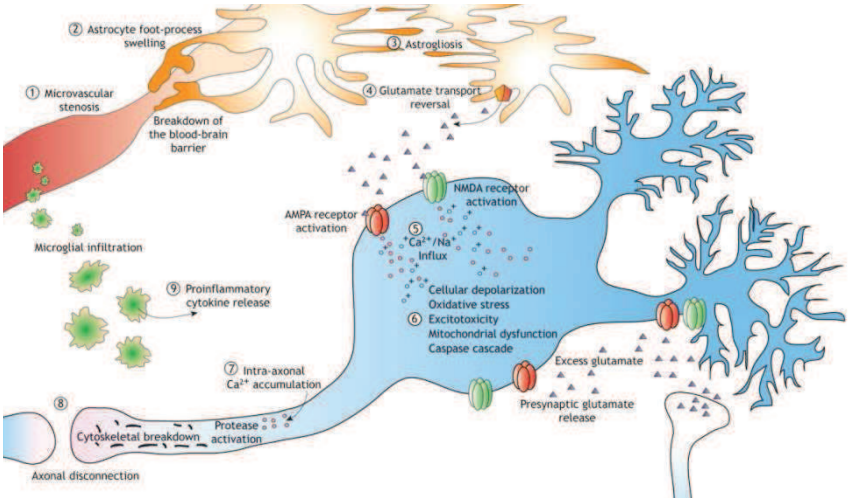

Figure 3. *The major pathways associated with the progression of secondary injury after a traumatic brain injury and cascades associated with calcium influx. Figure from Park E et al.* [30]

1.1.3 Mitochondrial damage

Mytochondria play an important role in the survival as well as the death of neurons. Both patients[31] and experimental models[32] of TBI show a reduction of oxidative metabolism related to mitochondria dysfunction. Mithocondria have the ability to buffer post-traumatic cytosolic Ca^{2+} overload. However, once the sequestration capacity of the mitochondria is exhausted or oxidative damage to the mitochondrial membrane has occurred, the oxidative phosphorylation is uncoupled from ATP production, with consequent release of Ca^{2+} back to the cytosol[33], production of free radicals and reactive oxygen species (ROS)[34], such as superoxide, hydrogen peroxide and hydroxyl radical. Radicals react with virtually any cellular component (carpohydrates, amino acids, DNA and phospholipids), causing direct damage. Besides being the major source of ROS production, mitochondria are also targets of oxidative stress. Overproduction of ROS in mitochondria is one of the elements inducing the collapse of membrane potential and the production of membrane transition pores, leading to the leakage of mitochondrial elements into the cytosol: the release of Hydrogen ions (H^+) dramatically reduce the pH, activate lysosomal enzymes contributing to the protein disruption; the release of cytocrom c, normally present between internal and external membrane of mitochondria, once release in the cytosol trigger the activation of apotosis[35,36].

1.1.4 Mechanisms of cell death

TBI associated cell death has been extensively associated with necrotic cell death[37,38]. Necrosis is characterized by mitochondrial swelling, nuclear picnosis followed by DNA fragmentation, loss of cell membrane integrity and an uncontrolled release of products of cell death into the intracellular space. The massive release of cellular components prevents a rapid clearance of debris from nearby phagocytes leading to the activation of inflammatory response and amplification of brain damage. Cell necrosis is the prevalent mechanisms of cell death in the core of the lesion, initiating within minutes after injury. Apoptosis, the programmed cell death, occurs predominantly in the penumbral regions starting hours after the primary injury and can last for months. Apoptotic dying cells have different morphological features compared to necrotic cells: chromatin is condensated and then fragmented, the cell shrinks and loses its contact with neighboring cells, the cytosol is fragmented in vescicolar bodies (blebs) that are rapidly phagocytes. Intracellular compounds are not released in the extracellular space thus there isn't activation of inflammatory response[39].

The first description of apoptotic events after experimental traumatic brain injury was performed by Rink and colleagues[40], while Clark and co-worker firstly showed apoptotic cell death in TBI patients[41]. Distinct regional and temporal pattern of apoptotic cell death have been described up to two months in cortex, hippocampus, white matter and thalamus of brain

injured rats[42] showing a long term involvement of different cerebral area in apoptotic pathways. The principal activator pathways and regulator of apotosis are illustrated in figure 4 and describe below.

Apoptosis is finely tuned by bcl-2 family proteins: bcl-2, bcl-xL, bcl-w are inhibitors while bax, bad, bcl-xS and bak are promoter of apoptosis. In normal healthy conditions the anti-apoptotic proteins are found in the outer membrane of mitochondria where they inhibit its permeabilization by sequestrating bax and bak and interacting with the permeability transition pore complex[43,44]. After pro-apoptotic stimuli, bax and bak undergo conformational modifications and fully enter in the outer membrane of mitochondria, thereby creating a transition pore[45]. It has been shown that after TBI the tumor suppressor gene p53 is highly upregulated[46] driving the downstream bax upregulation. The fate of mitochondria is determined by the balance between pro and anti-apoptotic signals. Once the death effectors become dominant, mitochondria will invariably direct the cell to apoptosis through caspase dependent or independent pathways.

Caspases are cysteine proteases that cleave aspatate residues of specific substrates and are the main player in the execution phase of apoptosis. They are constitutively expressed in the brain and are activated by intrinsic and extrinsic stimuli[36,47]. The *extrinsic pathway* is triggered by the activation of membrane death receptors such as (tumor necrosis factor (TNF) receptor, CD95/Fas and DR4/5 after the

binding with TNF, FasL and Apo21/TRAIL respectively. These signals lead to the formation of the death inducing signaling complex (DISC) that, together with the adaptor molecules FADD and TRADD, binds caspase-8 inducing its activation by auto-cleavage[48] that subsequently cleaves caspase-3. The intrinsic pathway is activated by different signals, like elevated intracellular Ca^{2+} levels, ROS, glutamate, DNA damage or mitochondrial release of cytocrome c[49]. These signals induce the activation of the “apoptosome” complex, which further contains the cytosolic protein Apaf-1 and pro-caspase-9. The apoptosome activates caspase-9 that finally lead to the activation of caspase-3[50]. Once the executioner caspase-3 is activated, the apoptotic process is irreversible: caspase-3 dismantle the cell by cleaving homeostatic, cytoskeletal, repair, metabolic and cell signaling proteins and induce DNA fragmentation by activating caspase-activated deoxyribonuclease (CAD) by cleaving the inhibitor protein ICAD.

Caspase-independent apoptosis is mainly driven by the mitochondrial flavoprotein AIF, that is required for the functioning of the respiratory chain. Under pro-apoptotic stimulation, AIF is released from intermembrane space and translocates to the nucleus promoting DNA degradation[51].

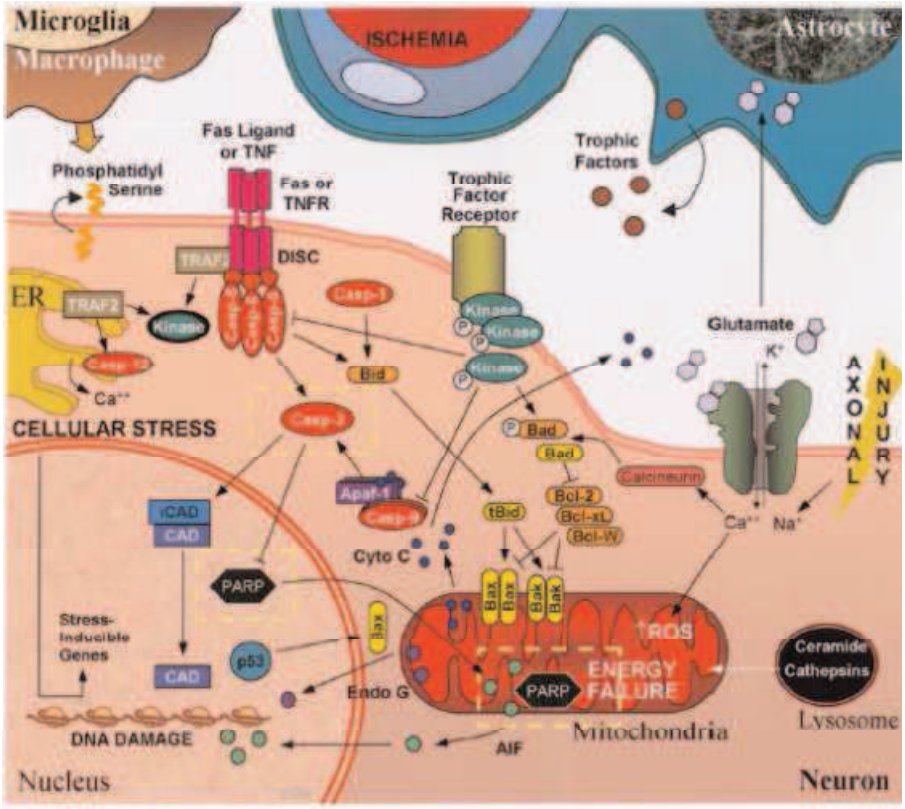

Figure 4 *– Schematic representation of the initiation and regulation of neuronal apoptosis after TBI. Figure from Zhang X et al.*[52]

1.1.5 Neuroinflammation after TBI

Once considered immunologically privileged, it is now known that the brain, like the other organs, is vulnerable to inflammatory response. A large body of data demonstrate that TBI elicits a marked local and systemic inflammatory response[15,53,54]. Both local and systemic events with specific temporal activation interact in a complex dynamism with redundant and overlapping cascades. The initiation and orchestration of inflammation in TBI is complex and multifactorial encompassing pro- and anti-inflammatory cytokines, chemokines, adhesion molecules, complement factors, reactive oxygen and nitrogen species, and other undefined factors. Neuroinflammation within the injured brain has long been considered to further harm the injured brain and contribute to the extension of tissue damage. However, the accumulated findings of experimental and clinical research support the notion that inflammation may differ in the acute and delayed phase after TBI and it is needed for injury resolution and that clear beneficial effects can be achieved if neuroinflammation is controlled, rather then switched off, in a time-regulated manner[55,56]. However the mechanisms underlying this beneficial/harmful dichotomy are mostly unknown.

Injury to the cerebral vasculature breaks the BBB enabling the recruitment of circulating neutrophils, macrophages and lymphocytes to the injured site, with different kinetics of infiltration/activation (figure 5)[15,57,58].

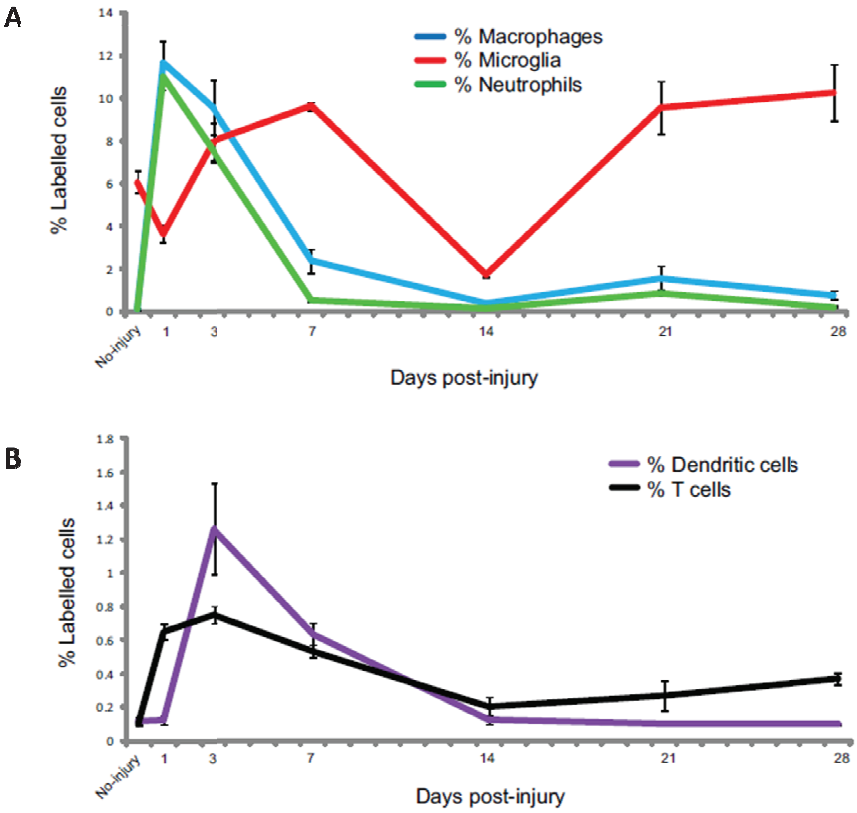

Figure 5. Temporal profile of immune cells in traumatized brain. *Cytometry quantification of: A) neutrophils ($CD45^+/Gr\text{-}1^+$ cells), machophages ($CD45^{high}/CD11b^+$ cells) and microglia ($CD45^{low}/CD11b^+$ cells) or B) T cells ($CD45^+/CD3^+$ cells) and dendritic cells ($CD45^+/Cd11c^+$) in the injured brain after CCI. Figure from Jin X et al.* [59]

Neutrophil recruitment has been shown to increase over the first 24 hours after experimental TBI[59,60] and it is dependent on both leukocyte CD11/CD18 [61] and endothelial intercellular adhesion molecule-1 (ICAM-1) [62]. Neutrophils trigger alteration of vascular permeability releasing a plethora of mediators such as ROS, proteases and pro-inflammatory cytokines, resulting in edema formation with consequent brain tissue loss[63].

Lymphocyte infiltration peaks at 24h after TBI[59,64]. Their role after acute brain injury is largely unknown: both detrimental[64,65] and beneficial[66] effects have been reported. However the early kinetic of infiltration and removal/disappearance of neutrophils and lymphocytes after TBI render these cell populations not the ideal target of therapeutic manipulations. Much more interest have received **microglia/macrophage** populations since they have both acute and prolonged activation after TBI and contribute to the evolution of brain damage with both neurotoxic and neuroprotective effects. Macrophages are the systemic myeloid component circulating in the blood that infiltrate into the brain parenchyma after acute brain injury. Microglia are the immune cells resident in the brain, constantly surveying perturbations in the cerebral microenvironment. Since both microglia and macrophages have a common myeloid origin, they share phenotypic markers that render activated microglia and infiltrated macrophages indistinguishable in the injured central nervous system (CNS)[67], thus they will be all referred as M. The main features of M activation after TBI will be described in the next paragraph.

1.1.5.1 Role of microglia/macrophages

Microglia are dynamic cells, with ramified morphology characterized by many processes that elongate and retract to survey the surrounding microenvironment[68]. Microglia express different pattern recognition receptors (PRRs) that detect small

molecular motifs present on pathogens (pathogen-associated molecular patterns, or PAMPs) or molecules released after damage (damage-associated molecular pattern molecules, or DAMPs). The recognition of exogenous pathogen-associated or endogenous danger-associated molecular patterns released by damaged neurons enable microglia to quickly react to hazardous signals[69]. Another important activator of microglia is represented by extracellular ATP massively released by the injured tissue activating the purinergic P2Y receptors on microglia[68]. After activation, microglia contract their processes assuming an ameboid-like shape typical of the active state, followed by proliferation and migration toward the site of injury[70]. Once there, microglial cells fuse around the lesion to form a barrier between the healthy and damaged tissue[68], suggesting that microglia may represent a primary defense after injury. Activated microglia together with blood-born macrophages recruited at the injured site, can secrete a variety of pro- and anti-inflammatory cytokines and chemokines, as well as neurotrophic factors[71]. There is growing evidence that M play ambivalent roles in inflammation[72–74]. In response to TBI, M can adopt diverse, complex activation states, enabling them to participate in the cytotoxic response, but also in immune regulation and injury resolution[72,75,76].

The activation states of M can be classified in two main phenotypes: classical activation, or M1, with pro-inflammatory and detrimental properties, and the alternative one, M2, with beneficial and protective functions. Among M2-activated M,

three subsets can be distinguished with different properties: M2a with pro-regenerative functions (mainly growth stimulation and tissue repair), M2b with immunoregulatory phenotype, and M2c involved in debris scavenging and healing functions[77,78]. After acute brain injury, both M1 and M2 activation states are present in the injured tissue[79,80]; however, the M2 phenotype vanishes very soon, favoring the balance towards the M1 phenotype which is responsible for the continued production of pro-inflammatory cytokines and exacerbation of injury[71,81].

Detrimental activation of microglia/macrophage

The M production of pro-inflammatory cytokines associated with neuronal cell death has long contributed to the view of detrimental effects of M activation after brain damage, thus strategies aimed at limiting M activation have long been pursued for TBI. A number of studies have shown protection after TBI and pharmacological inhibition of M activation. Minocycline, a derivative of antibiotic tetratcycline, administered intraperitoneally three times post TBI at 5 min (90mg/kg) and at 3 and 9h (45mg/kg) after injury attenuated microglial activation by 59% and reduced brain lesion volume by 58% decreasing TBI-induced locomotor hyperactivity and weight loss at 48h post-TBI and its effect lasted for up to 3 months[82]. Resveratrol treatment (a compound extracted from the skin of grapes, dose of 100 mg/kg at 5 minutes and 12 hours after mild TBI in mice) decreased M activation in cortex, corpus callosum and dentate gyrus of TBI mice, resulting in a

decreased expression of the pro-inflammatory cytokines IL-6 and IL-12 in hippocampus[83]. Propofol a generic anhestetic commonly used, administered to rats via the tail vein during TBI surgery (1.3 mg kg^{-1} min^{-1} after a bolus injection of 2 mg/kg), reduced M activation after TBI compared to isofluorane anesthetization. M inhibition was associated with decreased lesion volume with increased neuronal survival in the ipsilateral cortex and improved cognitive function at novel recognition test[84]. *In vitro* experiments revealed that propofol reduced microglia activation by limiting the activation of NADPH oxidase. M inhibition was obtained also using INO-1001, an inhibitor of the poly(ADP-ribose) polymerase-1 (PARP1) which regulates the activation of M. When given ip 20-24h after TBI at dose 10 mg/kg, INO-1001 reduced M activation in the perilesional cortex and ipsilateral hippocampus, increasing neuronal survival in the perilesional cortex and improving performance on test of forelimb dexterity (Sticky Tape and Vermicelli Tests) conducted 8 weeks after TBI[85].

M activation after TBI was reduced also by non pharmacologic modulation: caloric restriction (50% of daily food intake) lasting three months prior to TBI in adult rats reduced M activation associated with a decreased expression of TNFα and Caspase-3 in the ipsilateral cortex. These microglia effects resulted in an attenuated secondary injury on neuronal loss induced by TBI[86]. Finally 60 min of hyperbaric oxygen therapy (100% O_2 at 2.0 absolute atmospheres) used at 1h or 8h after

TBI in rats, reduced the TBI-induced microglial activation, TNFα expression and neuronal apoptosis at 72h post-TBI[87].

Altogether these data support the idea that M activation and recruitment after TBI is mainly deleterious to the brain and that a widespread M inhibition would protect cerebral tissue from deleterious secondary damage.

Beneficial activation of microglia/macrophage

Together with the detrimental effect after acute brain injury, increasing evidences support the idea that inflammatory mediators and immune cells can also have a neuroprotective effect and promote neurogenesis and lesion repair after CNS injuries[88,89]. Recruited macrophages and activated microglia have been proposed as beneficial through different mechanisms including glutamate uptake[90], removal of cell debris[91,92] and production of neurotrophins and anti-inflammatory cytokines[77,93,94]. It has been demonstrated that following transient occlusion of middle cerebral artery[95] and in the post-ischemic hippocampus[96] in rats, microglial expression of TGF-β1 mRNA does occur, identifying microglia as the major source of this anti-inflammatory and neuroprotective cytokines. In addition, post-ischemic proliferation of microglia represents an endogenous source of the neuroprotective factor IGF-1 and selective ablation of proliferating M after ischemic damage is associated with a increased production of pro-inflammatory cytokines, an increase in the size of ischemic lesion and in the

number of apoptotic cells, predominantly neurons[97], revealing a protective effect of microglia activation after acute brain injury.

Another important mechanism of protection induced by activated M is driven by their crosstalk with the glial scar[98]. After acute brain injury, activated microglia and infiltrating M1 macrophages produce pro-inflammatory cytokines and contribute to the formation of free radicals and ROS, leading to intense astrogliosis forming scar. M1 polarized macrophages has been shown to express higher levels of chondroitin sulfate proteoglycans (CSPGs, the main component of scar) compared to M2 polarized cells[99]. In the acute stage, glial scar is necessary to seal the lesion site and protects the spared neurons. Strategies aimed at inhibiting astroglial scar formation exacerbated the magnitude and duration of inflammatory activation, increasing tissue loss and aggravating neuronal functions[100,101], indicating that production of CSPGs is a purposeful mechanism associated with beneficial outcome. Conversely in the sub-acute/chronic stages glial scar affect neural stem/progenitor cell differentiation, axon outgrowth and oligodendrocyte maturation and remyelination[102]. M2 macrophages have been shown to be endowed with extracellular matrix remodeling properties, expressing the matrix metalloproteinase (MMP) 13 that can degrade CSPGs creating a permissive environment for axonal regeneration[103].

Altogether these data provide evidences that M may adopt different and evolving functions after acute brain injury and that a widespread M suppression would inhibit also their protective

and pro-regenerative effects. Thus strategies aimed at directing the M response toward a beneficial phenotype would prove effective benefit for TBI patients.

1.1.6 Neurorestorative processes

The aim of repair is to rewire or restore the damaged or missing parenchyma, yielding new functional tissue. Brain repair can be considered as the ability of the CNS to remodel itself in response to insults that impair tissue homeostasis. Beside damage, TBI induces endogenous neurorestorative events[104]. The brain retains neurogenic zones (namely subgranular zone (SGZ) in the hippocampus and subventricolar zone (SVZ) throughout the lateral walls of the lateral ventricles) with neural stem cells that can differentiate into functional neurons[105,106]. In experimental models of TBI, it has been observed an increased proliferative response in the hippocampal neurogenic zones beginning as early as two days post-injury[107], with a peak in the first week after injury and a return to baseline levels by 35 days[108]. The proliferation in the dentate gyrus is age-related, with greater cognitive recovery in juveniles[107]. TBI-induced neurogenesis has been observed also in SVZ area[109], and newly generated neuroblasts migrated toward the site of injury[110]. The mechanisms by which recruitment of neuroblasts to injury sites occurs in the adult brain is not know, as well as whether they can replace cortical neurons lost in TBI. However the highly pro-inflammatory microenvironment and the inhibitory milieu represented by

gliotic scar hamper the possibility of neuronal replacement[111], rendering neurogenesis highly insufficient to counteract tissue loss. Together with neurogenesis, axonal sprouting from surviving neurons may be associated with spontaneous motor recovery after TBI[112,113], but, again, it is highly affected by axonal growth-inhibitory environment[114].

Like neurogenesis/axonal sprouting, other endogenous restorative processes take place in the traumatized brain. Vasculogenesis after TBI has been shown to be induced as revealed by the increased production of angiogenic factor VEGF and its receptor VEGFR2, together with an high capillary density positive to BrdU, thus confirming the presence of newly formed vessels after injury[115].

All these data provide evidences that neurorestorative events are stimulated by endogenous growth factors, may continue for weeks and months and may contribute to functional and structural recovery. However, these spontaneous brain restorative processes are largely ineffective in counteracting the progression of damage and cumulative increases in injury lasts over time. Today clinical trials on TBI patients have primarily targeted neuroprotection, while trials directed specifically at neurorestoration have not been conducted. Providing the injured tissue with a facilitatory milieu that enhances neuroregeneration is an important additional therapeutic strategy that could benefit TBI patients.

1.2 Mesenchymal stromal cells for TBI

1.2.1 Biology of MSCs

The last 50 years in the field of stem cell biology have been stimulated by increasing interest in “mesenchymal stem/stromal cells” (MSCs)[116–118]. They were firstly described by Friedenstein and coworkers in the 1960s and 1970s as a minor population of cells in rodent bone marrow (BM) which adhered rapidly to plastic, with a fibroblast-like appearance, able to form clonal colonies *in vitro* and to differentiate towards the osteogenic lineage both *in vitro* and *in vivo*[119]. Today MSCs have been isolated from almost all human tissues[120]: adipose tissue[121–124], skeletal muscle[125], liver[126], synovial membrane[127], umbilical cord blood (CB)[128,129], periosteum[130], dental pulp[131], peripheral blood[132], placental tissue[133,134], amniotic fluid[135], tendon[136] and menstrual blood[137–139].

According to the *consensus* set out by the International Society for Cellular Therapy (ISTC)[140], the minimum criteria required to define human MSCs are that *in vitro* they must: 1) be plastic-adherent; 2) express the cell surface antigens CD105, CD73 and CD90; 3) not express the cell surface antigens CD45, CD34, CD14, CD11b, CD79α, CD19, or HLA-DR and 4) differentiate into osteoblasts, adipocytes and chondroblasts under standard *in vitro* differentiating conditions. In this widely adopted proposal, the authors also recommend designating these cells more appropriately as “mesenchymal stromal cells” given that not all of them are stem cells[140,141]. These criteria are applicable specifically to human MSCs and

cannot be entirely extended to cells isolated from other species, which may differ both in the marker expression and in some of their general characteristics and potential[142,143].

The use of MSCs for neurological disorders has been firstly driven by the MSC ability to trans-differentiate toward the neurogenic lineage[144,145] with the aim to reconstruct brain tissue. To date it is widely accepted that neuronal replacement is not the main mechanism of action of MSC therapy. In the following chapters different putative MSC mechanisms of protection and repair after acute brain injury will be reviewed.

1.2.2 Possible mechanisms of protection by MSCs in TBI

The aim of protective strategies is to limit injury progression and rescue threatened tissue. The heterogeneity of pathologic cascades of the secondary injury offer the potential for multitarget therapeutic intervention. MSCs might prove an ideal candidate[16] since they simultaneously affect multiple secondary injury mechanisms (figure 6).

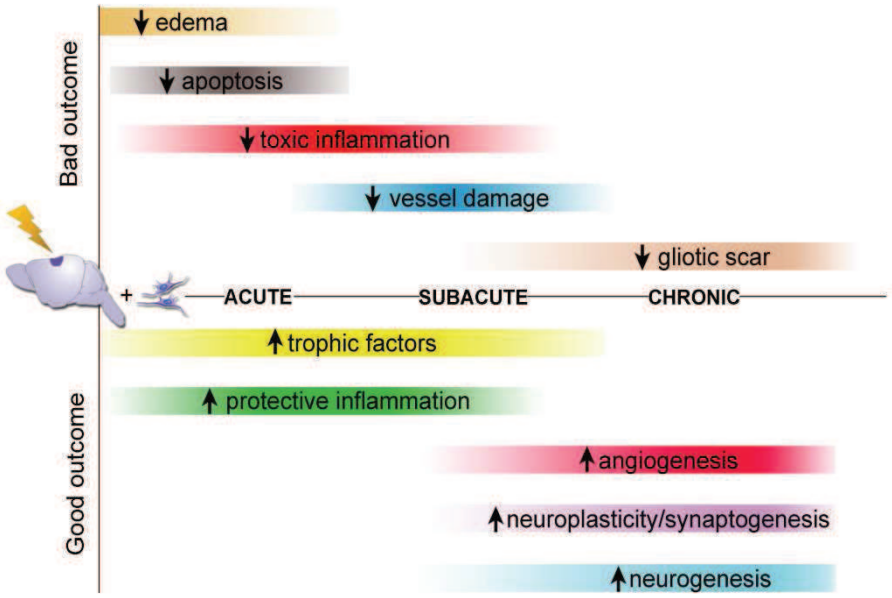

Figure 6.*Toxic and protective events affected by MSCs and their time course after TBI.*

1.2.2.1 Trophic factors

There is experimental evidence of beneficial effects in tissue repair and regeneration after transplantation of MSCs despite a rare/transient presence of transplanted cells in the host tissues. This prompted the proposal that the restorative outcome might be due to mechanisms other than *in vivo* differentiation of injected cells and replacement of host defective cells[116,118,146–149]. Thus a new mechanism was proposed in which the transplanted MSCs release bioactive trophic molecules that might reprogram the surrounding host environment through paracrine actions. In support of this, it has been shown that the conditioned medium generated from the

culture of MSCs (*i.e.* the medium containing the set of molecules secreted by these cells) injected in animal models of disease can recapitulate the beneficial effects of their cellular counterpart in tissue protection and repair[150–154].

Several MSC secreted pleiotropic molecules are involved in these effects, including cytokines/chemokines, growth factors, extracellular matrix proteins and tissue remodelling enzymes[155]. *In vitro* studies have described the secretome profile of MSCs isolated from BM, the best characterized source[156–158]. Among the secreted factors released *in vitro* under basal conditions, neurotrophins *i.e.*brain-derived neurotrophic factor (BDNF), glial cell-derived neurotrophic factor (GDNF), nerve growth factor (NGF) and neurotrophin-3 (NT-3) are of great interest for TBI[159,160]. *In vivo* studies have investigated the effect of MSCs on neurotrophin production after TBI, confirming that these neurotrophins increase after MSC treatment in the injured brain. The question remains, however, whether the infused cells are directly or indirectly responsible for the changes. Commonly used biochemical methods (western blot or ELISA) cannot distinguish between the human (in case of xenotransplant) and the rodent isoforms of neurotrophins, so when measuring these proteins after TBI and MSC treatment, it is not possible to distinguish the contribution of infused MSCs from that of host cells. Thus, increased production could be related to direct secretion by MSCs and/or to an indirect effect of MSCs on endogenous cell populations.

Kim and colleagues[161] injected two million-human MSCs intravenously 24 hours after TBI, modeled by controlled cortical impact (CCI) in rats. At 48 hours the homogenized injured hemisphere of TBI MSC rats showed increased amounts of NGF, BDNF and NT-3. NGF plays a key role in neuronal plasticity and prevents neuronal apoptosis[162]. BDNF is involved in neural development[163], neurogenesis[164,165] and in synaptic plasticity processes[166]. NT-3 supports the survival and differentiation of existing neurons and induces neurite outgrowth[167,168]. The survival cellular signaling of these neurotrophins is mediated by binding to the neurotrophic tyrosine kinase receptor (Trk). NGF preferentially binds TrkA, BNDF binds TrkB and NT-3 binds TrkC[169]. Autophosphorylation of the cytosolic domain induces the phosphorylation of Akt and ERK signal transducers which, in turn, through NF-κB, induces the transcription of genes responsible for cell survival[47,169]. The increased expression of neurotrophins in TBI MSCs treated animals found by Kim and colleagues was in fact related to increased phosphorylation of Akt (but not of ERK) at two days, together with reduced cleavage of caspase-3 (the main signal for cell apoptosis) at eight days. The molecular modifications induced by MSCs resulted in the functional recovery of motor function[161].

Our group and others have reported similar results on the increase in neurotrophin production with intravenous (iv)[170] or intracerebroventricular (icv)[171,172] transplantation of MSCs after TBI, showing increased production of BDNF[170,172] or NGF[170,171]

induced by infused/endogenous cells. We used MSCs from human CB, observing for the first time an effect on neurotrophin production similar to that described with BM MSCs[172].

In addition to the direct and indirect production of neurotrophins, a third mechanism for the increase in neurotrophins induced by MSCs was shown by Mahmood et al.[173]. Three millions human MSCs transplanted seven days after CCI, directly in the lesion cavity or impregnated in scaffolds, increased the production of tissue plasminogen activator (tPA) in the boundary zone 15 days after surgery. tPA regulates the final production of plasmin, a serine protease known for its part in the degradation of fibrin blood clots. In the brain, plasmin plays a pivotal role in the activation of BDNF and NGF, by cleaving pro-BDNF and pro-NGF into active forms[174,175]. Thus, focusing on the increase in neurotrophins, MSCs may: 1) directly produce and/or 2) induce other cells to produce and/or 3) produce, or induce the production of, proteases that can cleave neurotrophin precursors. In principle the paracrine action means that trophic factor alone can be applied, although up to now single-drug therapy has always failed in clinical trials. MSCs act like poly-pharmacy and improve the neurological outcome by creating a growth-promoting niche, this pleiotropic activity more than any single released factor possibly being the key to MSC therapeutic efficacy.

1.2.2.2 Microvesicles

MSCs may also exert their paracrine effects through the release of extracellular membrane vesicles that mediate the horizontal transfer of signals and molecules from one cell to another even over long distances. MSCs, like other many cell types[176], release circular membrane fragments known as *microvesicles*, either constitutively or in response to activation stimuli. These microvesicles, depending on their size and process of release, are generally classified as exosomes (multivesicular bodies of 30-120 nm and released from the endosomal compartment) or shedding vesicles (100-1000 nm, derived from direct budding of the cell plasma membrane). They are capable of transferring specific proteins, lipids and nucleic acids (mRNA, microRNA and DNA) thus serving as a unique mechanism for the intercellular trafficking of complex biological messages[177]. However, the complete biochemical composition of microvesicles remains to be determined and seems to vary depending on the cell source[176]. Recent studies have reported on the miRNA[178] and protein[179] composition of microvesicles from human BM MSCs, indicating that they include several molecules representative of the multiple properties of MSCs.

In spite of the fact that the physiological role and the biogenesis of MSC-derived microvesicles are still only partially understood, they appear to have a vital role in cell-to-cell communication[180]. In terms of the microvesicle-mediated therapeutic effects of MSCs, human MSC-derived microvesicles

injected in SCID mice with glycerol-induced acute kidney injury (AKI) had the same efficacy as their cellular counterparts on the functional and morphological recovery of AKI[181] and protected SCID mice from lethal cisplatin-induced AKI[182]. Exosomes released by human umbilical CB MSCs were reported to alleviate carbon tetrachloride (CCl_4)-induced fibrotic liver[183]. Lai and colleagues have documented the beneficial effects after the injection of MSC-derived microvesicles in animal models with cardiovascular diseases[184–187]. Zhu and collaborators, have reported that human BM MSC-derived microvesicles were therapeutically effective in C57BL/6 mice with *E. coli* endotoxin-induced acute lung injury[188]. Microvesicles have also been used to deliver selected factors and target specific therapeutic signals. For instance, Katsuda and collaborators administered adipose tissue-derived MSC exosomes carrying enzymatically active neprilysin, a trivial beta amyloid-degrading enzyme in Alzheimer rodents[189]. Exosomes were transferred into N2a cells overproducing Aβ and seemed to reduce both secreted and intracellular Aβ levels, suggesting therapeutic possibilities for adipose tissue-derived MSCexosomes in Alzheimer disease.

Alvarez-Erviti and collaborators succeeded in delivering functional siRNA to the mouse brain by systemically injecting targeted exosomes[190]. Xin and colleagues have reported that systemic administration of rat BM MSC-derived exosomes after acute brain injury in ischemic rats (induced by middle cerebral artery occlusion) significantly improved functional recovery

compared with control rats[191] and raised miR-133b in the ipsilateral hemisphere.

It was shown earlier that miR-133b is specifically expressed in midbrain dopaminergic neurons and regulates the production of tyrosine hydroxylase and the dopamine transporter[192]. The use of knock-in and knock-down technologies to up- or down-regulate miR-133b, demonstrated that exosomes from MSCs mediate the miR-133b transfer to astrocytes and neurons, which in turn regulates gene expression and is involved in neurite remodelling and functional recovery after stroke[193]. The bilipid membrane composition enriched with membrane-bound proteins suggests that microvesicles may become a therapeutic agent, homing the injured brain and treating TBI. To our knowledge the effects of microvesicles generated by MSCs after TBI have not been examined and detailed studies are needed to see whether MSC-derived microvesicles mimic the phenotype of their parent cells and provide a protective effect on TBI.

1.2.2.3 Immunomodulation

It has been widely demonstrated that MSCs can exert immunomodulatory properties: they are able to modify the function of different immune cells *in vitro*, including proliferation of T cells after stimulation by alloantigens or mitogens, as well as activation of T cells by CD3 and CD28 antibodies. MSCs inhibit the generation and function of monocyte-derived dendritic cells *in vitro* and may also modulate B-cell functions,

affecting the cytotoxic activity of natural killer cells by inhibiting proliferation and cytokine secretion (reviewed by Uccelli et al.[194]). Despite the general agreement on the MSC's effects on the modulation of the functions of these immune cells, little is known about the mechanisms involved. For example, inhibition of T-cell proliferation by MSCs appears to depend on cell-to-cell contact and on the release of soluble factors. MSC-derived molecules that may have immunomodulatory activity on T-cell responses include transforming growth factor-β (TGF-β), indoleamine 2,3-dioxygenase (IDO), prostaglandin E2 (PGE-2), interleukin (IL)-10, NO, miRNA (reviewed in Uccelli et al[194]), as well as exosomes(see above). However, this aspect of the MSC biology calls for further study. Characterization of the composition and functions of the MSC secretome will undoubtedly provide important information towards a better understanding.

In TBI pathology, the effects of MSCs on immune responses have been investigated by different groups. Zhang and colleagues[195] transplanted four million syngenic MSCs intravenously two hours after weight-drop injury in rats, reducing the infiltration of neutrophils (MPO^+) and $CD3^+$ lymphocytes and the infiltration/activation of macrophages/microglia (Iba^+) three days after injury. These anti-inflammatory effects were accompanied by a reduction of apoptotic cells in the pericontusional cortex and early improvement of sensorimotor function. Analysis of cytokine levels in the injured cortex showed decreases in the expression

of the pro-inflammatory mediators IL-1β, IL-6, IL-17, tumor necrosis factor-α (TNFα) and interferon (INF)-γ in the acute phase (12-72h) and increased expression of the anti-inflammatory cytokines IL-10 and TGF-β1 24-72h after TBI, indicating attenuation of the inflammatory response and a shift toward an anti-inflammatory microenvironment. There was also lower expression of the chemoattractant mediators monocyte chemotactic protein-1 (MCP-1, also known as CCL2, chemoattractant for monocytes, macrophages and microglia), macrophage inflammatory protein-2 (MIP-2, also known as CXCL2, secreted by monocytes/macrophages is chemotactic for polymorphonuclear leukocytes[196]) and RANTES (also known as CCL5, chemotactic for T cells, eosinophils and basophils and active in recruiting leukocytes into inflammatory sites), all indicating less recruitment of inflammatory cells from the circulatory system. The authors indicated the tumor necrosis factor-stimulated gene-6 (TSG-6) as one possible mediator of the attenuated innate immune response after MSC treatment. TSG-6 is an anti-inflammatory protein induced by TNFα and IL-1[197] and was upregulated in MSC-treated rats, together with a decrease in the expression of NF-kB, an important transcription factor that regulates many genes involved in the inflammatory response. Similarly, Watanabe and colleagues focused on TSG-6 and MSC-mediated protection[198]. CCI injured mice were injected with ten million MSCs or TSG-6 protein (50 μg/mouse) intravenously six hours after TBI. Both treatments induced a comparable inhibition of neutrophil infiltration and a decrease in

the expression of MMP-9 in the injured cortex at 24 hours, leading to less BBB leakage on day three. These data support the idea that MSC are able to attenuate the inflammatory response and one possible mediator is TSG-6.

Another important MSC-mediated immunomodulatory effect is the ability to modulate the activation state of microglia/macrophages (M, resident or infiltrated immune cells in the brain) toward a beneficial and protective phenotype after cerebral insults. MSCs have been used in different *in vitro* or *in vivo* models[199–202], with the aim to skew pro-inflammatory M1 polarization towards M with an M2 immunosuppressive and pro-regenerative profile. After TBI, Walker and colleagues[203] reported that iv injection of human BM-derived multipotent adult progenitor cells (MAPC, 10^7/kg, two injections 2 and 24 hours after CCI) raised the percentage of T regulatory cells in the periphery (spleen and blood) and the ratio of M2/M1 microglia in the brain. They reported that direct contact between MAPC and splenocytes is required for modulation of parenchymal microglia and attributed a central role to extraneurologic organs, by which transplanted cells act as “remote bioreactors” that boost systemic anti-inflammatory cytokine production, thereby affecting the resident microglia and the infiltrated macrophages in the peri-injury area[204].

These data indicate that immunomodulation is a real healing/protective mechanism of action of MSCs after TBI, involving modulation of both the local and systemic inflammatory responses. In the present thesis a deeper analysis

of MSC effect on M polarization has been further investigated. The results are shown in chapter 3.

1.2.2.4 Brain repair

As discussed in the paragraph 1.1.6, the brain possess a restorative potential after acute injury, however these mechanisms are largely insufficient to counteract the lesion progression. Providing the injured tissue with a facilitatory milieu that enhances neuroregeneration is an important additional therapeutic strategy for TBI patients. The roles of MSC in neurogenesis, synaptic plasticity and axonal sprouting and in angiogenesis/vascular repair are discussed under the heading below.

Neurogenesis

The beneficial effects on functional and structural damage after MSCs infusion in TBI models are not the result of replacement of injured or dead cells with exogenous cells. The first experimental study using MSCs for TBI was published in 2001 by Chopp and collaborators[205] and showed that MSCs infused intravenously 24 hours after TBI improved motor function in rats. Analysis of their fate showed that less than 0.005% of transplanted MSCs migrated to the injured cerebral area and only 5% expressed the neuronal marker NeuN and 7% the astrocytic marker glial fibrillary acidic protein (GFAP). The same group then worked to increase the engraftment rate in the traumatically injured area by intra-arterial (ia)[206] or

intracerebral (ic)[207] injection or by culturing MSCs with neurotrophic factors[206]. *In vivo* neuronal differentiation occurred in a negligible number of MSCs, with no relationship between route of administration and degree of efficacy. In the context of TBI no evidence has ever been provided that neurons newly generated from MSCs become functionally active. In some studies functional improvement was induced by MSCs in the absence of any neuronal differentiation[208,209] and there is ample evidence that MSC-treated animals show long lasting protection even when no transplanted cells are found in the brain injured tissue[161,171,172]. These findings, along with evidence that transplanted MSCs induce early (days-weeks) functional improvement suggest that the beneficial effects are due to stimulation of endogenous neuroreparative processes through a paracrine action, so neuron replacement is not the primary mechanism of MSC-induced protection.

The observation that MSCs may promote endogenous restorative processes through interaction with local neural cells is consistent with several studies showing the induction of local neurogenesis after MSC infusion in acute brain injured rodents. Mahmood et al. found an increased proliferation rate (5-bromo-2-deoxyuridine (BrdU) positive cells) in the svz and sgz of traumatized rats treated with MSCs[210]. This effect was regularly detected after iv, ic or icv MSCs[210].

Synaptic plasticity and axonal sprouting

Neuroplasticity is a major compensatory mechanism following acute brain injury. Through axonal sprouting, undamaged axons can reconnect neurons whose links were injured or disrupted or can establish new networks with undamaged neurons. Thus adjacent viable regions of the cortex might function vicariously after injury[211]. The ability of MSCs to foster synaptic processes after TBI was demonstrated by infusing a fluorescent dye (DiI) into the contralateral cortex five weeks after injury and measuring its transport from the injection site to the injured hemisphere through the corpus callosum (CC) one week later[212]. MSC-treated animals had a greater axonal fiber length that was directly correlated to performance in behavioral tests, indicating that neuroplastic processes are enhanced by MSCs, and can promote neuronal connectivity by directing axonal projections, neurite outgrowth and elongation in the injured cortex. Reduction of the growth inhibitory molecule Nogo-A is linked to MSC promotion of neuroplastic processes[213]. Nogo-A is a myelin-derived inhibitor of axonal outgrowth highly expressed in scar tissue after TBI[214]. Acute glial activation is needed to clear excessive glutamate release and remove dying cells and cellular debris after injury, limiting damage progression[71,215]. However, excessive glial scar in chronic stages inhibits remodeling[216,217]. Our group has demonstrated that CB derived-MSCs inhibit glial scar formation around the traumatized area five weeks after injury, with smaller lesion volume and better functional recovery[172]. Thus, inhibition

of gliotic scar and reduction of inhibitory molecules by MSCs render the damaged tissue more permissive to neuroregenerative and neuroplastic processes, fostering recovery and better outcome. Whether MSCs are directly involved in promoting plasticity of the injured neurons and proliferation of the endogenous cells in the svz or whether this is due to the interaction between MSCs and glial cells which, in turn, will be induced to secrete neurotrophins, still needs to be clarified.

Angiogenesis/vascular repair

The human brain is highly susceptible to ischemic damage. Global and regional cerebral ischemia/hypoperfusion have been observed in animal models of TBI and in brain-injured patients, after analysis of *post mortem* tissue[218–220]. Therapeutic strategies to restore blood flow and brain oxygenation are therefore particularly important for the vulnerable perilesional tissue that can be preserved from secondary damage and death if oxygen and glucose delivery is adequate.

Strategies aimed at restoring cerebral blood flow (CBF) after TBI may act on different mechanisms including vascular preservation from secondary damage and angiogenesis/vascular remodeling. MSC effects on CBF have been examined in live animals by magnetic resonance imaging (MRI)[221]. CBF was quantified by arterial spin-labeling done longitudinally on rats up to six weeks post TBI. TBI induced an early, persistent reduction of CBF (< 30 mL/100 g/min) in the

mechanically damaged and remote regions. MSCs restored and preserved CBF in the brain regions adjacent to and further from the lesion at chronic stages (three to six weeks), showing their ability to improve hemodynamics and moderate post-TBI hypoperfusion. *Post mortem* analysis showed MSCs boosted vascular density in the pericontusional area when administered in the acute phase (24 h)[222,223], or in the sub-acute phase (seven days)[224] after TBI and even at two months[225]. In principle a rescue effect on pre-existing vessels and/or the promotion of neoangiogenic processes may be responsible for the increase in vessel density, though the effects after delayed administration of MSCs clearly support a regenerative action on brain vasculature. Gene expression microarray analysis on MSCs *in vitro* detected the expression of genes involved in angiogenic processes that could potentially sustain both neurovascular repair at early stages and neovascularization later[226]. The vascular network improved when MSCs were transplanted ic[223,225] suggesting a paracrine effects of infused MSCs. However, neurovascular protection has also been seen after systemic infusion of MSCs[227], presumably due to a different mechanism. In this setting the tissue inhibitor of matrix metalloproteinase-3 (TIMP-3) has been identified as a critical factor. Iv injected MSCs are mainly trapped in the lungs where, interacting with pulmonary endothelial cells, they secrete TIMP-3 which significantly reduces BBB leakage. Thus, MSCs do sustain the brain vasculature and promote angiogenesis, acting

as local bioreactors, as well as interacting with extra-neurologic organs, providing a “remote” systemic control.

1.2.3 Challenges involved in the therapeutic use of MSCs

1.2.3.1 MSC heterogeneity

The protection observed in experimental models varies widely in different studies. Next to conceptual issues and methodological differences between injury models and laboratories, heterogeneity of MSC populations may contribute to disparate outcomes. This heterogeneity is at various levels. The first is *donor-to-donor heterogeneity*. Every MSC donor intrinsically differs genetically, physiologically, etc., and this may for example affect the patterns of MSC gene expression, differentiation capacity and secretion of bioactive molecules. Donor age also clearly contributes to differences in BM-derived MSC, though heterogeneity has also been observed in MSCs isolated from age- and sex-matched donors[228,229]. Another level of heterogeneity is between MSC populations isolated from different human tissues. MSCs from different sources cannot be considered entirely equivalent in terms of their immunophenotype, secretory and proteomic profile, differentiation potential and immunomodulatory ability[228]. One explanation may relate to the *in vivo* origin of MSCs from different tissues, which can influence the commitment, phenotype and functions of the cells differently. However, there are only a few studies directly comparing gene and protein expression and potential of cells isolated from different sources

and comparison is further complicated because different laboratories use non-standardized isolation and cultivation methods.

A further level of heterogeneity is *intra-population heterogeneity*. MSCs isolated from a specific source still tend to be heterogeneous populations which, when cultured, may contain both undifferentiated stem/progenitor cells and more mature cell types, with different functional abilities[148,230,231].In the case of BM MSCs this heterogeneity has been detected using different experimental approaches, including transcriptome or immuno-staining analysis as well as assays aimed at investigating differentiation abilities[228]. The question of MSC heterogeneity is even more complex since culture and expansion conditions can introduce experimental artefacts, modifying the expression of natively expressed markers and promoting the expression of new ones, altering the original cellular phenotype and functions. Additional work is urgently needed to identify the specific properties of each MSC sub-population and to understand the determinants of intrinsic MSC heterogeneity. This is vital in order to reduce experimental and clinical variability, predict MSC *in vivo* potency and develop successful MSC-based treatment transferable to the clinic.

1.2.3.2 Autologous or allogenic transplant and immunosuppression

The choice of rodent or human MSC sources in the experimental regenerative field is still in its early years. On the

one hand, rodent MSCs allow syngenic or allotransplants which are the conditions faced in the clinical setting (matched human MSCs transplanted in patients). On the other hand, human MSCs candidates for clinical use need to be tested in rodent models to assess their efficacy, long-term effects, and safety and to obtain regulatory approvals. The minimum criteria required to define MSCs[140] are applicable specifically to humans and cannot be entirely extended to cells isolated from other species. Rodent MSCs differ from human MSCs in marker expression and in some of their general characteristics and potency[142,143]. If rodent MSCs are fundamental as proof of concept studies, human MSCs offer a higher predictive value but their efficacy and mechanisms of action need to be fully addressed in the experimental setting in order to move to clinical trials.

Autologous stem cells have been the treatment of choice in TBI trials so far. Harvesting patient-specific MSCs poses timing, logistic and standardization constraints. To interact promptly with pathological pathways of secondary damage and to foster restorative processes, MSCs have to be transplanted in the acute phase. This limits the possibility of autologous transplantation. The transplantation of bank-stored good manufacturing practices (GMP)-grade certified MSCs may overcome some of the logistic limitations associated with autologous MSCs in the organ transplant setting and allow institutions without GMP facilities or the capacity to isolate MSCs to participate actively in this field of research. While the

autologous MSC product can introduce differences in cell potency related to the patient's age and disease[232], allogenic cell transplant can be easily standardized and therefore provide more comparable results among different trials[233]. Allotransplant, however, poses the risk of host rejection due to immunological mismatch. Thus, immunosuppression becomes a critical question before cell therapy can move to clinical application.

Data from acute brain injury models, including TBI[161,172,221], stroke[234–236] and spinal cord injury[237–239], show the efficacy of allo- and xeno-transplanted MSCs with different immunosuppression strategies[240]. MSCs do not appear to retain intrinsic immunogenic properties, do not trigger alloreactivity and can survive and differentiate into allogenic or even xenogenic immunocompetent recipients *in vivo*[241]. Thus, MSCs have been proposed as "universal donor cells". However, emerging reports have challenged the limited immunogenicity of allogenic MSCs[242–244] and there are also conflicting findings regarding immunogenicity of differentiated BM-derived MSCs[244]. MSCs are rejected after xenotransplantation into the ischemic rodent myocardium and immunosuppression is needed to improve their efficacy and survival in the ischemic heart[245,246]. Transplantation of MSCs into the non-injured adult rodent brain can induce an inflammatory response leading to rapid and complete rejection of the transplanted cells, preventing plastic effects[247,248]. Consequently, the immunological impunity of MSCs *in vivo* is not fully supported.

Immunosuppression in TBI patients clearly has dangerous implications because it increases susceptibility to infection which is directly related to unfavorable outcomes[249,250]. Thus allogenic MSC therapy should be necessarily performed in immunocompetent patients. Today no direct comparison of MSC efficacy in immunocompetent and immunosuppressed hosts has been performed. This issue has been analyzed by our group and results are shown in chapter 2.

1.2.3.3 Cell administration route

Various routes of administration of MSCs have been employed in experimental models of TBI and protection has been reported after iv, ia, ic or icv infusion. Thus different routes can be taken into consideration after TBI. Below we discuss some of the advantages and limitations of each route. We also suggest that the biology of TBI and its heterogeneity could be important factors in deciding the route of choice.

Systemic MSC administration

Intravenous delivery

A number of preclinical studies[195,208,251] used iv infusion for cell delivery for TBI. The iv route has two important advantages: 1) it is minimally invasive and, compared to direct transplantation into the central nervous system, overcomes the risks of bleeding and tissue injury; 2) it can be done quickly thus allowing timely treatment. However, some limitations also need to be considered. An initial obstacle to iv delivery is the large

proportion of first-pass pulmonary sequestration. Many studies in animal models have shown that iv infusion of MSCs does not yield a large number of cells reaching the organ of interest, because the majority are trapped in the lungs[252,253]. Pulmonary sequestration is primarily related to the MSC size. Schrepfer and colleagues showed that the mean size of suspended mouse MSCs (15-19 μm) is bigger than the pulmonary capillaries so most of the iv-injected MSCs are trapped in the capillaries, preventing access to the intended organs[252]. Besides cell size, the expression of adhesion molecule by MSCs is another important factor in pulmonary sequestration. Pre-clinical studies have shown that MSCs interact with endothelial cells, engaging P-selectin and vascular cell adhesion molecule-1 (VCAM-1)[254]. The inactivation of VCAM-1 counter ligand (VLA-4/CD49d) on the MSC surface blocks the MSC-endothelial interaction resulting in a significant increase of MSCs in the arterial system[253].

Syngenic stem cell passage across the pulmonary circulation was investigated in anesthetized Sprague-Dawley rats, using silicone tubing catheters placed in the left internal jugular vein and common carotid artery to measure pulmonary passage of MSCs, MAPCs, neural stem cells (NSCs) and BM derived mononuclear cells (BMMCs) (average diameters respectively 18, 15, 16 and 7 μm) co-labeled with specific nanocrystals and infused iv. The labelled cells in the arterial circulation and in peripheral filter organs (lungs, spleen and kidney) were quantified by flow cytometry and infrared imaging,

respectively. MSC pulmonary sequestration was 30 times that of BMMCs[253]. Two independent studies found that after TBI, only 0.001% of iv injected cells was found in the brain or organ systems other than lung parenchyma two-three days after injection[251,255]. These studies strongly suggest that the efficacy after MSC iv infusion is very likely unrelated to the MSCs reaching the injured tissue but that infused MSCs act as remote "bioreactors" stimulating resident cells in lung (macrophages) and spleen (t-cells) to acquire an anti-inflammatory phenotype[204,251,255] thereby promoting resolution of the brain injury.

Intra-arterial delivery

The rationale behind ia administration is to bypass the pulmonary first pass effect, increasing delivery of infused cells to the target tissue. However, microvascular occlusions have been documented and CBF impairment (80-90% reduction in laser Doppler flow signal) have been shown to occur in 35% of treated animals[256]. More encouraging results were obtained by Lundberg and collaborators[257] who reported no thromboembolic complications after ia delivery. Human MSC presence in the brain was higher compared to iv administration, but the contusion model they used was not associated with any gross neurological symptoms, thus preventing the assessment of stem cell transplantation efficacy.

Additional studies are needed to establish if ia transplantation of MSCs gives more favorable effects than iv

injection. Furthermore, while this approach may be particularly interesting in ischemic stroke when an endovascular procedure may already be planned, in TBI patients who have intracranial pressure and perfusion pressure problems, all the complications associated with carotid puncture/manipulation must be carefully evaluated before considering this strategy as promising.

Focal MSC administration

Intracerebroventricular delivery

Icv cannulation in human TBI is invasive and may have significant complications; however, it is recommended by authoritative guidelines for intracranial pressure monitoring of severe TBI in the intensive care unit[258,259]. In these patients, this site would therefore be free from additional surgical complications and offer the advantage of focal administration directly in the region of interest.

In mice icv administration of human umbilical CB or BM MSC induced lasting improvement in sensorimotor and cognitive functions and reduced contusion volume one month after TBI[172,260]. Our data provide evidence that MSCs can also act as a local “bioreactor” in the brain. In our model icv injected MSCs are detected in the ventricles and at the lesion site for up to five weeks in TBI mice, but are confined to the ventricles in sham-operated mice[172] supporting a local action of MSCs on host tissue. However, further studies are necessary to see whether if direct contact between MSCs and the injured cells *in*

vivo is needed for protection, as previous evidence suggests that MSCs may act through the release of active molecules rather than through cell-to-cell contact[172,204].

Intracerebral delivery

The rationale for ic stem cell implantation is to maximize the MSCs load at the site of injury. However, increasing evidence that MSC engraftment is not required for therapeutic efficacy challenges this approach. Furthermore, BM MSC differentiation could trigger immune rejection[261,262] probably due to a switch in surface-MHC molecule composition during MSC differentiation, as described in the heart[262]. The invasiveness of the ic approach and the possibility of further tissue damage during cell transplantation make this strategy not a choice in the treatment of TBI at the present time.

The decision on the administration route is therefore fundamental in the definition of a clinical protocol. Issues to be considered are the type of injury, the biodistribution of injected cells and the cell type. Iv application offers easy access and the potential for broad distribution, but has the disadvantage of a large pulmonary first-pass effect, thus significantly reducing the cells delivered to the arterial circulation. Ia delivery can target the injured tissue better, but it can cause emboli, impairing blood flow and worsening the clinical outcome. Icv delivery may be the choice in a selected group of severe TBI patients.

1.2.3.4 Treatment timing and doses

The optimal timing of therapeutic MSC administration after TBI is a point of open discussion. There is ample evidence of the reciprocal interaction between infused MSC and endogenous cell population. After TBI, the severity and kinetics of the TBI-related metabolic cellular and molecular cascades are the determinants of the injured microenvironment that, with time, may be more or less permissive to MSC functions. At present, technical aspects related to patient stabilization and identification of the patient-matched allogenic MSCs set the lower limit of the window of treatment at approximately 12-24 hours post-TBI. No robust data are available to define the upper limit. However, considering the rapid evolution of secondary damage involving pericontusional cerebral tissue that could be rescued, it seems reasonable to treat the patient as soon as possible.

Preclinical studies in rodents report a wide range of TBI-to-transplantation intervals, with a preference in the acute phase (within 24 h). Both systemic[161,170,195,198,205,227,251,263–265] and central[171,172,207,209,210,266–268] administration of MSCs within the first 24 h after injury have resulted in improvements of functional and structural outcome in the chronic stages by multiple mechanisms of protection and repair.

Sub-acute transplantation (day 4-7 after injury) has shown protective effects too after systemic[221,269] or central infusion[173,212,226]. Acute and sub-acute transplantation have been recently compared directly [270] by transplanting MSCs one

or seven days after injury in the CC ipsilateral to the injured site. The results show that injection on day seven produces greater functional and structural improvements one month post-TBI than the injection on day one. Confirmatory study by independent research groups is needed to explore the therapeutic window further.

Repeated administration could be a strategy to obtain a "booster effect", combining acute and sub-acute doses through a multiple delivery system. Acute MSC infusion will allow the interaction with early pathologic pathways and sub-acute administration will replace any MSCs may have been damaged or eliminated, allowing the stimulation of protective and restorative processes. No data are available in TBI so far and this possibility needs to be explored in order to confirm a potential advantage and exclude any secondary side effects.

Finally, treatment in the chronic phases of TBI were investigated by Bonilla and co-workers. They showed that MSC ic infusion into the lesion core two months after injury improved sensorimotor deficits and promoted neurorestorative processes (increase of vessel density and endogenous neurogenesis)[225]; iv injection at the same time failed to induce any significant difference between MSC-treated and control animals[271]. These data suggest that at chronic stages MSCs can still act as a local bioreactor promoting endogenous brain restorative processes. A pivotal mechanism of iv MSC infusion is the modulation of the focal and systemic inflammatory environment, which is no longer affected by later treatment.

To conclude, no systematic analysis of transplantation timing and its effect on TBI sequelae has been done yet. More experimental data are needed to clarify the ideal time windows for MSC transplantation in order to identify the best approach in the clinical setting.

Like for the timing of transplantation, there is no a real consensus on the ideal MSC dose. Depending on the route of delivery and the animal model used, most experimental studies use the largest dose that does not affect the animal's health (e.g. obstructing vessels with cellular emboli in case of iv injection) or cell viability (e.g. inducing cell mortality due to constriction in the syringe needle).

Iv injections in rat models go from two[161,170,205,263], three[221] or four[195,251,265] million-MSCs, while in mouse models the range is between 300,000[224,269] and one[198] or two million[227] MSCs.

Smaller amounts are commonly used for cerebral infusion: the icv dose to mice never exceeds 150,000-200,000 MSCs [171,172], while MSCs transplanted directly in the lesion cavity in rats range from 3-400,000[209,272] to one[207,266–268] or five millions[225]. MSCs impregnated into scaffolds transplanted into the lesion core of rats have been seeded at dose of 10,000 cells[273], 64,000[274] or three millions[173,212,226,272,275,276] while the only dose tested in mice was 300,000 MSCs[224].

A vast range of MSC doses have been investigated up to now, but few studies have tried to identify a dose-response effect. Mahmood and co-workers directly compared different doses: one or two million MSCs injected iv in rats 24 hours after

injury had different effects on sensorimotor function, with significant improvement only after the highest dose[264]. However, they found no dose-dependent effect when infusing two, four or eight million MSCs in the same experimental setting[208].

To conclude, there is no agreement on dose-dependent effects of MSC infusion and more studies are needed to clarify the issue. However, translation of results from the experimental setting to the clinical context is rarely possible.

1.2.4 Clinical trials of MSC for TBI patients

ClinicalTrials.gov (http://clinicaltrials.gov/) is the world's largest registry and result database of publicly and privately supported clinical trials (CTs). It currently lists 160,935 trials in 185 countries. This dataset is maintained by the US National Library of Medicine at the National Institutes of Health.Reviewing the clinical trials in ClinicalTrials.gov, we found 4598 trials in response to the search criteria 'stem cell* OR stromal cell*'. The strategy used to refine the search and to identify only CTs focused on the use of MSCs in acute brain injury is detailed in figure 7A. Among the 4598 CTs, 81 involved acute neurological conditions and 23 used MSCs (figure 7). The majority of the CTs were conducted on stroke (12 CTs; 52%) and in spinal cord-injured patients (10 CTs, 43%); one (4%) was on intraparenchymal hemorrhage and none in TBI patients.

To date CTs with adult stem/stromal cells in TBI have all focused on acute interventions with an autologous source, thus

excluding the use of MSCs that need time and manipulation in order to be selected and expanded (figure 7B). There are four CTs with BM-derived mononuclear cells (BMMCs), a heterogeneous population that includes MSCs together with hematopoietic stem cells, lymphoid cells (lymphocytes, plasma cells), monocytes and macrophages. The main features of these trials are summarized in Table 1 and will be discussed briefly since we believe they are of interest even if slightly out of focus. One CT (NCT00254722) has been completed while the other three (NCT01575470, NCT01851083 and NCT02028104) are currently recruiting participants. Three of them are led by the same group (The University of Texas Health Science Center, Houston, USA).

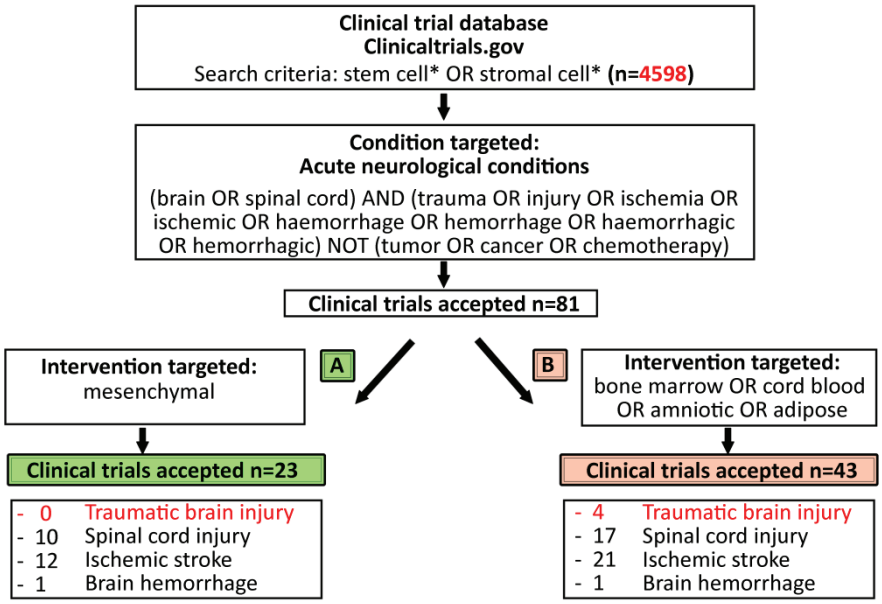

Figure 7. *Clinical trials (CTs) registered in ClinicalTrials.gov and focused on stem/stromal cells for acute neurological conditions on 12 February 2014. CTs were first selected by the search criteria 'stem cell* OR stromal cell*'. Then, in the "condition" field acute neurological injuries (e.g. TBI, spinal cord injury, stroke and brain hemorrhage) were targeted. CTs not involving neurological conditions/diseases and acute mechanism of disease were rejected. Last, adding "mesenchymal" in intervention field (A) resulted in 23 trials, but none were focused on TBI. Appling the wider intervention criteria 'bone marrow OR amniotic OR cord blood OR adipose' in the field 'interventions' (B) four TBI CTs were identified.*

Registration Number	Trial Name	Purpose	Phase	Start date	Status	Age (Years)	Regimen	Sponsor
NCT00254722	Safety of Autologous Stem Cell Treatment for Traumatic Brain Injury in Children	To determine if bone marrow derived mononuclear cells (BMMNC) autologous transplantation in children after isolated traumatic brain injury is safe and will improve functional outcome	1	November 2005	Completed	5-14	$6x10^6$ autologous BMMNC/kg body weight delivered intravenously within 48 hours after TBI	University of Texas Health Science Center, Houston
NCT01575470	Treatment of Severe Adult Traumatic Brain Injury Using Bone Marrow Mononuclear Cells	To determine if bone marrow harvest, BMMNC separation, and reinfusion in adults with acute severe TBI is safe and will improve functional outcome	1/2	March 2012	Recruiting	18-55	Intravenously administration. *Cohort 1:* $6x10^6$ BMMNC/kilogram body weight. *Cohort 2:* $9x10^6$ BMMNC/kilogram body weight. *Cohort 3:* $12x10^6$ BMMNC/kilogram body weight.	University of Texas Health Science Center, Houston
NCT01851083	Pediatric Autologous Bone Marrow Mononuclear Cells for Severe Traumatic Brain Injury	To determine the effect of intravenous infusion of autologous BMMNCs on brain structure and neurocognitive/functional outcomes after severe TBI in children	1/2	August 2013	Recruiting	5-15	Bone marrow harvest performed within 36 hours of injury, followed by a single intravenous infusion of autologous BMMNC ($6x10^6$ autologous BMMNCs/kg bodyweight)	University of Texas Health Science Center, Houston
NCT02028104	Stem Cell Therapy in Traumatic Brain Injury	To determine the effect of stem cell therapy on common symptoms in patients with chronic TBI	1	March 2010	Recruiting	6 months-65 years	BMMNC are administered intrathecally in traumatic brain injury patients. Data about dose are not available in ClinicalTrials.gov.	Neurogen Brain and Spine Institute, Mumbay, India

Table 1. Published and ongoing clinical trials applying stem/stromal cells in TBI.

The **NCT00254722** phase one non-randomized trial was carried out in Houston (Texas, USA) from April 2006 to November 2009. It was designed to evaluate the logistics, feasibility and safety of BMMC autologous transplantation in children after TBI. The secondary objective was to investigate whether the late functional outcome was improved by BMMCs compared to age and severity matched concomitant controls, in order to estimate the potential treatment effect size for future trial planning. Ten children (aged five to 14 years) with acute TBI (within 24 h) and a post-resuscitation GCS of 5-8 were recruited. Patients were treated with 6 million autologous BMMCs/kg body weight delivered intravenously within 48 h after TBI. The safety of the procedure was evaluated by monitoring systemic and cerebral hemodynamics during BM harvest; infusion-related toxicity was determined by pediatric logistic organ dysfunction scores, hepatic enzymes, Murray lung injury scores and renal function. One and six months post-injury, conventional MRI, neuropsychological and functional outcome measures were obtained. Infusion of BMMCs to acutely treat severe TBI in children appeared to be safe. There were no episodes of harvest-related depression of systemic or cerebral hemodynamics and no detectable infusion-related toxicity. MRI one and six months post-injury showed no significant decrease in grey matter, white matter or intracranial volume; there was no significant rise in cerebrospinal fluid volume during the study. Assessment of functional and neuropsychological outcome one and six months post-TBI

showed improvements in all scores examined. The dichotomized Glasgow outcome scale at six months showed 70% good to 30% bad outcome or death, which is similar to other major reports in pediatric severe TBI[277]. However, although structural preservation and improved functional outcomes were observed, the study was underpowered and not designed to assess therapeutic efficacy.

The safety of the protocol led to the controlled prospective, randomized, blinded phase II ongoing trial (August 2013 - June 2018) **NCT01851083**, designed to determine the effect of iv infusion of autologous BMMCs on brain structure and neurocognitive/functional outcomes after severe TBI in children (aged 5 to 15 years, post-resuscitation GCS 3-8, recruitment within 24 h of injury). BM was harvested within 36 h of injury, followed by a single infusion of BMMCs.

The **NCT01575470** (March 2012 - June 2014) is a phase I/II trial, designed as a dose-escalation study, consisting of four cohorts of five adult TBI patients cohorts (age 18 to 55 years, admission GCS 5-8). The investigator's primary hypothesis is that autologous BMMCs transplantation after TBI is safe (harvest- and infusion-related toxicity). The secondary hypothesis is that: 1) functional outcome measures will improve after BMMC infusion, 2) BMMC infusion will reduce BBB permeability, 3) BMMCs are neuroprotective and preserve grey and white matter structures assessed by diffusion tensor MRI.

The trial **NCT02028104** (March 2010 - January 2015) is a phase I study lead by Neurogen Brain and Spine Institute

(Mumbay, India). The purpose is to evaluate the effect of stem cell therapy on common symptoms in patients with TBI. BMMCs are administered intrathecally in six months to 65-year-old patients. Unlike the other CTs mentioned so far patients with chronic TBI are enrolled in this trial.

Considering the overall analysis of CTs focused on TBI, some observations can be made. First, most of them are safety trials and are not powered to detect functional measures of efficacy. However, valid estimates can be made from these findings to allow controlled phase II trials. Second, none of them uses pure MSCs but rely on an autologous source, using the more heterogeneous population of BMMCs. Finally, the patients are mainly children, who have greater neurologic plasticity with a unique injury pattern compared to adults.

1.3 Scope of the thesis

The multiple pathological cascades activated after TBI and their extended nature offer the possibility for therapeutic interventions possibly affecting multiple injury mechanisms simultaneously. MSC therapy matches this need, being a bioreactor of a variety of molecules able to interact and modify the injured brain microenvironment.

Compared to autologous MSCs, bank stored GMP-graded allogenic MSCs appear to be a realistic choice for TBI in a translational perspective, due to the need of delivering cell therapy in the acute phase of the pathology and promptly interact with the damaged tissue and maximize neuroprotective and restorative processes. Allogenic transplant poses the risk of host rejection due to immunological mismatch and introduces the critical issue of immunosuppression. In TBI patients immunosuppression is associated with an increased susceptibility to infections which is directly related to unfavorable outcomes and thus deserves careful consideration. Today no direct comparison of MSC efficacy in immunocompetent and immunosuppressed hosts has been performed. In this thesis we analyzed whether long-term efficacy of human bone marrow MSCs in traumatized mice brain is dependent or not on immunosuppressive treatment (Chapter 2), in order to address this important preclinical issue. By observing a similar degree of protection in immunocompetent compared to immunosuppressed mice our data represent a forward step towards the definition of a clinical

protocol and provide a strong rational to further investigate the potential of human BM MSC in TBI.

In the second part of the thesis, using the same MSC source and the same injury/treatment protocol we focused on mechanistic insight of MSC effect after injury and analyzed the interaction between infused MSCs and resident/recruited immune cerebral cells. In particular we investigated the effects of MSC treatment on the activation and functional changes of microglia/macrophages after TBI and how these phenotypical changes are related to microenvironmental beneficial effects (Chapter 3).

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Chapter 2

Immunosuppression does not affect human bone marrow mesenchymal stromal cell efficacy after transplantation in traumatized mice brain

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2.1 Abstract

The need for immunosuppression after allo/xenogenic mesenchymal stromal cell (MSC) transplantation is debated. This study compared the long-term effects of human (h) bone marrow MSC transplant in immunocompetent or immunosuppressed traumatic brain injured (TBI) mice.

C57Bl/6 male mice were subjected to TBI or sham surgery followed 24 h later by an intracerebroventricular infusion of phosphate buffer saline (PBS, control) or hMSCs (150,000/5µl). Immunocompetent and cyclosporin-A (CsA) immunosuppressed mice were analyzed for gene expression at 72h, functional deficits and histological analysis at five weeks.

Gene expression analysis showed the effectiveness of immunosuppression (INFγ reduction in CsA treated groups), with no evidence of early rejection (no changes of MHCII and CD86 in all TBI groups) and selective induction of Treg (increase of Foxp3) only in the TBI hMSC group. Five weeks after TBI, hMSCs had comparable efficacy, with functional recovery (on both sensorimotor and cognitive deficits) and structural protection (contusion volume, vessel rescue effect, gliotic scar reduction, induction of neurogenesis) in immunosuppressed and immunocompetent mice.

Therefore, long-term hMSC efficacy in TBI is not dependent on immunosuppressive treatment. These findings could have important clinical implication since immunosuppression in acute TBI patients may increase their risk of infection and not be tolerated.

2.2 Introduction

The adult brain, once considered immunologically privileged, is subject to considerable immune surveillance[1]. The dynamic interaction between resident and recruited immune/inflammatory cell populations and the injured tissue enables the brain to respond to transplanted stem cells. As a consequence, the majority of cell types transplanted to the injured brain suffer poor survival[2,3]. In the experimental setting, high doses of immunosuppressant are needed, to improve efficacy and graft survival after allogenic- or xeno-transplantation[4–7]. Immunosuppression has potential toxic side effects for the acute brain injured patient [8]. Therefore, a primary goal for translational research would be to assess the patient's need for immunosuppression.

Recent findings suggest that mesenchymal stromal cells (MSCs) are a good source for transplantation strategies in acute brain injury[9–11]. Data from acute brain injury models, including traumatic brain injury (TBI)[12–14], stroke[15–17] and spinal cord injury[18–20], show the efficacy of allo- and xeno-transplanted MSCs with different paradigms of immunosuppression[21]. The promising preclinical data of MSCs transplantation in rodent TBI models has led to the launch of a clinical TBI trial with human autologous bone marrow derived stem cells (www.clinicaltrials.gov: NCT00254722). However, harvesting patient-specific tissue poses logistic, timing and economic constraints and can introduce differences in cell potency related to the patient's age and disease, possibly limiting their

therapeutic potential[22]. There would be clear advantages if allogenic donor MSCs could be used for transplantation without the need for immunosuppression. MSCs do not appear to retain intrinsic immunogenic properties, do not trigger alloreactivity, suppress proliferation of T-cells *in vitro*[23] and can survive and differentiate into allogenic or even xenogenic immunocompetent recipient *in vivo*[24]. Thus, MSCs have been proposed as "universal donor cells". However, this has been challenged. First, although they may retain their immunosuppressive properties *in vitro*, allogenic murine MSCs could be immunogenic in immunocompetent animals[25,26]. Second, MSC are rejected after xenotransplantation into the ischemic rodent myocardium and immunosuppression is needed to improve their efficacy and survival in the ischemic heart[27,28]. Third, transplantation of MSCs into the non-injured adult rodent brain can induce an inflammatory response leading to rapid and complete rejection of the transplanted cells, preventing plastic effects[2,29]. Consequently, the immunological impunity of MSCs *in vivo* is not fully supported, and a dedicated study is needed to assess whether long-term efficacy of MSCs in traumatized mice brain is dependent or not on immunosuppression.

To answer these questions we intracerebroventricularly (icv) transplanted hMSCs isolated form bone marrow in immunosuppressed and immunocompetent traumatic brain injured mice. The study was designed to determine whether immunosuppression with cyclosporine A (CsA) affects the

efficacy of hMSCs transplanted into the traumatically injured mouse brain.

2.3 Materials and Methods

2.3.1 Isolation and culture of hMSC

The local institutional review board approved the study and informed consent was obtained from healthy donors. hMSCs were isolated from bone marrow of healthy donors and expanded *ex vivo* as previously described[30]. Our good manufacturing practices (GMP) facility (Laboratory "Stefano Verri", San Gerardo Hospital, Monza, Italy) produces GMP-graded MSC using Platelet Lysate (PL) 5% as expanding medium. PL is currently available in most transfusion centers and it is produced accordingly to standardized clinical grade procedures in a closed system. In our operating procedure each PL was obtained from a single allogenic platelet unit stored at -40°C. The standard platelet unit had a mean volume of 50±5 ml with a mean platelet concentration of 1.2±0.4 x 10^6 /ml. The lysate was thawed, spun and the supernatant removed, aliquoted and frozen at -20°C until use.

Total nucleated cells were isolated from the washouts of sealed bone marrow collection bags and filters. Cells were plated, without further separation, at 800 x 10^3 cells/cm^2 in complete medium consisting of Dulbecco's Modified Eagle's Medium (DMEM, Lonza Basel, Switzerland) supplemented with 5% freshly thawed PL, 2 mM L-glutamine (LiStarFish, Milano, Italy) and 1% penicillin/streptomicyn (Sigma-Aldrich, St. Louis,

MO, USA). After 24 hours the non-adherent cells were removed. Adherent cells were trypsinized after reaching 70-80% confluence and seeded at 100-200 cells/cm^2. The final cell products are bona fide hMSCs as defined by the International Society of Cell Therapy and have been subject to all quality controls required for clinical use. Release criteria included: lack of detectable microbial contamination (aerobic or anaerobic bacteria, fungi, and mycoplasma) according to European Pharmacopoeia, cell viability >90%, endotoxin levels in the final product <5 EU/kg, cell characterization with high expression (>80%) of CD73, CD90, CD105 and lack (<10%) of CD14, CD34, CD45 and normal karyotype and inability to grow without anchorage in a semisolid fluid. Cell lots were cryopreserved and thawed right before use. hMSCs used for the experiments were between passage (P) 3 and 5.

2.3.2 Phenotypic characterization of hMSCs

Expanded hMSCs were characterized by the following monoclonal antibodies, according to the manufacturer's instructions: phycoerythrin (PE)-labeled anti-CD14, anti-CD90, anti-CD105 (eBioscience, San Diego, CA, USA); PE-labeled anti-CD45, anti-CD73, anti-MHCII (Becton Dickinson (BD), Franklin Lakes, NJ, USA); fluorescein isothiocyanate (FITC)-labeled anti-CD34 (IQ product, Groningen, The Netherlands); FITC-labeled anti-MHC class I (BD). Samples acquired by FACScalibur (BD) were analyzed with CellQuest Software (BD).

2.3.3 Multilineage differentiation

The osteogenic and adipogenic differentiating ability of hMSCs was determined at $P3^{31}$ and, evaluated respectively after induction conditions, by Alizarin Red (Sigma-Aldrich) and Oil Red O (Sigma-Aldrich) staining.

2.3.4 Proliferation assay

Peripheral blood mononuclear cells (PBMC) were stimulated with 5 µg /mL of phytohemagglutinin (PHA) (Irvine Scientific, Santa Ana, CA, USA) and co-cultured with different doses of irradiated (35 Grey) hMSCs in the 96-well plates; 48 hours after co- culture, cells were pulsed for 16 hours with [3H]-thymidine at 1 µCi/well (Perkin Elmer, Waltham, MA, USA) then harvested. [3H]-thymidine incorporation was measured using a Multipurpose Scintillation Counter (Beckman Coulter, Brea, CA, USA).

2.3.5 Animals

Procedures involving animals and their care were conducted in conformity with the institutional guidelines at the IRCCS – Institute for Pharmacological Research “Mario Negri” in compliance with national (Decreto Legge nr 116/92, Gazzetta Ufficiale, supplement 40, February 18, 1992; Circolare nr 8, Gazzetta Ufficiale, July 14, 1994) and international laws and policies (EEC Council Directive 86/609, OJL 358, 1, Dec. 12, 1987; Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, (Eighth Edition) 2011). Male

C57Bl/6 mice (20–24g, Harlan Laboratories, Italy) were housed in a specific pathogen-free vivarium (room temperature 21±1°C, 12h light–dark cycle, free access to food and water). All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.3.6 Study design and blinding of *in vivo* studies

A) Immunosuppression and early rejection were evaluated on a total of 48 mice equally divided into six experimental groups: 1. sham operated mice given phosphate buffered saline, 24 hours after surgery, (SHAM PBS); 2. sham operated mice given hMSCs (SHAM hMSC). 3. TBI mice given PBS (TBI PBS); 4. TBI mice given hMSCs (TBI hMSC). 5. TBI mice given PBS and CsA (TBI PBS CsA) 6. TBI mice given hMSCs and CsA (TBI hMSC CsA). Mice (n=8) were euthanized 3 days post-surgery for real time reverse transcription (RT)-PCR analysis.

B) hMSCs protection on brain function and structure in immunocompetent and immunosuppressed mice was evaluated on 72 mice. First, to exclude confounding factors related to any direct neuro-protective/toxic effects of CsA we assessed the effects of our CsA immunosuppressive protocol on anatomical and functional damage one week after TBI (two groups of mice were used: TBI PBS and TBI PBS CsA, n=6). Since there was no difference in anatomical or functional damage between the two groups (see Results and Fig.5), immunocompetent TBI mice (TBI PBS) were considered the appropriate control for all further experiments.

C) Long term effects were evaluated on 60 mice divided into five equal experimental groups (1. SHAM PBS, 2. SHAM hMSC, 3. TBI PBS, 4. TBI hMSC, 5. TBI hMSC CsA). Mice (n=12) were used for behavioral analysis up to five weeks post-injury. After euthanasia, brains were processed and contusion volume (n=12), hMSCs distribution (n=12), vessel density (n=8), gliotic scar (n=8) and endogenous neurogenesis (n=8) were quantified.

Mice were assigned to surgery and treatment groups with surgery and treatment distributed equally across cages and days. Investigators who did behavioral and post mortem analysis were blinded to the treatment allocation. Fig.1 illustrates the experimental design.

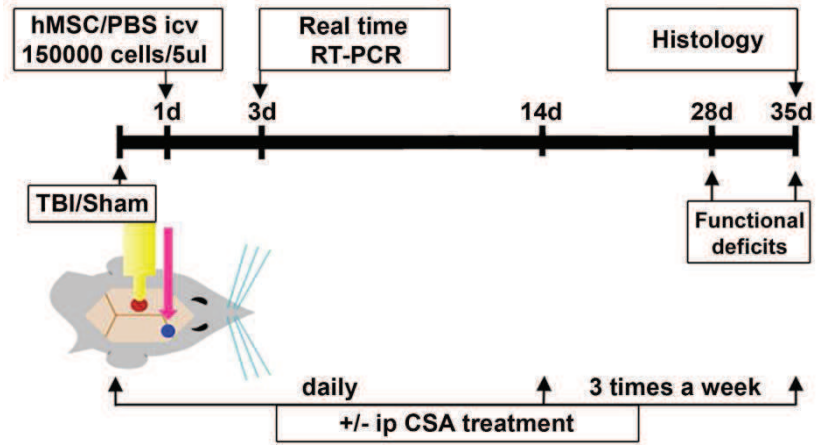

FIGURE 1. Experimental design. *hMSCs or PBS (control) were infused icv in the contralateral ventricle 24 hours after TBI or sham surgery. To assess the need for immunosuppression TBI mice transplanted with hMSCs or PBS were given immunosuppressive treatment with cyclosporin A (CsA, 10 mg/kg ip, daily for the first 15 days, then three times/week) or no treatment. Behavioral tests, histology and real time RT-PCR analysis were done at the time points indicated.*

2.3.7 Experimental brain injury

Anesthetized mice (sodium pentobarbital 65 mg/kg, intraperitoneal ip), were placed in a stereotaxic frame, and craniectomy was followed by induction of controlled cortical impact (CCI) brain injury as previously described[12]. Our injury model uses a 3 mm rigid impactor driven by a pneumatic piston, rigidly mounted at 20° from the vertical plane and applied perpendicularly to the exposed dura mater over the left parieto-temporal cortex at a velocity of 5 m/s and 1 mm depth. The craniotomy was then covered with a cranioplasty and the scalp sutured. Body temperature was maintained at 37°C during all surgical procedures. Sham-injured mice received identical anesthesia without brain injury.

2.3.8 Immunosuppression

Immunosuppressed mice received an ip injection of CsA (Sandimmun, Novartis, Italy, 10 mg/kg). The first dose was given one hour after surgery, and thereafter daily for the first 15 days, then three times/week as previously described[12].

2.3.9 Hoechst staining, cell preparation and transplantation

hMSCs were stained with 5 µg/mL Hoechst-33258 (Sigma-Aldrich) for 1 hour at 37°C and resuspended in PBS before transplantation. The hMSCs concentration was adjusted to 150,000 cells/5 µL PBS. As reported previously[12] this dose corresponds to the highest concentration associated with

viability greater than 90% after passage through the stereotaxic needle. Twenty-four hours after surgery hMSCs were transplanted icv (stereotactic coordinates: 0 mm caudal to bregma, 1 mm lateral to the midline and 3 mm beneath the dura mater) in anesthetized mice over 5 min.

Control mice were transplanted with PBS alone (5 μL) following the same procedures.

2.3.10 Real time RT-PCR

Three days after surgery ipsilateral cortical areas were dissected out, immediately frozen on dry ice, and stored at -80°C until analysis[32]. Total RNA was obtained using the Trizol reagent (Gibco BRL, MD)[3] and 1.5 mg was reverse-transcripted (TaqMan Reverse transcription reagents, Applied Biosystems, Foster City, CA, USA). Real time RT-PCR was conducted according to the manufacturer's.

We analized the expression of the following genes was analyzed: interferon-γ (INFγ), major histocompatibility complex II (MHCII), CD86, forkhead box P3 (Foxp3). Beta-actin was used as reference gene, according to the manufacturer's ΔΔCt method (Applied Biosystems) and relative gene expression levels were determined. Sequences of primers used are listed in Table 1.

Gene	NCBI ref Sequence	Forward primer	Reverse primer
β-actin	NM_007393.3	GCCCTGAGGCTC TTTTCCAG	TGCCACAGGATTC CATACCC
INFγ	NM_008337.3	TTGGCTTTGCAG CTCTTCCT	TGACTGTGCCGTG GCAGTA
MHCII	NM_001042605	CCAACGCGACCT CATCTCTAA	AGGGCGGTTGCC CAGTA
CD86	NM_019388.3	GTTACTGTGGCC CTCCTCCTT	CTGATTCGGCTTC TTGTGACATA
Foxp3	NM_001199347.1	CCAGGGAGCCA GCTCTACTCT	GTTGCTGTCTTTC CTGGGTGTAC

TABLE 1. Real time RT-PCR. *Real time RT-PCR were designed using Primer Express 2.0 software (Applied Biosystems) based on GenBank accession numbers.*

2.3.11 Behavioral tests

Sensorimotor deficits were evaluated by neuroscore[12,33] and beam walk[12,34,35] tests the day after surgery (before transplantation) and at five weeks. Cognitive function was assessed four weeks after surgery using the Morris water maze[12,36].

Neuroscore: animals were scored from 4 (normal) to 0 (severely impaired) for each of the following indices: 1) forelimb function; 2) hind limb function and 3) resistance to lateral pulsion as previously described[12,33,35]. The maximum score per animal is 12.

Beam walk: this test measures the number of foot-faults of a trained mouse walking twice on an elevated and narrow

wooden beam (5 mm wide and 100 cm length). The number of foot-faults was normalized on the baseline value obtained at day 1, before transplantation. Values lower than 1 indicate improvement[12,34].

Morris water maze: a circular pool (1 m diameter) filled with water (18–20°C) made opaque by nontoxic white paint and a fixed submerged platform (1 cm below the water surface) was used. The learning task consisted of 8 trials/day for 3 consecutive days for a total of 24 trials. Latencies to reach and climb onto the platform were recorded (Ethovision XT 5.0; Noldus Information Technology, Wageningen,The Netherlands) for each trial with a maximum of 60 sec per trial. Cognitive performance was obtained by averaging the latencies of 24 trials over 3 days[12,36].

2.3.12 Contusion volume

At five weeks, perfused brains were obtained[12,37] and cryosectioned at 20 µm. Twelve coronal sections 400 µm apart (from bregma +0.8 mm to bregma -3.6 mm) were stained with cresyl violet (Sigma-Aldrich). The Analytical Image System (Imaging Research Inc, Brock University, St Catharines, Ontario, Canada) was used for image acquisition and contusion volume was calculated as previously described[12].

2.3.13 Assessment of hMSC presence

Transplanted Hoechst positive cells were often packed or clustered so no cell count was possible. Therefore five weeks

after surgery two independent investigators, blinded to the allocation of surgery and treatment, made a semi-quantitative assessment of hMSC distribution and cluster size in the whole brain. Thirty-two 20 µm coronal sections per brain (from bregma +2 mm to bregma -3.6 mm) spaced 200 µm were analyzed using an Olympus BX61 microscope (Olympus, Tokyo, Japan). Hoechst fluorescent hMSCs were identified by their emission when excited at 352 nm (DAPI filter). Autofluorescent signals (emission under FITC or TRITC filters) were excluded. Data are expressed as hMSC presence score obtained from the sum of distribution and cluster size score. Distribution ranged from 1 to 8. Cluster size score ranged from 1 to 4. Scores were attributed according to Table 2.

Score	**Distribution**
0	No cells
1	at least 1 cell in 2 adjacent slices
2	at least 1 cell/slice in 2 non adjacent slices
3	at least 1 cell/slice in 3 non adjacent slices
4	at least 1 cell/slice in 4 non adjacent slices
5	at least 1 cell/slice in 5 non adjacent slices
6	at least 1 cell/slice in 6 non adjacent slices
7	at least 1 cell/slice in 7 non adjacent slices
8	at least 1 cell/slice in 8 non adjacent slices
Score	**Cluster size**
0	No cells
1	0< area <0.08 mm^2
2	0.08 < area <0.16 mm^2
3	0.16 < area <0.24 mm^2
4	area > 0.24 mm^2

TABLE 2. *Assessment of hMSC presence.*

2.3.14 Immunohistochemistry

Immunohistochemistry was done on 20 μm brain coronal sections using mouse anti- GFAP (1:2000; Millipore, Billerica, MA, USA), rat anti-CD31 (1:100; BD), goat anti-doublecortin (DCX, 1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) to measure astrogliosis, vessel density and neurogenesis respectively. Biotinylated secondary antibodies (1:200, Vector Laboratories, Burlingame, CA, USA) were used. Positive GFAP, CD31 or DCX cells were stained by reaction with 3,3-diaminobenzidine-tetrahydrochloride (Vector Laboratories) as previously described[38]. Negative controls were run for each reaction.

2.3.15 Acquisition and quantification

Three brain coronal sections per mouse (0.4-1.6-2.8 mm posterior to bregma) were used to quantify CD31 vessel density (nine frames per section) and GFAP stained area (eight frames per section). Two coronal sections (0.0 and 0.4 mm anterior to bregma) were used to quantify DCX-stained area (12 frames per section). To ensure operator-independent sampling, we selected anatomical reference points, namely the contusion edge/cortex boundary for CD31 and GFAP and ventricle ependimal wall for DCX, and acquired the same focal plan for all the samples[12,39].

An Olympus BX61 microscope equipped with a motorized stage and managed with AnalySIS software (Olympus) was used for unbiased sampling of the region of interest. For CD31

(Fig.2A), a first row of 10x magnification fields was positioned at the edge of the contused cortical tissue, and a second and a third row were positioned aligned to the first row. No space between fields was left and fields did not overlap. For GFAP (Fig.2B), a first row of 40x magnification fields was positioned at the edge of the contused cortical tissue, and fields were separated by 361.2μm, while a second row of fields was positioned aligned to first row at a distance of 722.4μm (distance between centres of fields). For DCX (Fig.2C), 40x magnification fields were positioned along the entire boundary of the ventricle with no vertical gap or overlapping between each field. The GFAP and DCX immunostained area was measured by segmentation of stained area using Fiji software[40]. Briefly, to subtract the background signal, a minimum threshold was applied based on the highest grayscale (0-256) value of background. GFAP and DCX staining was expressed as positive pixels/total assessed pixels and indicated as staining percentage area[12]. Vasculature density was assessed as previously described[41] with some modifications. Digitalized images were overlayed with a grid (15×15 μm per single square). The vascular network, visualized with anti-CD31 immunostaining was quantified by counting the number of vessels crossing the grid using Fiji software. Two independent investigators blinded to the identity of the samples performed immunohistochemical analysis of brain sections and quantification procedures.

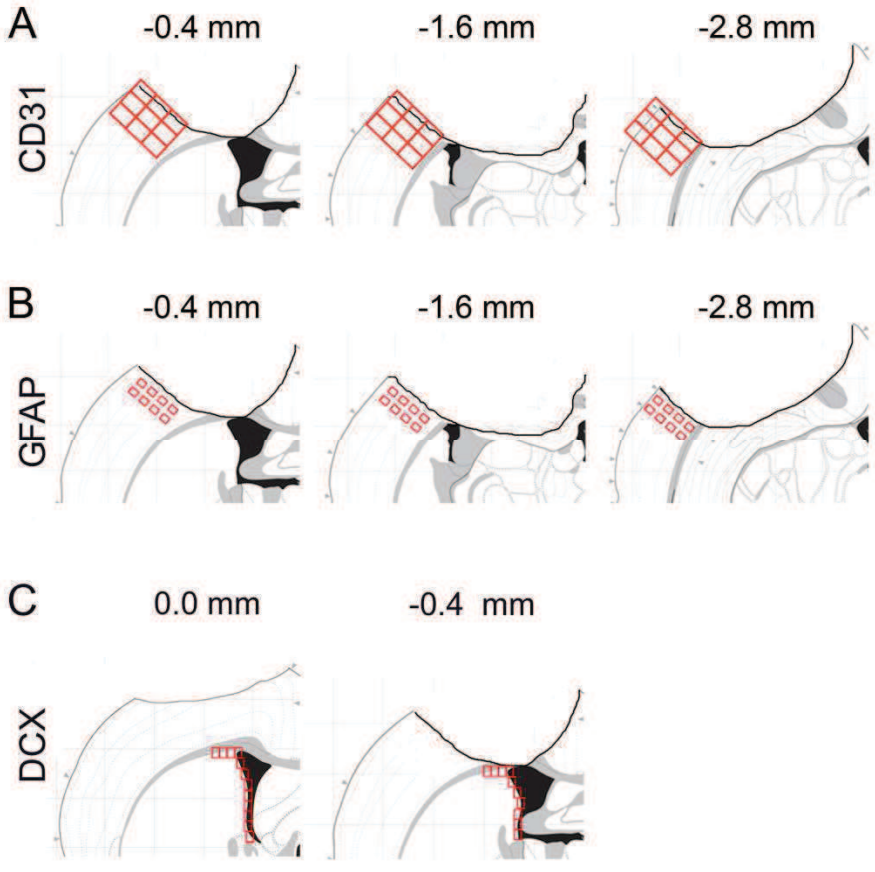

FIGURE 2. Slice selection and tissue sampling for quantification of immunostaining. *Fields for CD31 (A), GFAP (B) quantification were positioned within the contused tissue at defined distances. For DCX (C), fields were positioned along the entire boundary of the ventricle with no gap between each fields.*

2.3.16 Statistical Analysis

We used standard software package (GraphPad Prism Software Inc., San Diego, CA, USA, version 4.09 and all data are presented as mean and SD. For lymphocyte proliferation, gene expression analysis (INFγ, MHCII, CD86, Foxp3), global learning, contusion volumes, hMSC distribution and histological analysis (CD31, GFAP, DCX expression) groups were compared by one-way analysis of variance (ANOVA) and Bonferroni post hoc test. For sensorimotor and cognitive deficits, groups were compared by Kruskal-Wallis and two-way ANOVA for repeated measurements (RM) followed by Dunn or Tukey post hoc test. The Kolmogorov-Smirnov test was used to check normal distribution.

2.4 Results

2.4.1 hMSCs *in vitro* characterization

Flow cytometric analysis showed that hMSCs from healthy donors were positive for stromal cell-associated markers (e.g. CD105, CD90, CD73, and MHC I) and negative for hematopoietic markers (e.g. CD45, MHC II, CD14, CD34) (Fig.3A). These cells were also able to differentiate into osteoblasts (Fig.3B), and adipocytes (Fig.3C), as indicated by Alizarin Red staining and Oil Red O staining respectively. Finally they inhibited lymphocyte proliferation after mitogenic stimuli (i.e. PHA) in a dose-dependent manner (Fig.3D).

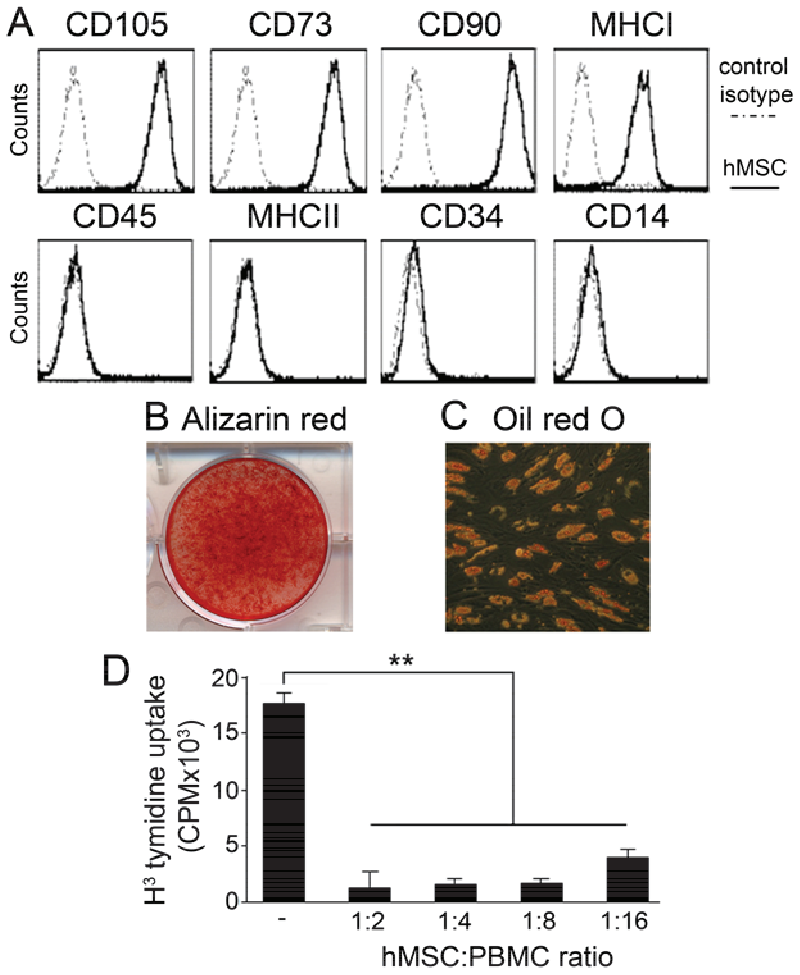

FIGURE 3. Characterization of hMSCs. *hMSCs were characterized by FACS analysis (A). Isotype control staining (dotted line) was compared with the specific antibody staining profile (continuous line). The osteogenic (B) and adipogenic (C) differentiation capacity of hMSCs was detected respectively by calcium deposition with Alizarin Red and by morphological appearance of fat droplets with Oil Red O staining. PBMC co-cultured with different concentrations of hMSCs proliferated significantly less than controls (D). Data are expressed as mean+SD, n=5, one way ANOVA followed by Bonferroni post test; **p<0.01, vs. PBMC stimulated with PHA in absence of hMSC.*

2.4.2 hMSC-induced immune response

Immunosuppression was analysed by INFγ gene expression at 72 hours (Fig.4A). All immunocompetent groups expressed similar levels of INFγ in the pericontusional cortex, but TBI PBS CsA and TBI hMSC CsA mice had significantly lower expression (respectively 84% and 68% less then the TBI PBS group, $p<0.05$) indicating the effectiveness of immunosuppression. Early rejection of transplanted hMSCs was analysed by MHCII (Fig.4B) and CD86 (Fig.4C) gene expression. TBI induced up-regulation of these genes compared to the sham groups. Transplanted hMSCs did not further rise MHCII and CD86 gene expression levels (mean±SD: MHCII: TBI PBS: 7.4±2.5, TBI hMSC: 7.72±2.9, TBI PBS CsA: 6.6±1.3, TBI hMSC CsA: 7.6±1.5; CD86: TBI PBS: 2.3±0.8, TBI hMSC: 2.5±1.0, TBI PBS CsA: 2.2±0.6, TBI hMSC CsA: 2.4±0.8), thus, suggesting negligible *in vivo* acute immunogenicity.

Induction of T-reg cells was analyzed by Foxp3 gene expression. TBI down regulated Foxp3 expression compared to sham groups (TBI PBS: 50% less than sham PBS, $p<0.001$, Fig.3D). Foxp3 expression was significantly up-regulated in the TBI hMSC group compared to TBI PBS (22%, $p<0.05$), TBI PBS CsA (32%, $p<0.01$) and TBI hMSC CsA (24%, $p<0.05$) groups.

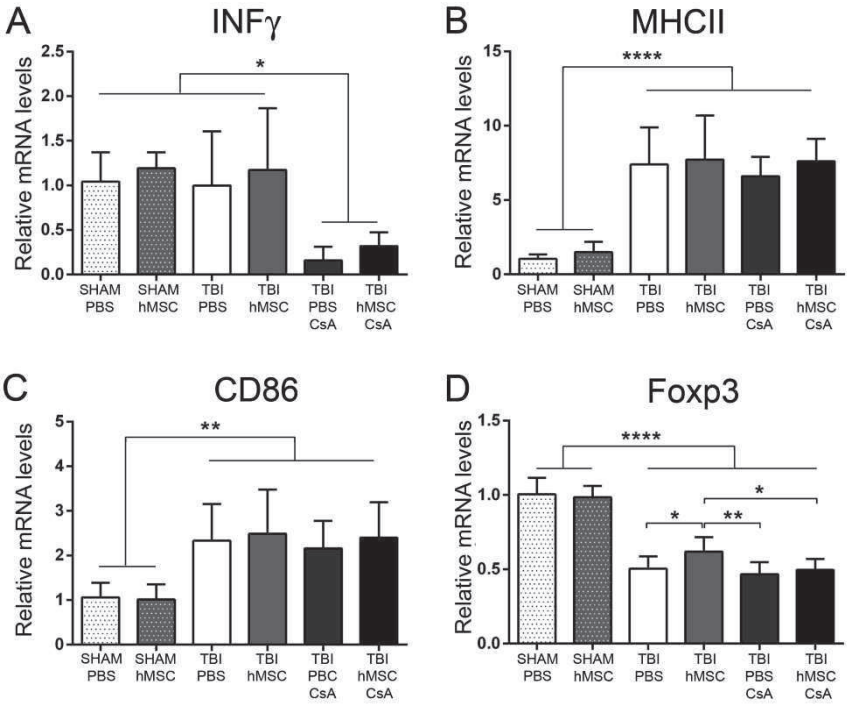

FIGURE 4. Immunosuppression and early rejection. *INFγ gene expression at 72 hours (A): all immunocompetent groups expressed similar levels of INFγ, while TBI PBS CsA and TBI hMSC CsA mice had significantly lower INFγ expression. MHCII (B) and CD86 (C) gene expression: TBI induced an up-regulated these genes compared to sham groups. Transplanted hMSCs did not further increase MHCII and CD86 gene expression. Induction of Treg cells was analyzed by Foxp3 gene expression. TBI down-regulated of Foxp3 expression compared to sham mice (D). hMSCs up-regulated this gene in TBI hMSC but not in the TBI hMSC CsA group compared to TBI PBS. Data are expressed as mean+SD, n=8; one-way ANOVA followed by Bonferroni post test; *p<0.05, ***p<0.001).*

2.4.3 hMSC protection of brain function and structure after TBI in immunocompetent and immunosuppressed mice

We first analyzed the effect of the CsA immunosuppressive protocol on anatomical and functional damage after TBI to exclude possible confounding factors related to direct neuro-protective/toxic effects of CsA. One week after TBI there were no differences in contusion volume or neuroscore between TBI PBS CsA and TBI PBS mice (Fig.5). Thus, for all further experiments immunocompetent TBI mice (TBI PBS) were considered the appropriate control for assessing whether hMSC efficacy was dependent or not on immunosuppression.

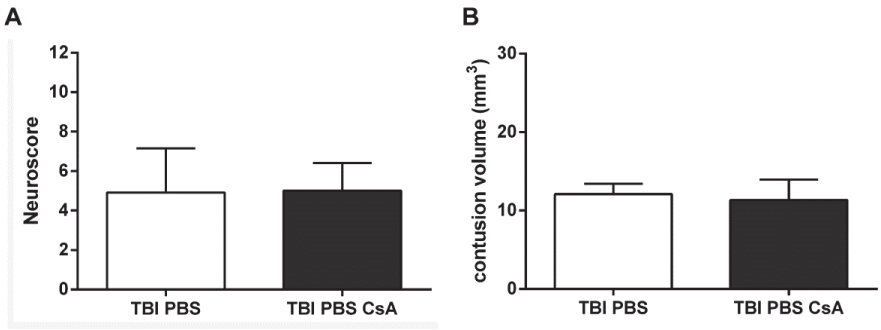

Fig.5 Effects of chronic CsA treatment on anatomical and functional damage 7 days after TBI. *Immunocompetent (TBI PBS) and immunosuppressed (TBI PBS CsA) mice were evaluated for functional (neuroscore, A) and anatomical (contusion volume, B) damage 7 days after TBI. No differences could be detected in the two groups. Data are expressed as mean+SD, n=6, unpaired t-test.*

2.4.3.1 Sensorimotor functions.

At five weeks post-injury, clear impairment in sensorimotor function was evident from the neuroscore in TBI PBS compared to sham groups (Fig.6A, $p<0.001$). These deficits were significantly less in TBI mice transplanted with hMSCs, both in the absence (TBI hMSC) or presence (TBI hMSC CsA) of immunosuppression (Fig.6A, $p<0.001$). The beam walk test also showed a similar degree of functional improvement in both transplanted groups compared to TBI PBS mice at five weeks post-injury (Fig.6B, $p<0.05$ at 4 weeks).

2.4.3.2. Cognitive function

Four weeks after surgery, TBI PBS mice showed clear learning dysfunction compared to the sham groups (Fig. 6C, $p<0.001$). This deficit was attenuated in TBI mice transplanted with hMSCs, without (TBI hMSC) or with (TBI hMSC CsA) immunosuppression. The beneficial effect was evident starting from day 2 of learning in TBI hMSC (Fig.6C, $p<0.01$) and from day 3 in TBI hMSC CsA mice (Fig.6C, $p<0.05$). Overall learning performance showed no differences in the two hMSC treated groups (Fig. 6D, $p<0.05$).

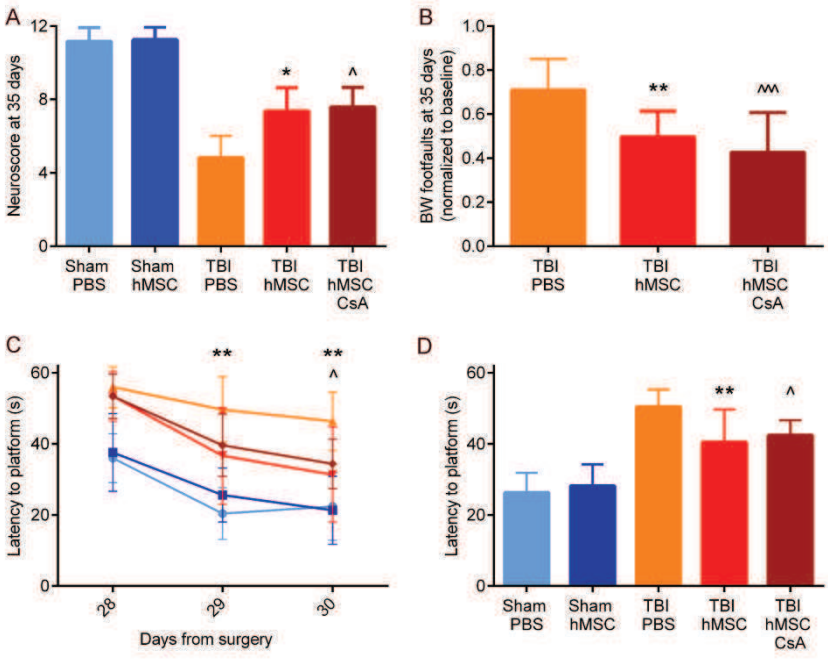

FIGURE 6. Protective effects of hMSCs on sensorimotor and cognitive functions. *Neuroscore (A): TBI PBS mice showed significant sensorimotor dysfunction compared to sham mice at five weeks. TBI hMSC and TBI hMSC CsA groups had significant comparable improvement of neurologic deficits at five weeks. Beam walk (B): both hMSC transplanted groups showed a significant improvement of foot-faults (normalized to baseline value) compared to TBI PBS group at five weeks after injury. In the Morris water maze test (C), done four weeks after surgery to investigate hMSC's effect on cognitive functions, the TBI PBS group showed a clear cognitive deficit compared to sham groups. hMSCs significantly improved cognitive performance after TBI leading to shorter escape to the platform from day 2 (TBI hMSC) or 3 (TBI hMSC CsA) of training. Global learning (D) reflected the degree of cognitive improvement. Data are expressed as mean±SD, n=12-14, A-C: Kruskar wallis followed by Dunn's post test; D: one-way ANOVA, followed by Tukey post test; *p<0.05, **p<0.01, TBI PBS vs. TBI hMSC; ^p<0.05, ^^^p<0.001 TBI PBS vs. TBI hMSC CsA.*

2.4.3.3. Anatomical damage

Five weeks after surgery the contusion volume was significantly smaller in TBI hMSC (mean±SD: 17.8±2.7 mm3) and TBI hMSC CsA (mean±SD: 16.4±2.4 mm3) compared to TBI PBS mice (mean±SD: 21.0±2.7 mm3). The contusion volume did not differ in TBI hMSC and TBI hMSC CsA mice, indicating a similar treatment effect (Fig.7).

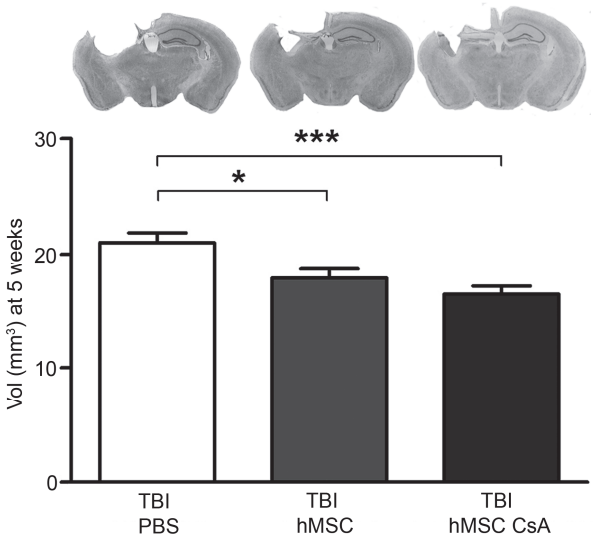

FIGURE 7. Anatomical damage. *Contusion volume five weeks after surgery was significantly reduced by hMSCs in the TBI hMSC and TBI hMSC CsA groups compared to TBI PBS mice. The contusion volume was the same in the two hMSC treated groups. Data are expressed as mean+SD, n=12, one-way ANOVA followed by Bonferroni post test: *p<0.05, ***p<0.001.*

2.4.4. hMSC distribution

In sham and TBI mice, hMSCs were mostly located in the contralateral ventricle (injection site, Fig.8A-C, box 1). An important finding was that, while in sham mice hMSCs were never found in the parenchyma, in TBI the cells were also seen in the injured cortex (Fig.8B-C, boxes 2-3). The presence score was significantly higher in both TBI transplanted groups than sham animals ($p<0.05$, Fig.8D). No significant difference was found between the two transplanted TBI groups.

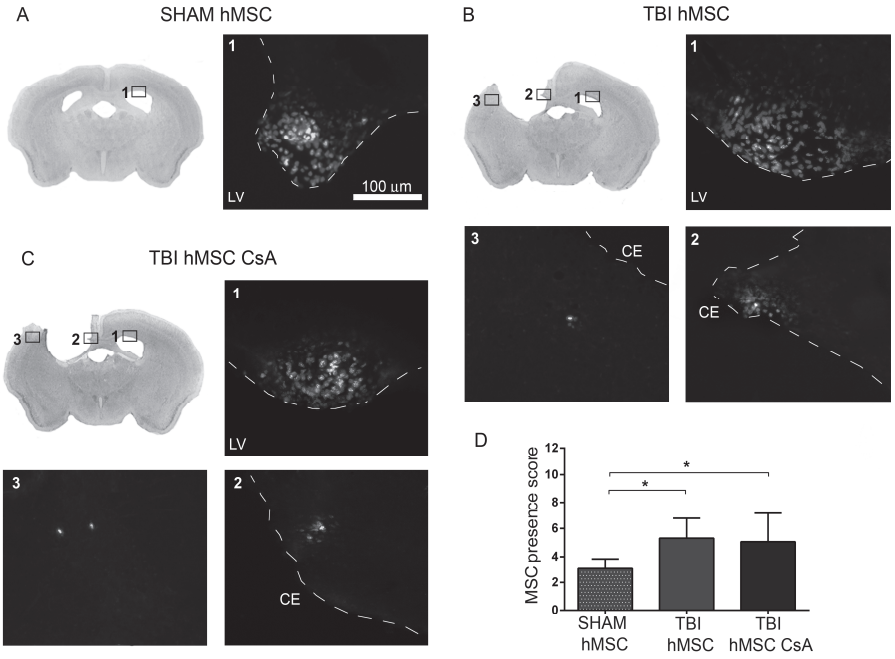

Fig.8 Distribution of hMSCs in sham operated and TBI mice at 5 weeks after injury. *hMSCs were infused icv to TBI/sham injury contralaterally. In sham animals, hMSCs (hoechst stained) were exclusively found clustered and confined into the ventricles (A, box 1: periventricular area). In both TBI hMSC treated groups (B and C), hMSCs were also found in the lesioned tissue (box 2: contusion edge, box 3: injured parenchyma). Semi-quantitative analysis of hMSC presence showed an increased amount of hMSCs in TBI mice irrespective of the immunosuppressive treatment (D, mean+SD, n=8, one way ANOVA, followed by Bonferroni post test, *p<0.05). LV=lateral ventricle; CE=contusion edge.*

2.4.5 Long-term brain environmental changes in immunocompetent and immunosuppressed TBI mice

Five weeks after surgery CD31 immuno-staining in the peri-contusional cortex showed an increase in vessel density in TBI hMSC (+19%, $p<0.01$) and TBI hMSC CsA (+20%, $p<0.01$) compared to TBI PBS mice (Fig.9A-D), indicating that hMSCs can promote vascular repair in immunocompetent and immunosuppressed mice. GFAP immunostaining showed that hMSCs reduced gliotic scar formation in TBI hMSC (-34%, $p<0.05$) compared to TBI PBS mice (Fig.9E-H). This reduction was close to significance in TBI hMSC CsA (-23%, $p=0.068$) compared to TBI PBS mice (Fig.9H). Finally, the effect on endogenous neurogenesis was assessed at five weeks on the basis of DCX expression in the subventricular zone (svz) of the ipsilateral hemisphere. hMSCs increased the expression of DCX in svz, comparably in immunocompetent (+79%, $p<0.05$) and immunosuppressed (+82%, $p<0.05$) mice (Fig. 9I-L).

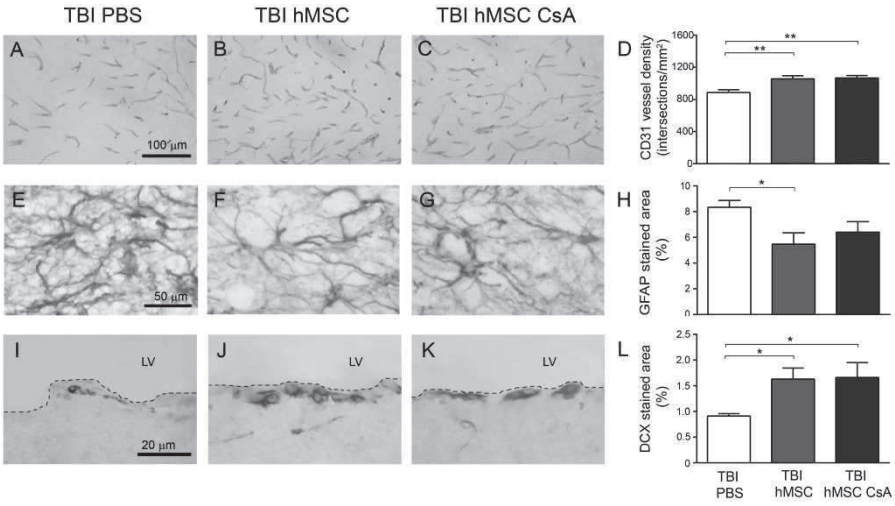

FIGURE 9. hMSCs induce comparable long-term brain environmental changes in immunocompetent and immunosuppressed TBI mice. *Microphotographs show immunoreactivity for the endothelial marker CD31 (A-C), GFAP (E-G) and DCX (I-K) five weeks after injury in the peri-contusional cortex of TBI PBS, TBI hMSC and TBI hMSC CsA mice. Quantification indicated: D) significantly greater CD31 vessel density in both hMSC transplanted groups compared to TBI PBS mice, H) significantly smaller GFAP stained area in the scar region in TBI hMSC compared to TBI PBS group. This effect was close to significance (p=0.068) in TBI hMSC CsA mice. L) significantly greater DCX stained area in TBI hMSC and TBI MSC CsA compared to TBI PBS mice (D). Data are expressed as mean+SD, n=8, one-way ANOVA p<0.01, followed by Bonferroni post test; *p<0.05, ** p<0.01. LV=lateral ventricle.*

2.5 Discussion

We report a comprehensive evaluation of the effects of xenogenic hMSC transplantation for TBI in the presence or absence of immunosuppression. Xenogenic hMSCs were similarly effective in immunocompetent or immunosuppressed mice in improving neurological functional and structural recovery and promoting protective and reparative mechanisms. This finding is important considering that chronic immunosuppression in TBI patients can lead to infective complications and may not be tolerated[8].

Preclinical studies confirmed the potential of hMSCs to enhance recovery after brain injury in different experimental models. Beneficial effects are mainly attributed to their paracrine neuroprotective effects[14,42] and their immunomodulatory functions[43,44]. The interaction of transplanted hMSCs with the injured microenvironment leads to the attenuation of the progression of the lesion[12], vascular rescue[45,46] and stimulation of endogenous reparative mechanisms[45,47]. To interact promptly with pathological pathways of secondary damage and to foster restorative processes, hMSCs have to be transplanted in the acute phase. This limits the possibility of autologous transplantation since isolation and *in vitro* expansion of hMSCs requires weeks. The transplantation of bank-stored GMP-grade certified hMSCs poses the risk of host rejection due to immunological mismatch. Thus, immunosuppression becomes a critical question before cell therapy can move to clinical application.

Immunosuppression clearly has dangerous implications because it increases susceptibility to infection which is directly related to unfavorable outcome[48,49]. So a major goal would be to avoid immunosuppression. Although many studies have demonstrated the efficacy of MSCs in immunosuppressed and even in immunocompetent rodent models of TBI[12–14,45,50], the specific contribution of immunosuppressive treatment to MSCs efficacy has never been addressed directly. We xenotransplanted hMSCs into mouse hosts, a condition that amplifies immunological mismatch-related problems.

The immunosuppressive agent CsA specifically inhibits lymphocytic cyclophilin, inducing a downstream block of interleukin (IL)-2 expression (a cytokine that is essential for T-cell activation and proliferation). CsA treated mice showed a reduction of INFγ (largely produced by mitogenically or antigenically activated T lymphocytes) confirming the effectiveness of immunosuppression. The lack of INFγ increase in immunocompetent TBI compared to sham mice was not surprising since in the same TBI model, INFγ peaks at 4 hours post-injury and returns to baseline by 24 hours[51]. Early xenogenic rejection was ruled out by measuring the expression of MHCII and its co-receptor CD86. Expression of both genes was not affected by hMSCs in immunocompetent or in immunosuppressed animals.

We have previously described the lack of correlation between the long term presence of icv transplanted hMSCs in the injured parenchyma and the degree of functional and

anatomical recovery[12], indicating that their presence in the contusion is not necessary for protection. Here we analyzed the distribution and cluster density to obtain an indicator of xenogenic rejection at chronic stages. The similar hMSC presence scores in immunosuppressed and immunocompetent TBI mice indicates that in our condition hMSCs can escape the normal processes of xenogenic rejection.

Next we noted that hMSCs induced T-reg (Foxp3+ cells) expansion, shifting the local microenvironment toward a more tolerogenic phenotype. Since this population is regulated by IL-2 which is inhibited by CsA, the Foxp3 increase was seen only in immunocompetent mice. A similar peripheral effect on splenic T-regulatory cells (CD4+/CD25+/Foxp3+) was reported after intravenous delivery of human multipotent adult progenitor cells in immunocompetent traumatized mice[52]. These data illustrate the low immunogenic potential and immunoregulatory properties of hMSCs after TBI.

Since the aim of our work was to establish whether hMSC efficacy is affected by immunosuppressive treatment, the TBI PBS group was considered the control and we did not explore the direct contribution of CsA on long term TBI PBS mice. Experimental data show that CsA can give protection when administered in the acute phase in TBI (for a complete review see Lulic et al.[53]) and two clinical trials demonstrate the safety and tolerability of low doses (1-5 mg/kg) of CsA in the acute phase (8-12 hours of injury) for severe TBI patients[54,55]. Acute and rapid exposure of injured neurons to CsA is needed to

obtain neuroprotection[53,56]. Instead chronic administration used to obtain immunosuppression, is associated with increased risk of infections, nephrotoxicity and hepatotoxicity, all factors that negatively affect outcome in TBI patients[8,57–59].

First we demonstrated similar effects of hMSCs in immunosuppressed and immunocompetent mice on all outcome measurements (i.e.: neuroscore, beam walk, Morris water maze, contusion volume). Next, we investigated the effect of hMSCs in immunocompetent and immunosuppressed mice on gliotic scar formation in the peri-lesioned tissue, on vessel density and on host stem cell proliferation in the svz. These data extend our previous findings using cord blood MSC in the same TBI model, where there was a selective reduction of astrocytic activation in the GFAP subpopulation not engaged in trophic function[12]. We also saw a clear rescue effect on vessel density associated with induction of neurogenesis in TBI mice treated with hMSCs, with no differences between immunosuppressed and immunocompetent groups. Further studies are needed to establish the extent to which newly generated neural progenitors (DCX positive cells) contribute to the observed functional protection. However, the recent failure of the citicoline monotherapy trial underlines once again the need for strategies that simultaneously affect regional and widespread injury in TBI[60]. We found that in both the immunosuppressed and immunocompetent recipients, hMSCs interacted with the injured tissue through multiple mechanisms, promoting protective and remodeling processes.

These results provide urgently needed experimental evidence that the long-term presence and efficacy of hMSCs in the injured brain is not impaired in the absence of immunosuppressive treatment. Thus hMSCs isolated and expanded from donors, tested for their functional capabilities and stored as an “off the shelf” cell medicinal product could be made immediately available for a TBI patient, with no delay to therapy.

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Disclosure

No competing financial interests exist.

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Chapter 3

Bone marrow mesenchymal stromal cells drive protective M2 microglia polarization after brain trauma

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3.1 Abstract

Microglia/macrophages (M) are major contributors to post-injury inflammation but they may also promote brain repair in response to specific environmental signals that drive classic (M1) or alternative (M2) polarization.

We investigated the activation and functional changes of M in TBI mice receiving intracerebroventricular human bone marrow mesenchymal stromal cells (MSCs) or saline infusion. MSCs up-regulated *Ym1* and *Arginase-1* mRNA ($p<0.001$), two M2 markers of protective M polarization, at 3 and 7d post-injury and increased the number of Ym1+ cells at 7d post-injury ($p<0.05$). MSCs reduced the presence of the lysosomal activity marker CD68 on the membrane surface of CD11b positive M ($p<0.05$), indicating a reduced phagocytosis. MSC-mediated induction of the M2 phenotype in M was associated with early and persistent recovery of neurological functions evaluated up to 35 days post-injury ($p<0.01$) and reparative changes of the lesioned microenvironment.

In vitro, MSCs directly counteracted the pro-inflammatory response of primary murine microglia stimulated by TNFα+IL17 or by TNFα+INFγ and induced M2 pro-regenerative traits, as indicated by the down-regulation of *iNOS* and up-regulation of *Ym1* and *CD206* ($p<0.01$) mRNA.

In conclusion we found evidence that MSCs can drive the M transcriptional environment and induce the acquisition of an early, persistent M2 beneficial phenotype both *in vivo* and *in vitro*. Increased *Ym1* expression together with reduced *in vivo*

phagocytosis suggests M selection by MSCs towards the M2a subpopulation, which is involved in growth stimulation and tissue repair.

3.2 Introduction

Traumatic brain injury (TBI) is the leading cause of mortality and disability among young people in high income countries[1]. Mechanical “primary” injury is responsible only for a part of the subsequent neurologic damage. In the following days/weeks, waves of toxic cascades convert at-risk into dead brain tissue, with a major impact on the final outcome[2]. Besides these events, however TBI also induces lasting neurorestorative processes[3,4], which contribute to the spontaneous recovery. Thus, promotion of endogenous restorative mechanisms may represent an interesting therapeutic approach. A critical review of TBI trial failures has highlighted the need to focus on strategies that simultaneously affect multiple injury mechanisms and foster repair[5,6]. In this context, mesenchymal stromal cells (MSCs) that interact with parenchymal brain cells in multiple ways, are promising candidates[7–10].

Mononuclear phagocytes (microglial cells, perivascular macrophages and blood-born macrophages, all referred to here as M) hold a prominent role in tissue surveillance and the response to changes in brain homeostasis. Activated M are consistently detected in the peri-contusional tissue after TBI[11,12], but their specific contribution to the progression of injury is far from being completely elucidated[13–15]. In response to TBI, M are capable of adopting diverse, complex activation states, allowing them to participate in the cytotoxic response, but also in immune regulation and injury resolution[13,16,17]. These states can be classified in four main phenotypes: classically

activated M1 phenotype, with cytotoxic properties; alternative activated M2a phenotype, with pro-regenerative functions; M2b immunoregulatory phenotype; M2c deactivated phenotype. The activation state of M is reflected by the expression of cell surface antigens with recognized functions[18,19] that can be used to characterize changes of microglial phenotypes over time after acute brain injury and to describe, *in vivo* the environmental changes related to a specific M state[20].

We recently showed long term protection (on sensorimotor and cognitive functions and anatomical damage up to 5 weeks) of human MSCs transplanted 24h after TBI in mice[21]. Infused MSCs are able to reprogram the local inflammatory microenvironment from detrimental to beneficial, favoring endogenous neurorestorative mechanisms[9,21]. Using the same protocol of injury and MSC infusion in the same experimental model, we assessed *in vivo* M activation and functional polarization after human bone marrow MSC treatment. Similarly to our previous study we choose the intracerebroventricular (icv) infusion of MSCs. Notably in severe TBI patients icv cannulation is recommended by authoritative guidelines for intracranial pressure monitoring. Thus this approach would allow to inject MSCs focally without exposing the patient to additional surgical procedures potentially harmful.

We investigated: 1) the M phenotype following MSC transplantation to injured mice; 2) the involvement of M and their phagocytic activity in the protection induced by MSCs. In addition we assessed the direct ability of MSCs to drive a

phenotypic switch in primary microglial cultures under inflammatory conditions.

3.3 Materials and Methods

3.3.1 Isolation and culture of human MSCs

The local institutional review board approved the study and informed consent was obtained from healthy donors. MSCs were isolated and expanded *ex vivo* from bone marrow aspirates, as previously described[22]. Briefly, total nucleated cells were isolated from the wash-outs of sealed bone marrow collection bags and filters. Cells were plated, without further separation, at $800x10^3$ cells/cm^2 in DMEM (Lonza Basel, Switzerland) supplemented with 5% freshly thawed PL, 2 mM L-glutamine (LiStarFish, Milano, Italy) and 1% penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO, USA). After 24 hours the non-adherent cells were removed. Adherent cells were trypsinized after reaching 70-80% confluence and seeded at 100-200 cells/cm^2. MSCs at passage 3 were screened by flow cytometry for the expression of CD34, CD45, CD73, CD90, CD105, CD105, HLA-ABC, and CD11b. MSCs were also tested for their capacity to differentiate into adipocytes and osteoblasts. Cells were used for the experiments between P 3 and 5, preparation from individual donors (n=2) have been injected into individual sham-operated/TBI mice.

3.3.2 *In vivo* studies

3.3.2.1 Animals

Procedures involving animals and their care were conducted in conformity with the institutional guidelines at the IRCCS – Institute for Pharmacological Research "Mario Negri" in compliance with national (Decreto Legge nr 116/92, Gazzetta Ufficiale, supplement 40, February 18, 1992; Circolare nr 8, Gazzetta Ufficiale, July 14, 1994) and international laws and policies (EEC Council Directive 86/609, OJL 358, 1, Dec. 12, 1987; Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, (Eighth Edition) 2011). The protocol used and details of this report are also in accordance with ARRIVE guidelines. Male C57Bl/6J mice (20–24 g, Harlan Laboratories, Italy) were housed in a SPF *vivarium* at a constant temperature (21 ±1°C) with a 12h light–dark cycle and free access to food and water.

3.3.2.2 Experimental brain injury

Anesthetized mice (sodium pentobarbital, 65 mg/kg ip) were placed in a stereotaxic frame and subjected to craniectomy followed by induction of CCI brain injury as previously described[9]. Briefly, a 3 mm rigid impactor driven by a pneumatic piston and rigidly mounted at an angle of 20° from the vertical plane, was applied perpendicularly to the exposed *dura mater* over the left parieto-temporal cortex (AP: -2.5 mm, LL: -2.5 mm) at a velocity of 5 m/s and depth of 1 mm. The craniotomy was then covered with a cranioplasty and the scalp

sutured. During all surgical procedures, mice body temperature was maintained at the 37°C. Sham-operated mice received identical anesthesia without brain injury.

3.3.2.3 MSC preparation and transplantation

MSCs were resuspended in PBS, before transplantation. Cell number was evaluated by light microscopy. Viability of MSCs was evaluated by the trypan blue exclusion test and cell concentration was adjusted to 150,000 cells/5 µL PBS. In a set of experiments MSCs were labeled with PKH26 red fluorescence cell linker (Sigma-Aldrich) according to manufacturer's instructions in order to visualize cell localization and interaction with host tissue.

Twenty-four hours after surgery, a hole was drilled in the scalp in anesthetized mice, contralateral to the injured side at coordinates 0 mm caudal to bregma, 1 mm lateral to the midline, and 3 mm beneath the *dura mater*. MSCs were infused icv over 5 min and the needle was left in place afterwards for another 5 min. Control mice were infused with PBS alone (5 µL) following the same procedures. No animals died after transplantation.

3.3.2.4 Sensorimotor deficits

Sensorimotor deficits were evaluated by neuroscore and beam walk tests[9,21,23] before injury (day 0) and at 7, 21 and 35 days post TBI. For neuroscore, animals were scored from 4 (normal) to 0 (severely impaired) for each of the following

indices: 1) forelimb function, 2) hind limb function and 3) resistance to lateral pulsion, as previously described[9,24]. The maximum score per animal is 12. The beam walk test measures the number of foot-faults of a trained mouse walking twice on an elevated, narrow wooden beam (5 mm wide and 100 cm long). The best score is 0[9,23].

3.3.2.5 Real time RT-PCR

On day 3 or 7, mouse ipsilateral cortical areas (including all the tissue above the rhinal fissure [25] were dissected out, rapidly frozen on dry ice, and stored at 80°C until analysis. Total RNA was obtained from tissue specimen using Trizol reagent (Gibco BRL, MD) [26]. Samples of total RNA (1.5 µg) were treated with DNAse (Applied Biosystems, Foster City, CA, USA) and reverse-transcripted with random hexamer primers using Multi-Scribe Reverse Transcriptase (TaqMan Reverse transcription reagents, Applied Biosystems). Real time RT-PCR was conducted according to the manufacturer's instructions. The expression of the following genes was analyzed: *CD11b*, *TNFα, CD86, CD68*, *Ym1*, *Arginase-1*, *CD206*, *SOCS3, CCL2*, *IL-1β, IL-10, IGF1, VEGF*, *GFAP. β-Actin* was used as reference gene and relative gene expression levels were determined according to the manufacturer's ΔΔCt method (Applied Biosystems). Primers were designed to selectively match mouse but not human sequences using Primer Express 2.0 software (Applied Biosystems) or Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/) based on GenBank accession numbers (Table 1).

Gene	NCBI ref Seq	Forward primer	Reverse primer
β-actin	NM_007393.3	GCCCTGAGGCTC TTTTCCAG	TGCCACAGGATTC CATACCC
CD11b	AK089521	GAGCAGCACTGA GATCCTGTTTAA	ATACGACTCCTGC CCTGGAA
TNFα	NM_013693.2	AGACCCTCACAC TCAGATCATCTTC	TTGCTACGACGTG GGCTACA
CD86	NM_019388.3	GTTACTGTGGCC CTCCTCCTT	CTGATTCGGCTTC TTGTGACATA
CD68	AK002264	GGATTGGATTGA GGAAGGAACTG	GCCGCATGGCAGA GATG
Ym1	NM_009892.2	TCTGGTGAAGGA AATGCGTAAA	GCAGCCTTGGAAT GTCTTTCTC
Arg-1	NM_007482.3	CATGGGCAACCT GTGTCCTT	TCCTGGTACATCT GGGAACTTTC
CD206	NM_008625.2	CCCAAGGGCTCT TCTAAAGCA	CGCCGGCACCTAT CACA
SOCS3	NM_007707.3	TTTCTTATCCGCG ACAGCTC	GGATGCGTAGGTT CTTGGTC
CCL2	NM_011333.3	GCCTGCTGTTCA CAGTTGC	ATTGGGATCATCTT GCTGGT
IL1β	NM_008361.3	AGTTGACGGACC CCAAAAGA	GGACAGCCCAGGT CAAAGG
IL-10	NM_010548.2	CGGCTGAGGCGC TGT	TGCCTTGCTCTTAT TTTCACAGG
IGF1	NM_001111274.1	CACACTGACATG CCCAAGAC	CCTTTCCTTCTCCT TTGCAG
VEGF	NM_009505.4	CCAGACCTCTCA CCGGAAAG	CTGTCAACGGTGA CGATGATG
GFAP	NM_001131020.1	GAAAACCGCATC ACCATTCC	TCGGATCTGGAGG TTGGAGA

3.3.2.6 Brain transcardial perfusion

Seven or 35 days after injury mice were deeply anesthetized with Equitensin (120 µl/mouse ip) and transcardially perfused with 20 ml of PBS, 0.1 mol/l, pH 7.4, followed by 50 ml of chilled paraformaldehyde (4%) in PBS. The brains were carefully removed from the skull, and transferred to 30% sucrose in PBS at 4°C overnight for cryoprotection. The brains were then rapidly frozen by immersion in isopentane at -45°C for 3 min, sealed in vials and stored at -70°C until use.

3.3.2.7 Immunohistochemistry

Immunohistochemistry was done on 20 µm brain coronal sections using rat anti mouse-CD11b (1:700; kindly provided by Dr. Doni), rat anti mouse-CD68 (1:200; Serotec, Kidlington, UK), rat anti mouse-CD45 (1:800, BD Pharmingen, New Jersey, USA), rabbit anti mouse-Ym1 (1:400, Stem Cell Technologies, Vancouver, Canada). Positive cells were stained by reaction with DAB (Vector laboratories, CA, USA) as previously described[20]. For negative control staining, the primary antibodies were omitted and no staining was observed. Quantitative analysis was made in defined anatomic boundaries, acquiring the same focal plan throughout the samples[9,27]. Unbiased, operator non-dependent tissue sampling was done using a BX61 Olympus microscope equipped with a motorized stage and managed with AnalySIS software (Olympus, Japan). Quantification fields at 40x magnification were selected over three brain coronal sections per mouse, 0.4

mm posterior to bregma (12 fields), 1.6 mm posterior to bregma (12 fields) and 2.8 mm posterior to bregma (9 fields). The first row of fields was positioned at the contusion edge, spacing each field by 361.2 µm (distance between centres of the fields). A second and a third row of fields were positioned further from the lesion and aligned to the first row. Distance between each row was 722.4 µm. Immunostained area for each marker was measured using Fiji software[28] and expressed as positive pixels/total assessed pixels and reported as the percentage staining area for subsequent statistical analysis. Two independent investigators blinded to the identity of the samples did the immunohistochemical analysis of brain sections and quantification.

3.3.2.8 Immunofluorescence and confocal analysis

Immunofluorescence was done on 20 µm coronal sections as previously described[20,29]. Primary antibodies used were: anti-mouse CD11b (1:30000, kindly provided by Dr. Doni), anti-mouse Ym1 (1:400, Stem Cell Technologies, Vancouver, Canada), anti-mouse CD68 (1:200, Serotec, Kidlington, UK) and anti-mouse GAP-43 (1:500,[30]). Fluoro-conjugated secondary antibodies used were: Alexa 546 anti-rat, Alexa 594 or Alexa 647 anti-rabbit (all 1:500, Invitrogen, Carlsbad, CA). Biotinylated anti-rat or anti-rabbit antibodies (1:200, Vector Laboratories, Burlingame, CA) were also used followed by fluorescent signal coupling with a streptavidin TSA amplification kit (cyanine 5, Perkin Elmer, MA, USA). Appropriate negative

controls were run without the primary antibodies. None of the immunofluorescence reactions gave an unspecific fluorescent signal in the negative controls. Immunofluorescence was acquired using a scanning sequential mode to avoid bleed-through effects by an IX81 microscope equipped with a motorized stage and a confocal scan unit FV500 with 3 laser lines: Ar-Kr (488 nm), He-Ne red (646 nm), and He-Ne green (532 nm, Olympus, Tokyo, Japan) and a UV diode. Co-localization was analyzed over three-dimensional fields measuring 180x135x7 µm, obtained by stacking 31 confocal planes at 800x600 resolution, distanced by a z-axis step of 0.23 µm. Three-dimensional fields were positioned over the same sections as for immunohistochemical analysis (0.4 mm, 1.6 mm and 2.8 mm posterior to bregma) using the motorized stage under the control of xy Stage software (Olympus, Japan).

For each coronal section, four non-overlapping fields, over a 2x2 matrix, for CD68/CD11b co-localization in the cortex and three fields (1x3 matrix) for Ym1/CD68 co-localization in the hippocampus were aligned. Quantification of double positive voxels (co-localization) was performed with Imaris (Bitplane, Switzerland) using the ImarisColoc algorithm [31]. Signal intensity over a volume with no positive staining (background) was calculated for green and red channels and used as the lower signal threshold. Voxels that were over lower thresholds for both channels were co-localized. A co-localization channel (yellow) containing only co-localized voxels was generated and visualized by surface rendering (IsoSurface, Imaris) using the

thresholds applied for co-localization analysis. Analysis was performed by an operator blinded to the study. Co-localization is expressed as percentage of double positive voxels over total CD68 or Ym1 positive voxels.

3.3.2.9 Study design and blinding

C57Bl/6J male mice, divided into four equal experimental groups, were used: 1) SHAM PBS: sham-operated mice receiving PBS (5 µL, icv) 24 h after surgery; 2) SHAM MSCs: sham-operated mice receiving MSCs (150,000/5 µL icv) 24 h after surgery; 3) TBI PBS: TBI mice receiving PBS (5 µL icv) 24 h after surgery; 4) TBI MSCs: TBI mice receiving MSCs (150,000/5 µL icv) 24 h after surgery.

For real time RT-PCR analysis, mice were euthanized 3d or 7d (n=8) post-injury. For immunohistochemistry analysis, mice (n=6) were euthanized 7d or 35d post-injury.

Mice were randomly allocated to surgery (sham-operated or TBI) taking care to distribute them equally across experimental days and batches to avoid systematic errors.

All surgery and injuries were done by the same investigator, who was masked to the treatment of each mouse. At the end of the procedure, a second investigator assigned a masked code to each mouse (including the sham-operated group). MSCs or PBS treatment and all subsequent behavioral, biochemical and histologic evaluations were done by investigators unaware of the injury or treatment status of the animals. Sham-operated

mice were used since the contralateral hemiphere is not a proper control after TBI[9,32].

3.3.3 *In vitro* studies

3.3.3.1 Microglial cultures and *in vitro* polarization

Primary mouse microglial cells were isolated from mixed cultures of cortical and hippocampal astrocytes, established from P2 C57Bl/6J mouse pups and maintained in MEM supplemented with 20% FCS and 5.5 g/L glucose (glial medium). To promote microglial proliferation, one week after plating, the culture medium was supplemented with GM-CSF produced from X-63 GM-CSF cells. After 5-7d microglia were harvested by shaking mixed glial cultures, and seeded on polyornithine-coated glass coverslips (16 mm diameter, $1x10^5$ cells), or 60 mm tissue Petri dishes ($8X10^5$ cells). To minimize activation, half of the medium in which microglia were kept after shaking from mixed cultures was replaced with fresh low serum (1%) medium.

Two stimuli were used to drive M1 phenotypes microglia cells were exposed either to a combination of TNFα (200 U/ml) and IL-17 (500 U/ml) or to a combination of TNFα (200 U/ml) and INFγ (500 U/ml)[33]. Two hours after the toxic stimuli or in control conditions microglia-MSC co-cultures were obtained by plating MSCs on purified microglia in a microglia:MSCs ratio of 1:1 and were maintained in glial medium for 72 h. At the end of the treatment, microglia were washed and either harvested with TRIZOL (Invitrogen) for RT-PCR analysis or fixed with 4%

paraformaldehyde for immunocytochemistry. In a set of experiments MSCs were co-cultured with microglia indirectly, using a 0.3 µm pore size transwell (Corning Life Sciences, Pittson, PA).

3.3.3.2 Reverse transcriptase-coupled PCR

Total RNA was isolated from murine microglia or microglia-MSC co-cultures using Nucleo Spin miRNA kit (Macherey-Nagel) following the manufacturer's protocol. To remove any contaminating genomic DNA, total RNA was digested with DNase. cDNA synthesis was done using the SuperScriptIII™ RT-PCR system (Invitrogen) and Random Hexamers as primer. The resulting cDNAs were amplified using mouse specific TaqMan® Gene Expression Assay (Applied Biosystems). The mRNA expression was normalized to the label of mouse specific *GAPDH* mRNA. Mouse specific TaqMan Assays were tested on MSC cDNA to confirm the specificity for murine sequences and their inability to detect human sequences and relative gene expression levels were determined according to the manufacturer's ΔΔCt method (Applied Biosystems).

3.3.3.3 Cell fluorescence analysis

Pure microglial cultures and microglia-MSC co-cultures were fixed at room temperature for 25 min with 4% paraformaldehyde in 0.1 M phosphate buffer containing 0.12 M sucrose. Fixed cells were permeabilized with detergent and labeled with anti-CD206 monoclonal Abs (1:100, AbD Serotec,

USA) and anti-YM1 polyclonal Abs (1:100, Stem Cell Technologies, Vancouver, Canada), followed by Alexa-488 anti-rat Abs (1:500) and Alexa-568 anti-rabbit Abs (1:200) (Invitrogen, Carlsbad, CA). In one set of experiments MSCs co-cultured with microglia were identified by the mesenchymal cell marker CD105 (eBioscience, San Diego, CA, USA). The coverslips were mounted in 70% glycerol in phosphate buffer containing 1 mg/ml phenylenediamine. Cells were observed with a Leica SP5 confocal microscope.

3.3.4 Statistical analysis

We used a standard software package for statistical analysis (GraphPad Prism Software Inc., San Diego, CA, USA, version 6.0). All data are presented as mean±SD. For sensorimotor deficits (neuroscore and beam walk tests) groups were compared using two-way analysis of variance (ANOVA) for repeated measures followed by Tukey's test. For immunohistochemical analysis and *in vitro* studies of microglia in basal condition, groups were compared using the unpaired t-test. For gene expression analysis groups were compared by two-way analysis of variance (ANOVA) followed by Tukey's test. Probability values <0.05 were considered statistically significant. Assumptions of normality were checked using the Kolmogorov-Smirnov test.

3.4 Results

In vivo and *in vitro* experiments were conducted according to the experimental design shown in Figure 1.

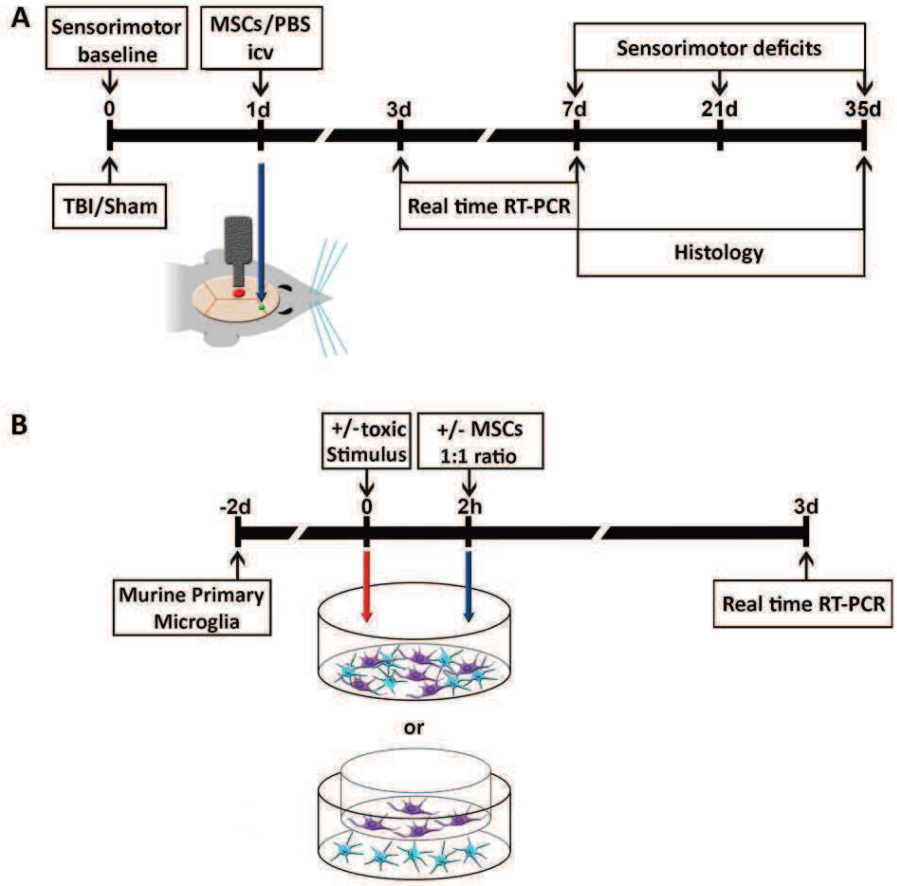

Figure 1. Experimental design of *in vivo* and *in vitro* experiments. *In vivo* experiments: TBI/sham surgery was done 1d before treatment. MSCs/PBS were infused in the contralateral ventricle. Sensorimotor deficits were evaluated at 0, 7, 21 and 35 d. Animals were sacrificed at 3d or 7d for real time RT-PCR or at 7 or 35 d for histological analysis. *In vitro* experiments: primary murine microglial cells were cultured for 48h then at the time points indicated in the plan were exposed to: 1) pro-inflammatory stimuli (TNFα/IL17 or TNFα/INFγ) for M1 classical activation; 2) MSCs; 3) pro-inflammatory stimuli followed by either direct or indirect (transwell) MSC co-culture. Unexposed cultures served as controls. After 72h, co-cultures were analyzed by real time-PCR or immunohistochemistry.

3.4.1 MSC infusion induces protection on sensorimotor deficits 7d after TBI

Using the same protocol of injury and MSCs infusion, we recently demonstrated that MSCs improve sensorimotor and cognitive dysfunctions induced by TBI and reduce contusion volume at 5 weeks after surgery[21]. Here we analyzed sensorimotor deficits at 7, 21 and 35 days after TBI, to evaluate early time points and confirm efficacy later on. Significant protection after MSC infusion was observed with the neuroscore at every time point considered (7d: TBI PBS: 2.37±1.06, TBI MSCs: 4.75±1.58 $p<0.001$; 21d: TBI PBS: 3.62±0.91, TBI MSCs: 5.75±1.58; 35d: TBI PBS: 4.00±1.60, TBI MSCs: 5.75±1.03 $p<0.01$, Figure 2A) and with the beam walk test at 21 and 35 days (21 d: TBI PBS: 31.75±3.73, TBI MSCs: 25.50±5.88 $p<0.05$; 35d: TBI PBS: 34.37±6.30, TBI MSCs: 20.75±8.65 $p<0.001$, Figure 2B).

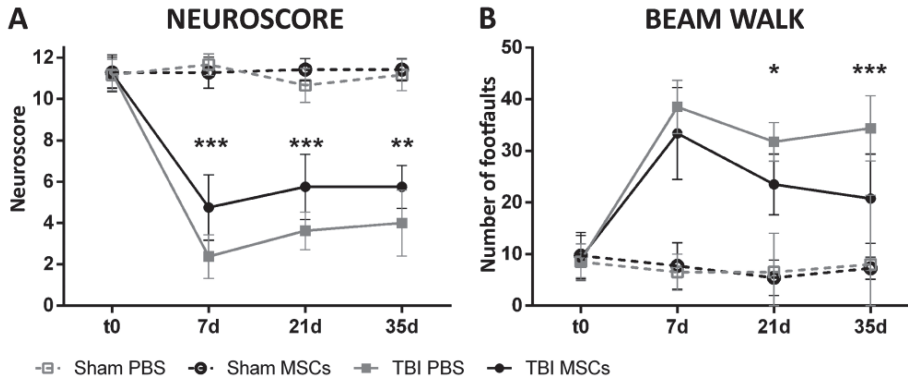

Figure 2. Effects of MSC infusion on sensorimotor deficits. MSC infusion induced early and persistent improvement of sensorimotor deficits as measured by Neuroscore (A) or beam walk (B) tests. Neuroscore test showed sensorimotor improvement in TBI MSCs mice from 7d on (A), while beam walk test showed a significant improvement of TBI MSCs from 21d on

(B). Data are mean+SD, n=8, 2-way ANOVA for RM followed by Tukey's test.

3.4.2 MSCs drive the up-regulation of M2 gene expression at 3 and 7 d after TBI

Three and 7d after TBI, we measured the mRNA expression of *CD11b, TNFα and CD86* (markers of M activation), *CD68* (a marker associated with the lysosomal activity of myeloid cells) and *Ym1*, *Arginase-1*, *CD206, SOCS3* (four markers of M2a or 2c polarization). Compared to sham-operated mice, *CD11b, TNFα* and *CD86* showed induction at both times after TBI (*CD11b:* 3.08±0.77 at 3d; 2.92±0.46 at 7d; *TNFα:* 12.51±3.00 at 3d; 12.73±6.47 at 7d; *CD86:* 2.42±0.67 at 3d; 3.67±0.78 at 7d). No further increase were detected in TBI MSCs mice at both times (*CD11b*: 3.10±0.80 at 3d; 3.39±0.56 fold at 7d; *TNFα:* 11.51±2.96 at 3d; 11.43±5.50 at 7d; *CD86:* 2.65±0.81 at 3d; 4.38±1.30 at 7d; Figure 3A-C). Similar changes were observed for CD68 (7.92±1.85 at 3d, 6.05+1.17 at 7d) with no significant changes after MSC infusion (9.27±2.85 at 3d; 7.73±2.45 at 7d; Figure 3D). *Ym1*, *Arginase-1* and *SOCS3* were significantly up-regulated at 3d (*Ym1*: 29.06±12.50; *Arginase-1*: 165.1±59.23; *SOCS3:* 2.74±0.25) but not at 7d (*Ym1*: 3.23±2.22; *Arginase-1*: 32.45±30.21; *SOCS3:* 1.57±0.86) after TBI. Of note, TBI mice injected with MSCs showed a more marked and lasting increase in the *Ym1* and *Arginase-1* but not *SOCS3* transcripts than TBI PBS mice (3d: *Ym1*: 49.40±11.18, +70%; *Arginase-1*: 286.00±106.70, +73%; *SOCS3:* 3.06±0.69, +11%; 7d: *Ym1*: 30.73±19.75, +850%;

Arginase-1: 460.20±342.10, +1300%; *SOCS3:* 1.49±0.55, -5%; Figure 3E-F-H). Compared to sham-operated mice *CD206* was transiently induced in TBI PBS at 3 but not at 7d (3d: 1.61±0.30, 7d: 1.68±0.95), *CD206* induction was more lasting after MSC infusion (3d: 1.66±0.33, 7d: 2.33±0.67; Figure 3G). MSCs in sham-operated mice did not change mRNA expression for any of the assessed genes.

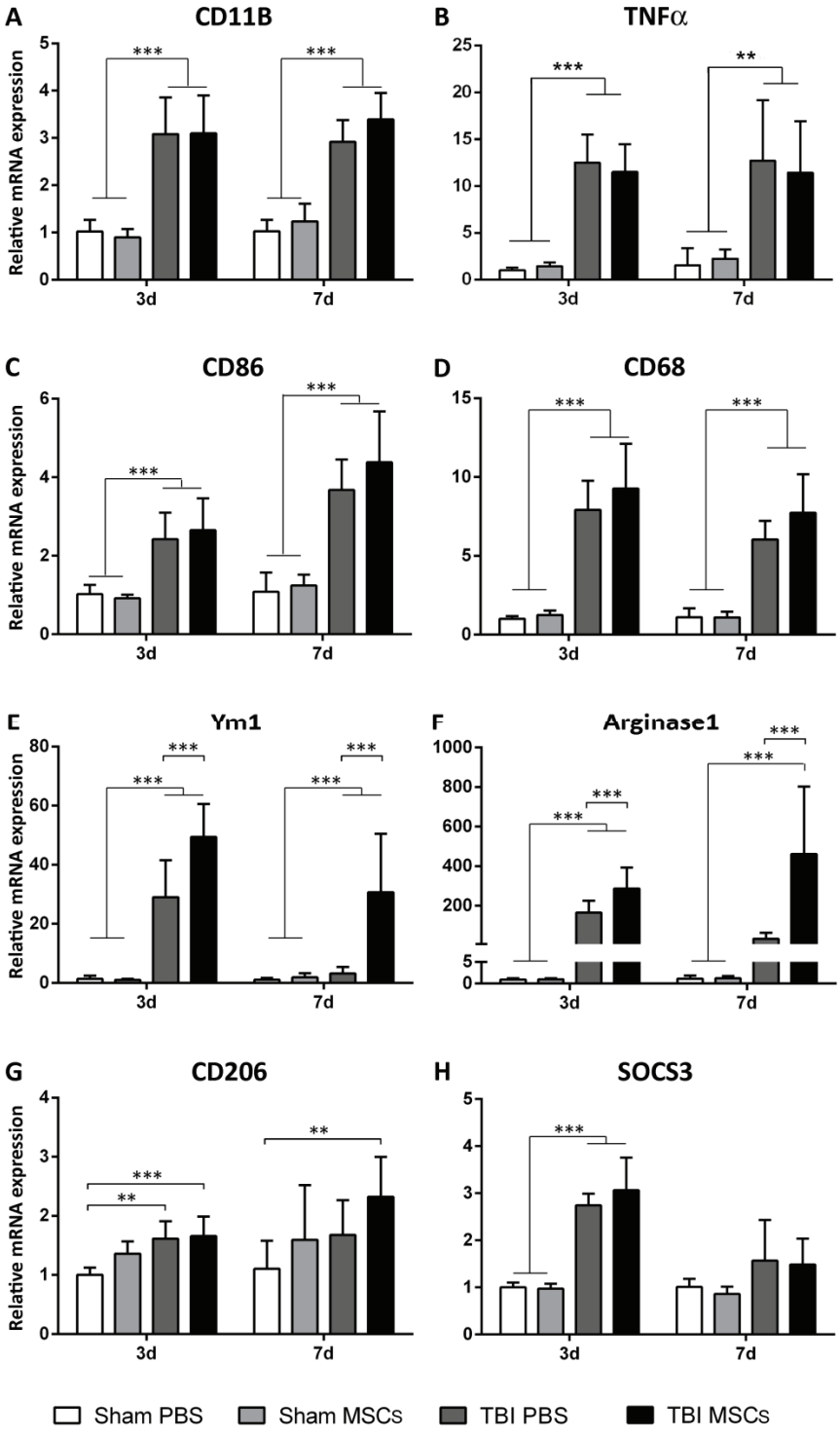
A
CD11B
B
TNFα
C
CD86
D
CD68
E
Ym1
F
Arginase1
G
CD206
H
SOCS3
Relative mRNA expression
3d
7d
Sham PBS
Sham MSCs
TBI PBS
TBI MSCs

Figure 3. mRNA expression of genes related to microglia activation and polarization in brain cortices, 3d and 7d after surgery. *CD11b*, *TNFα*, *CD86* and *CD68* were significantly up-regulated in TBI compared to sham-operated mice both at 3d and 7d after surgery with no difference between TBI PBS and TBI MSCs mice (A-D). *Ym1*, *Arginase-1, SOCS3* and *CD206* were significantly up-regulated in TBI compared to sham-operated mice at 3d but not at 7d after surgery. MSC infusion significantly increased *Ym1* and *Arginase-1* expression in TBI MSCs compared to TBI PBS (E, F) at both time points considered. No difference in *SOCS3* mRNA expression was found between TBI PBS and TBI MSCs groups. At 7d TBI MSCs mice showed increased expression of *CD206* than sham PBS (E). Data are expressed as the fold induction compared to the sham PBS group. Data are mean+SD, n=8. ***p*<0.01, ****p*<0.001 vs. sham or TBI PBS. 2-way ANOVA followed by Tukey's test.

3.4.3 MSC infusion induces increased CD45^{high} cell infiltration and reduces M lysosomal activity in the ipsilateral cortex 7d after TBI

We measured the number of CD45^{high} positive cells 7d after TBI by immunohistochemistry. CD45^{high} cells were significantly increased in TBI MSCs mice compared to mice receiving PBS (Figure 4A), indicating an increased infiltration of peripheral immune cells mediated by MSC [34].

We measured the expression and co-localization of CD11b and CD68 7d after TBI by immunohistological techniques. Neither marker quantitatively changed its protein expression after MSC administration (Figure 4B-C), in line with gene expression data (Figure 3A-D). Analysis of three-dimensional images revealed that CD68 was always expressed by CD11b+ cells, as previously described [35]. Since during lysosomal activity CD68 is localized at the plasma membrane [36,37], we assessed the extent of CD68 co-localization with CD11b, the latter being

a surface marker of myeloid cells. We found that the percentage of CD68 and CD11b double-positive voxels was 56.04±10.56 in TBI PBS mice and that it significantly decreased to 35.98±10.71 following MSC infusion (Figure 4D'-E'-F), thus suggesting a reduction in the phagocytic activity.

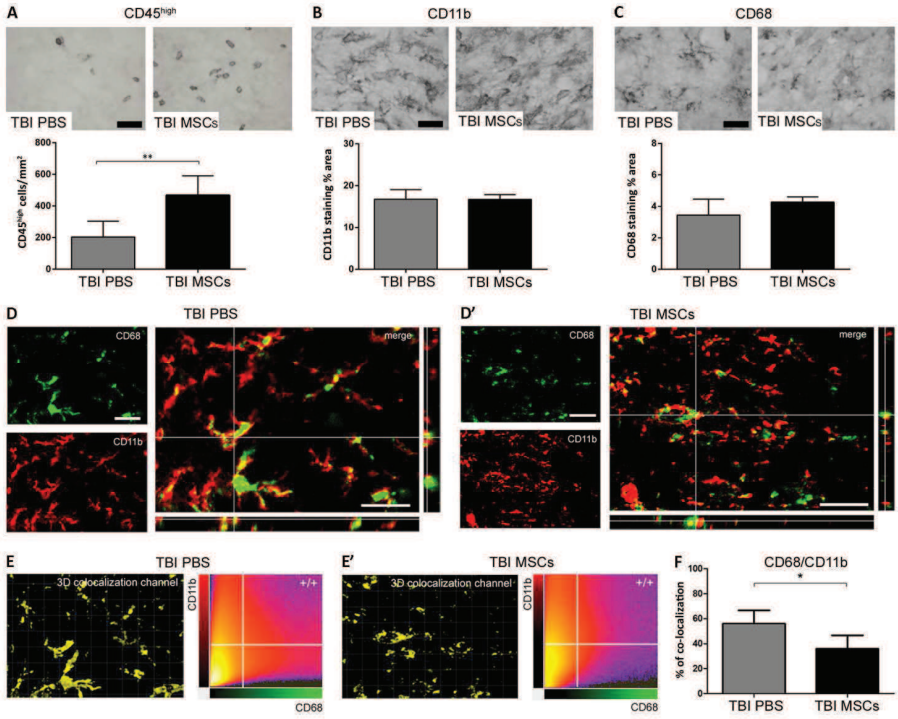

Figure 4. Immunohistochemical analysis of CD45, CD11b and CD68 and quantification of their co-localization in the injured cortex. Representative micrographs of CD45 (A) CD11b (B) and CD68 (C) immunostainings 7d after TBI and their quantifications. The number of $CD45^{high}$ cells was significantly increased in TBI MSCs mice, whereas no differences were observed either in CD11b or CD68 expression. Representative micrographs of CD68 (green) and CD11b (red) co-localization in TBI PBS (D) and TBI MSCs (D') mice. In TBI PBS mice, CD68 often co-localized with the membrane marker CD11b (D, merge), while in TBI MSCs mice it remained mainly located in the cytoplasm, thus yielding less co-localization with CD11b (D', merge). Quantification of co-localized voxels in the three-dimensional confocal acquisitions (E, E') showed a reduction of CD68/CD11b positive voxels after MSC infusion indicating a

reduction of lysosomal activity in TBI MSCs than TBI PBS mice (F). Data are mean+SD, n=8 (A; B, E). *p< 0.05, unpaired t-test. Bars: 20 µm.

3.4.4 MSC infusion enhances the presence of M2 polarized cells within the lesion site 7d after TBI

Ym1 protein expression in the ipsilateral cortex was significantly up-regulated 7d after TBI in mice infused with MSCs (staining percentage area in TBI PBS: 0.10±0.06; TBI MSCs: 0.22±0.13; Figure 5A). We then measured Ym1 co-localization with CD68 by analyzing three-dimensional immunofluorescence images (Figure 5B, C). MSC infusion significantly reduced Ym1/CD68 co-localization (co-localized voxel percentage in TBI PBS: 68.14±11.53; TBI MSCs: 50.57±10.99, Figure 5D-F). Furthermore triple immunofluorescence analysis revealed a different pattern of cellular CD68/CD11b co-localization in $Ym1^+$ cells in TBI PBS versus TBI MSCs mice, the latter showing a decrease in CD68 membrane localization (Figure 5G, H).

A physical contact between M2 polarized cells co-expressing CD11b and Ym1 and the PKH26-labelled MSCs was observed in some but not every cases (Figure 6) indicating that M2-induced polarization by MSCs does not require direct cell-cell contact.

Ym1 and CD68 protein expression in TBI mice infused with MSCs was dramatically decreased at 35d after TBI compared to 7d (Ym1: -94%, CD68: -80%, data not shown) suggesting that these events played a role at early time points.

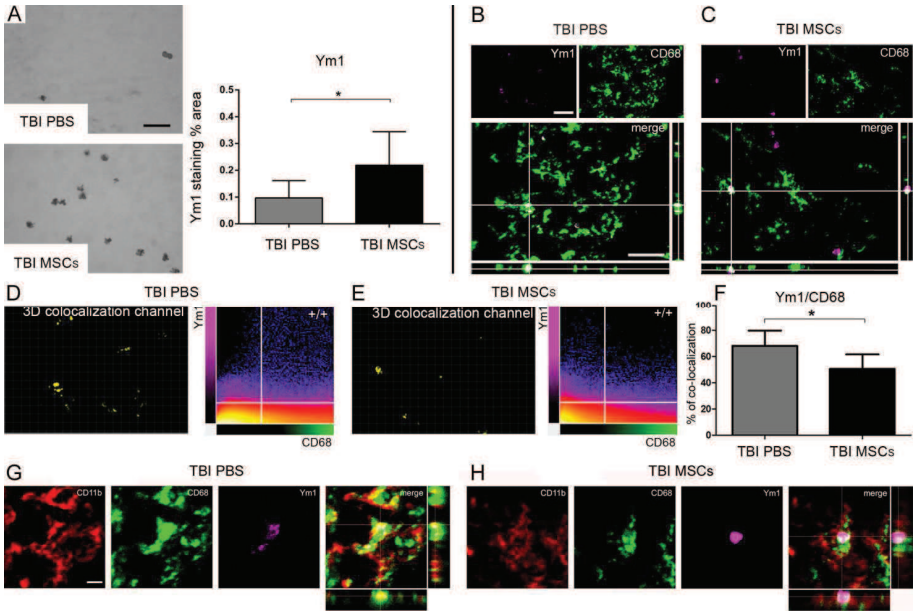

Figure 5. Immunohistochemical analysis of Ym1 in the injured cortex and quantification of its co-localization with CD68 in the injured hippocampus. Representative micrographs of Ym1 immunostaining 7d after TBI in PBS or MSCs treated mice (A) and its related quantification showing an increase of Ym1 expression in TBI MSCs compared to TBI PBS mice (A). Co-localization of Ym1 (purple) and CD68 (green) in TBI PBS (B) and TBI MSCs (C) mice. Bar: 20 µm. Quantification of co-localized voxels in the three-dimensional confocal acquisitions (D, E) showed a reduction of Ym1/CD68 positive voxels after MSC infusion (F).
Triple immunofluorescence for CD11b (red), CD68 (green) and Ym1 (purple) for TBI PBS (G) and TBS MSCs mice (H). In TBI PBS mice co-localization between Ym1 and CD68 (white) is observed in cells with strong co-localization between CD68 and CD11b (yellow, G). Xyz-view and 3D renderings of co-localized pixels (centre and right panels) do better illustrate this (G). In TBI MSCs mice cells with reduced Ym1/CD68 co-localization (white) show diminished CD68/CD11b co-localization (H). Xyz-view and 3D renderings of co-localized pixels are shown in centre and right panels in H. Bar: 5 µm. Data are mean+SD, n=8 (A, F). *p<0.05, unpaired t-test.

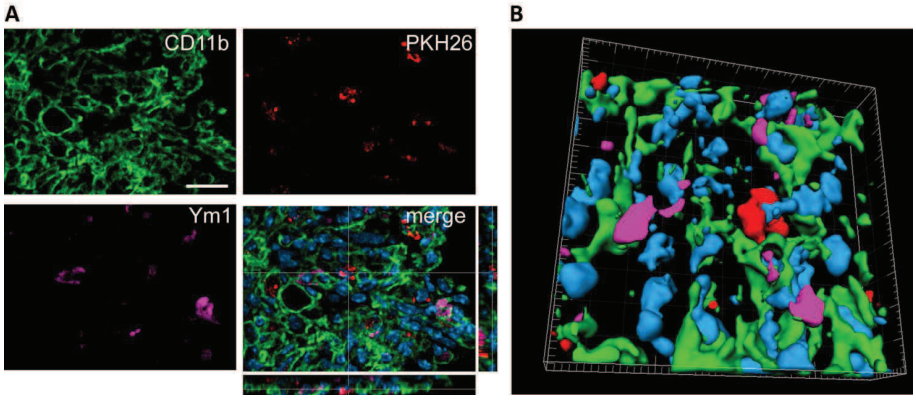

Figure 6. Localization of CD11b/Ym1 double positive cells with PKH26-labelled MSCs in the injured cortex. At 7d after TBI in mice infused with MSCs, the cells positive to CD11b (green) and Ym1 (purple) showed direct contact with infused MSCs (PKH26, red, A), as better depicted in the three-dimensional rendering (B). Bar = 20 μm.

3.4.5 MSC infusion induces early microenvironmental changes

We then assessed possible alterations of cytokine and growth factor gene expression after MSC infusion. TBI up-regulated the cytokines *CCL2*, *IL-1β* and *IL10* and the growth factor *IGF1* at 3d (*CCL2*: 28.88±6.10; *IL-1β*: 8.21±2.62; *IL10*: 252.40±61.82; *IGF1*: 3.73±1.25); and IL1*β* and IGF1 also at 7d (*IL-1β*: 7.39±2.36; *IGF1*: 9.88±4.95) after injury, compared to sham PBS animals (Figure 7A-D). More importantly, TBI MSCs mice showed significant up-regulation of *CCL2* (38.21±21.11; Figure 7A), *IL-1β* (11.21±3.89; Figure 7B) and *IL10* (969,96±392.72; Figure 7C) on day 7 compared to the TBI PBS group. No significant differences were found in *IGF1* (Figure 7D) gene expression at either times.

We analyzed possible early changes in *VEGF* and *GFAP* gene expression in the cortical contusion core and bordering

regions to clarify whether early acquisition of a beneficial M phenotype was associated with more general reprogramming of the microenviroment towards a pro-regenerative state, involving endothelial cells and astrocytes. As expected *VEGF* was lower in TBI PBS both 3d (0.46±0.15) and 7d (0.52±0.26) after surgery (Figure 7E), compared to sham-operated mice. MSC infusion did not cause significant changes in TBI mice at day 3 (0.47±0.13), but it increased *VEGF* expression, close to the levels observed in sham-operated animals at 7d (0.74±0.32; Figure 7E), suggesting a pro-angiogenic activity. In TBI mice, *GFAP* showed 8.64±2.62 and 16.01±5.18 fold induction respectively, at 3 and 7d compared to sham PBS mice, revealing a strong reaction of astrocytes to the mechanical insult. MSC infusion significantly reduced *GFAP* gene expression in TBI mice at both time points (3d: 6.78±1.17, 7d: 11.92±3.37; Figure 7F).

Interestingly, MSCs induced an increase in the immunofluorescence for GAP-43, a marker of axonal regeneration, in the contused cortex both at 7d and 35d after TBI (qualitative observation based on 4 mice per condition, Figure 8A, B). MSCs labelled with PKH26 prior to infusion were found only at 7d. At this time point, PKH26 positive cells localized either far from GAP-43 positive cells or in close proximity to neurons undergoing axonal regeneration (Figure 8C). The latter localization was particularly evident in those cases in which MSCs reached the subventricular zone niche (Figure 8D).

M2 polarized cells (Ym1 positive) were present in areas showing intense GAP-43 staining and in contact with GAP-43 positive neurons (Figure 8E, F).

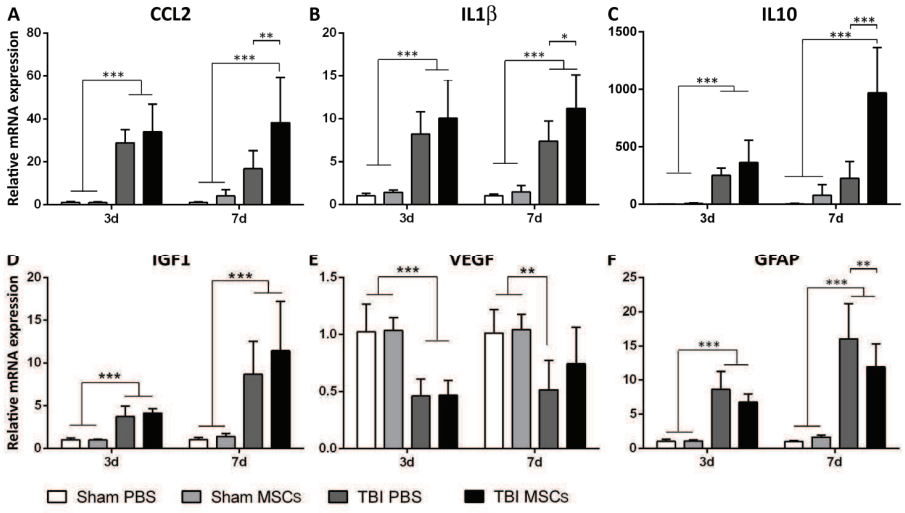

Figure 7. mRNA expression of cytokines and growth factors in brain cortices, 3d and 7d after surgery. *CCL2*, *IL1β*, *IL10* and *IGF1* were significantly up-regulated in TBI compared to sham-operated at 3d after surgery. The *CCL2*, *IL1β* and *IGF1* up-regulation in TBI mice persisted also at 7d after surgery (A-D). There was no difference in *CCL2*, *IL1β*, *IL10* and *IGF1* expression between TBI PBS and TBI MSCs groups at 3d (A-C). At 7d MSC infusion significantly increased *CCL2, IL1β* and *IL10* expression in TBI MSCs compared to TBI PBS (A-C), while no difference was found in *IGF1* expression (D). *VEGF* expression was down-regulated in TBI PBS mice compared to sham-operated groups both at 3d and 7d (E). TBI MSCs mice showed a trend toward an increase of *VEGF* expression at 7d restoring *VEGF* expression close to the levels observed in sham-operated animals. *GFAP* expression was significantly up-regulated in TBI compared to sham-operated mice both at 3d and 7d after surgery (F). MSC infusion induced a significant reduction of *GFAP* expression at 7d after surgery. Data are shown as fold induction compared with sham PBS group and are mean+SD, n=8. ***p*<0.01, ****p*<0.001, vs. sham or TBI PBS, 2-way ANOVA followed by Tukey's test.

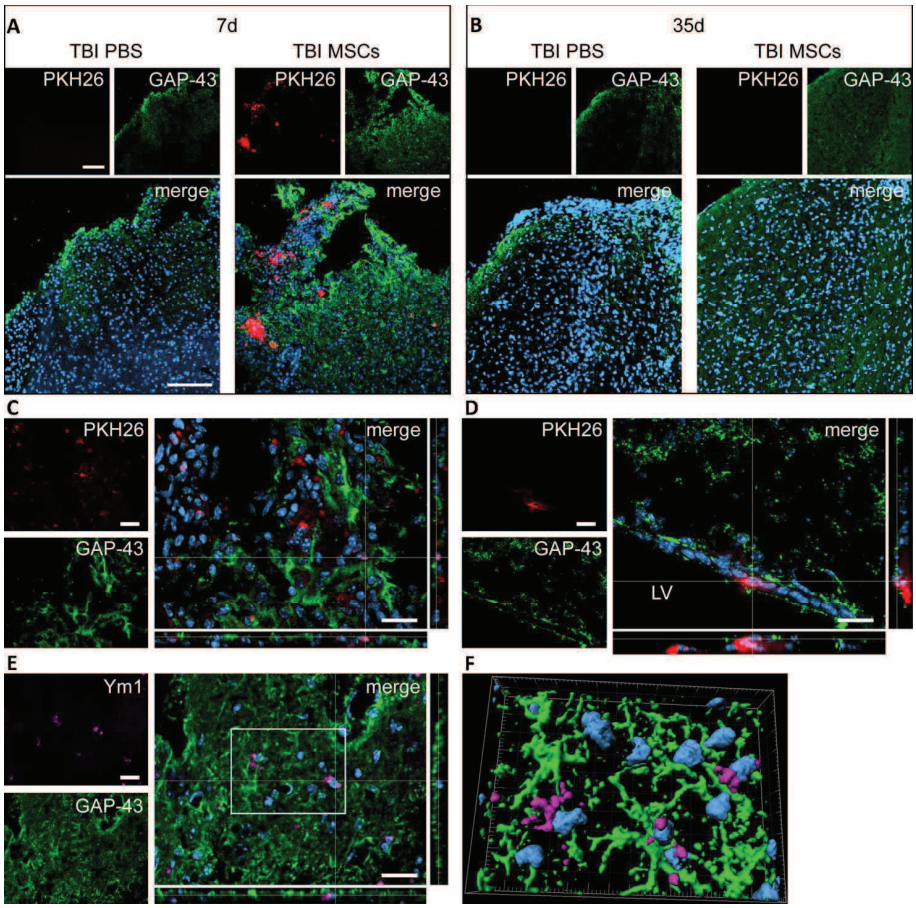

Figure 8. Localization of PKH26-labelled MSCs and immunofluorescence for GAP-43 and Ym1 in the injured cortex. Representative micrographs at low magnification showing the presence of PKH26-labelled MSCs (red) in cortical areas positive for GAP-43 (green) at 7d (A) or 35d (B) after TBI (nuclei are in blue, bar = 100 μm). PKH26 was visible only at 7d in mice infused with MSCs, while no PKH26 positivity was detectable at 35d in either PBS or MSCs treated mice. GAP-43 appeared to be increased at 7d and 35d in mice receiving MSCs. In TBI MSCs mice at 7d, PKH26 positive cells were found either in association with GAP-43 positive cells or in areas negative for GAP-43 (C). When MSCs reached the neurogenic niche in the subventricular zone, they localized close to GAP-43 positive cells (D). M2 polarized cells (Ym1 positive, purple) were located in areas positive for GAP-43 (E) and showed strong association with GAP-43 positive cells (three-dimensional rendering, F). Bars in C, D and E = 20 μm. LV= lateral ventricle.

3.4.6 MSC exposure switches microglia from M1 to M2 polarization *in vitro*

To establish a direct link between MSC infusion and changes in the activation state of M, we co-cultured primary murine microglial cells under basal or inflammatory conditions, with MSCs.

MSC exposure significantly up-regulated *Ym1* (Relative mRNA expression: 3.37±1.82) and *CD206* (Relative mRNA expression: 2.42±0.80) transcripts (Figure 9A, C) in microglia cells. CD206 transcript was similarly upregulated when microglia were co-cultured with MSC using the transwell system (Relative mRNA expression: 2.75± 0.25). Accordingly, immunofluorescence analysis showed that 3d after co-culturing with MSCs, a small proportion of microglial cells became Ym1 positive while the large majority had higher levels of CD206 protein expression than the pure microglial cultures maintained in basal conditions (Figure 9B, D). No major changes in CD68 protein expression could be observed after MSCs co-culturing with microglia, which is characterized *in vitro* by high basal CD68 expression.

Next we used 2 different pro-inflammatory stimuli (TNFα/IL-17 or TNFα/INFγ) to verify the ability of MSCs to directly revert the M1 phenotype acquired by microglia after an inflammatory challenge and to steer traits towards M2 polarization (see experimental plan, Figure 1B). As expected, exposure to both pro-inflammatory stimuli up-regulated *iNOS* transcript

(TNFα/IL-17: 24.06±5.68; TNFα/INFγ: 133.00±1.50; Figure 10A) compared to controls. When MSCs were applied to microglia 2h after the pro-inflammatory stimulus they reverted the *iNOS* up-regulation (TNFα/IL-17 + MSCs: 11.61±0.39; TNFα/INFγ + MSCs: 34.00±1.47; Figure 10A) and increased *Ym1* and *CD206* when considering the most effective pro-inflammatory challenge, namely TNFα/INFγ (*Ym1:* TNFα/INFγ: 0.36±1.09, TNFα/INFγ + MSCs: 4.32±1.86; *CD206:* TNFα/INFγ: 0.44±2.20, TNFα/INFγ + MSCs: 3.52±1.39; Figure 10B, C). Similar results were obtained following indirect co-culture of microglia challenged with TNFα/IL-17 with MSCs (iNOS expression: -60% in direct co-cultures; -49% in transwell system; CD206 expression: +300% in direct co-cultured; +460% in transwell system).

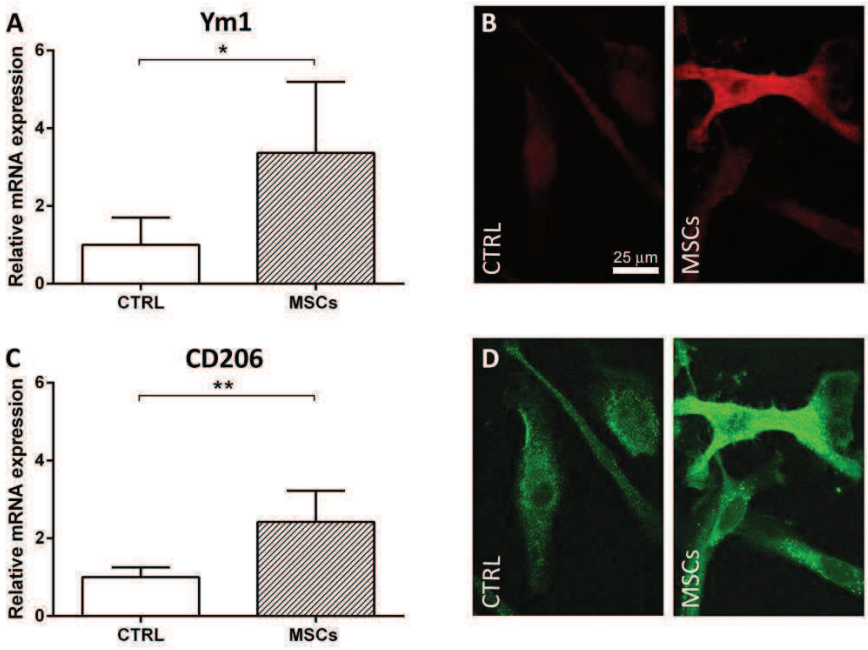

Figure 9. *In vitro* analysis of microglia markers after MSC exposure. Microglia expression of Ym1 (A-B) and CD206 (C-D) in control condition or in co-culture with MSCs for 72h. Quantification of mRNA expression indicates an up regulation of both M2 markers induced by MSC co-culture (A, C). Representative confocal images of pure microglial cultures and microglia co-cultured with MSCs stained for Ym1 (B) and CD206 (D). Data are mean±SD from 3 independent experiments.*p<0.05, ***p*<0.01, unpaired t-test.

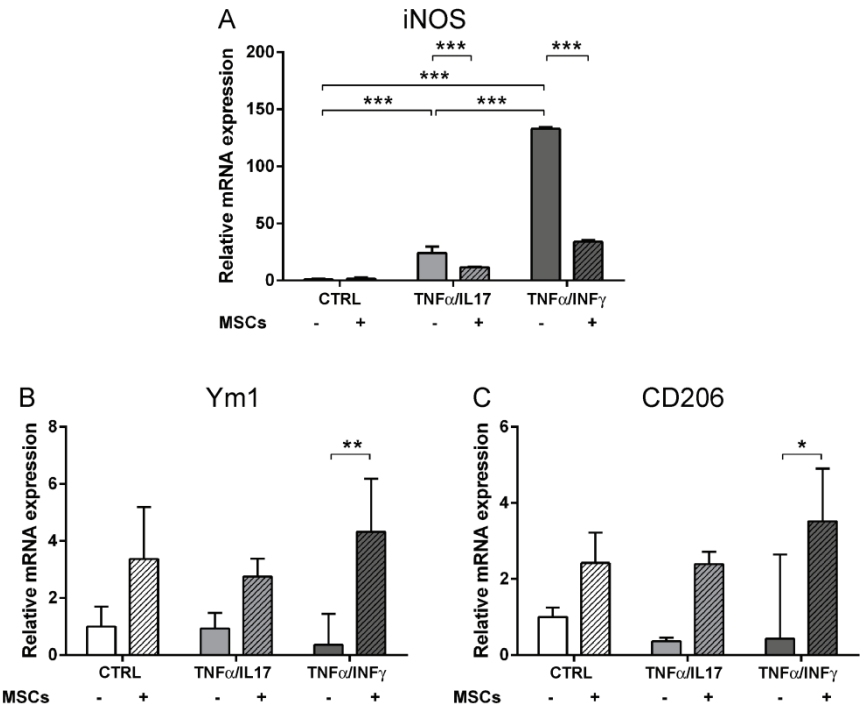

Figure 10. *In vitro* analysis of M1 and M2 polarization markers following exposure to pro-inflammatory stimuli and MSCs. mRNA expression for *iNOS* (A), *Ym1* (B) and *CD206* (C) in control or activated (exposed to TNFα/IL17 or TNFα/INFγ) microglia, maintained *in vitro* in isolation or co-cultured with MSCs. Data are mean±SD from 3 independent experiments.*p<0.05, **p<0.01, ***p<0.001 2-way ANOVA followed by Tukey's test.

3.5 Discussion

This study found that icv infusion of MSCs after TBI induces early and lasting acquisition of protective M2 traits by M. MSC-induced M2 polarization is indicated by: a) up-regulation of the M2 expression markers *Ym1*, *Arginase-1* and *CD206* in mice 3d and 7d after TBI and b) reduction of the lysosomal activity in M at the contusion core and bordering regions 7d after TBI. This phenotypic profile suggests selection of M towards the M2a subpopulation, characterized by pro-regenerative activity and reduced phagocytosis[38]. Induction of a M beneficial phenotype by MSCs is associated with other pro-regenerative changes of the lesioned microenvironment and with early and persistent recovery of neurological functions. These results were obtained infusing into the mouse injured brain human MSCs, the most relevant cells in a translational perspective[39]. Indeed, we recently reported the absence of innate inflammatory responses after human MSC infusion in immunocompetent mice[21]. Importantly, we have established a direct link between MSC activity and M phenotypical switch in an *in vitro* co-culture model, where MSCs were able to reverse the M1 pro-inflammatory phenotype acquired by microglia after 2 independent inflammatory challenges and to steer traits towards M2 polarization.

By interacting with other inflammatory cells, resident microglia and recruited monocytes retain a prominent role in post injury damage, contributing to both pathogenic and protective mechanisms. M can directly protect neurons[40] and

participate in hippocampal neurogenic processes after brain injury[41]. In addition, M eliminate invading neutrophils by phagocytosis[42] though they are a target for infiltrated T-cells, which can orchestrate their polarization state[43], allowing M to participate in the cytotoxic response, immune regulation, and injury resolution. The M2 phenotype is acquired early on after acute brain injury, but it vanishes very soon, favoring the balance towards the M1 phenotype that contributes to excessive inflammatory and immune reactions exacerbating injury progression[19,35]. Our results, in line with this evidence, show the transient up-regulation of M2 markers (at 3d but no longer at 7d post-TBI) in TBI PBS compared to sham-operated mice. More importantly, we showed that MSC infusion skews M at the injured site by enhancing the amount of “alternatively activated” M positive for M2 markers both 3d and 7d after TBI.

One previous study reported that intravenous administration of multipotent adult progenitor cells (MAPC) raised the percentage of T regulatory cells in the periphery (spleen and blood) and the ratio of M2:M1 macrophages in the brain. Remarkably it also reported that direct contact between the MAPC and splenocytes is required for modulation of parenchymal microglia after MAPC administration, thereby indicating a central role of the spleen in MAPC-mediated neuroprotection[44]. Conversely our data provide evidence that MSCs can also act as a local “bio-reactor” within the brain. In our model icv injected MSCs can be detected both in the ventricles and at the lesion site in TBI mice while they are

confined into the ventricles in sham-operated mice[21] supporting a local action of MSCs on host M.

M phenotypic changes is associated with an increased recruitment of $CD45^{high}$ positive infiltrating monocytes with no major changes in overall CD11b immunoreactivity in TBI mice, infused with MSCs. These data indicate a crucial role of infiltrating monocytes in switching the inflammatory environment towards a protective phenotype upon MSC administration.

In vitro experiments with MSCs and purified microglial cells show that MSCs directly up-regulate the M2 polarization and counteract the microglia reaction to an inflammatory challenge, by switching microglia from a cytotoxic to a beneficial phenotype. Moreover data obtained in a transwell system together with the analysis of the physical interaction between MSCs and the M2 cells in the injured brain strongly suggest that MSCs control M polarization through the release of active molecules rather than cell to cell contact[9,44]. Our data extend previous *in vitro* evidence obtained using lipopolysaccharide-stimulated BV2 cells and MSCs[45,46], showing reductions of pro-inflammatory mediators (such as iNOS, TNFα, INFγ) after MSC exposure.

To gain insight into the functional meaning of M polarization *in vivo* we analyzed the expression of CD68, a lysosomal glycoprotein associated with phagocytic function[36,47] and involved in lysosomal traffic[48,49]. CD68 was similarly expressed at both mRNA and protein levels in TBI mice infused or not with MSCs. However, the cellular distribution of the antigen differed

after MSC infusion. Indeed quantitative confocal analysis showed that the expression of CD68, which normally sits in the cytoplasm, was decreased at the membrane level after MSC transplantation. Notably, CD68 on the cell surface is associated with an active scavenging commitment, in line with surface functions, such as internalization of target ligands by elicited macrophages[37]. The decrease of CD68 on the cell membrane after MSC infusion suggests a functional switch of M towards a less active phagocytic cell population. The role of phagocytosis in the lesion progression is complex and, unlike other M markers, phagocytic activity does not seem clearly linked to a specific M1 or M2 polarization state. Cells belonging to the M2c class of protective polarization as well as M1 toxic cells all have phagocytic functions[38]. On the one hand, phagocytosis is needed to remove cell debris and dying cells, thus limiting the propagation of danger signals that can further exacerbate damage progression ("secondary phagocytosis"). On the other hand, under certain conditions such as inflammation, phagocytosis can target viable neurons, thus causing their death ("primary phagocytosis"). After injury, such detrimental phagocytic activity may result from exposure of eat-me signals on otherwise viable neurons as a result of subtoxic and reversible insult[50,51]. The ability of MSCs to influence and limit "primary phagocytosis" might be a key pathway to confer protection after TBI. The complexity of *in vivo* phagocytosis accounts for the apparently divergent results obtained *in vitro*

by Giunti *et al.*[45] where MSCs enhance the phagocytic activity of LPS-activated microglia.

To further characterize the functional state of M after TBI and MSC infusion we quantified Ym1 protein expression and Ym1/CD68 co-localization. MSCs significantly increased the number of Ym1+ cells but it reduced their expression of CD68, suggesting this M population may belong to the M2a subtype (characterized by the expression of Ym1, Arg1 and CD206), which is involved in growth stimulation and tissue repair[38] but not in phagocytic activity.

Gene expression in the pericontusional tissue showed that MSC administration was also associated to a more general reprogramming of the microenvironment likely involving other parenchymal cell populations. Actually MSC infusion reduces *GFAP* and attenuates down-regulation of *VEGF* and increase immunoreactivity of the proregenerative axonal regeneration marker GAP-43. These changes are consistent with data showing reduction of the gliotic scar and increase in vessel density described by our[9,21] and other groups[52–54]. Analysis of inflammatory cytokines and chemokines showed significant up-regulation of IL1β after MSC infusion at 7d after TBI. A similar IL1β increase was reported by Giunti *et al.*[45] in BV2 cells challenged with LPS, after MSC co-culturing. Furthermore data from a model of spinal cord injury indicate that IL1β may support the induction of M alternative activation[55], suggesting a role in injury resolution and brain repair besides being involved in the pro-inflammatory response. We also detected a

significant up-regulation of CCL2 after MSC infusion in TBI mice at 7d. CCL2 promotes chemotaxis of monocytes and hematopoietic progenitors to inflammation sites[56,57] and may therefore indicate increased mobilization and infiltration of peripheral immune cells by MSCs. Consistently we show here a significant increase in $CD45^{high}$ positive infiltrating monocytes in the injured tissue. This could explain the modified inflammatory milieu, potentially more prone to promote M2 polarization. Indeed, among infiltrating immune cells, as mentioned previously, lymphocytes are recognized orchestrators of M polarization. In particular T-regs and Th2 cells, through IL4 release, are able to induce the M2 phenotype[43,58,59]. Finally, CXC chemokines are recognized as regulatory linkers between inflammation and angiogenesis, providing fine tuning that leads to tissue repair[60]. Globally, these data indicate that MSCs besides inducing beneficial traits in M, act on other brain cell populations, skewing the balance of the microenvironment towards protection.

In conclusion MSCs induce an early, lasting acquisition of a protective M2 phenotype both *in vivo* and *in vitro*. Elevated M expression of Ym1 and reduction of phagocytosis suggest polarization towards the M2a subpopulation, involved in growth stimulation and tissue repair. Protective/pro-regenerative changes of the lesioned microenvironment occur early after injury and may contribute to lasting protective and remodeling processes.

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Chapter 4

General Conclusion

4.1 Summary

TBI is the leading cause of mortality and disability among young individuals. Today no single-agent pharmacologic treatment has been successfully translated to the clinical setting underlining the need to focus on strategies that affect simultaneously multiple injury mechanisms. A key issue in TBI care is the temporal progression of tissue damage. Long-lasting pathological cascades, activated after the mechanical injury, progressively affect the tissue surrounding the contusion-core. On the other side, next to toxic events, agiogenesis, neurogenesesis and brain plasticity processes are fundamental regenerative mechanisms that are induced after acute brain injury. Those are however largely ineffective in counteracting the lesion progression but they introduce the rational for developing strategies able to foster this endogenous response. Overall the challenge is to find therapeutic approaches able to affect at the same time both neuroprotective and neurorestorative pathways and maximize the ability to improve outcome in TBI.

In this perspective, MSCs are promising candidates since accumulating experimental evidence indicates that they affect simultaneously multiple mechanisms of protection and repair

after injury and an increasing number of studies show efficacy in different CNS pathologies.

In our work we have dealt with an important translational issue related to the optimization of the protocol to be used in clinical setting. The need of a quick intervention after TBI forces the choice towards an allogenic source of MSCs, opening the question of immunosuppression. However immunosuppression in TBI patients is associated with an increased susceptibility to infections which is directly related to unfavorable outcome. Our work has demonstrated the feasibility of this treatment, showing that, in a system where immunological mismatches are amplified (human MSCs xenotransplanted in mice), the long-term effects of MSCs never differed between immunocompetent and immunosuppressed TBI mice. These important results suggest the feasibility of allogenic cell transplantation in immunocompetent TBI patients without loosing MSC efficacy, an important step in the definition of a successful protocol transferrable to the clinic.

Looking at the long term effects of MSCs in TBI mice we have seen improvement of functional deficits (sensorimotor and cognitive function) associated with reduction of anatomical damage, increase of neurogenesis and a remodeling effect on pericontusional brain tissue, i.e. reduction of gliotic scar area and increase of vessel density, mechanisms favoring a pro-healing function and brain plasticity processes. These events underline the multiple aspects of MSC action, stimulating both protective and regenerative cascades.

In the second part of our work we focus on early events associated with TBI and MSC treatment, in particular we investigated the MSC-mediated modulation of resident/recruited immune cells. The data obtained show MSC ability to increase macrophage recruitment at the lesion site and to skew microglia/macrophage activation towards a M2 protective phenotype that is associated with a decrease of phagocytic activity and an increase of regenerative processes. Importantly, cell-cell contact doesn't seem to be necessary for this phenotypical switch, since M2 polarized cells were rarely found directly in contact with MSCs. Moreover with the *in vitro* experiments we have shown that MSCs have a direct effect on isolated microglia, favouring M2 polarization both in basal condition and after pro-inflammatory stimuli, and that the skew towards a protective phenotype is obtained both in MSCs/microglia co-culture in direct contact and in a transwell system. These data indicate that the mechanisms of action of cell therapy pass through the release of molecules with paracrine effects. Finally in traumatized brain, the phenotypical switch of microglia/macrophage is associated with the promotion of protective cerebral microenvironment, more prone to regenerative processes. These data indicate that MSCs are able to modulate the damaged microenvironment, both by a direct effect (i.e. secreting neurotrophins) or by bystander effects passing through microglia/macrophages, whose polarization towards protective phenotype promotes regenerative endogenous pathways. Altogether these direct

and indirect MSC-mediated phenotypical changes are responsible for the improvements of functional deficits.

4.2 Conclusions

Mesenchymal cell therapy is a valid strategy for TBI patients. The multitarget potential of MSCs fulfills the need of the traumatized brain in which multiple injury mechanisms are triggered after the mechanical impact. We have provided evidence of MSC and immune cell interaction *in vitro* and *in vivo* after injury, showing a causal relationship between MSC infusion and the acquisition of microglia/macrophages beneficial traits after TBI. Infused MSCs are able to reprogram the local inflammatory microenvironment from detrimental to beneficial, favoring protective/pro-regenerative changes of the lesioned tissue and contributing to permanent improvement of neurological function. Importantly we have also addressed an important preclinical issue, demonstrating that allogenic MSC infusion after TBI does not elicit adverse immune host reaction. We have shown that the efficacy of MSC is not affected by immunosuppression and that immunocompetent mice show a similar degree of protection compared to immunosuppressed mice, supporting the possibility of allogenic MSCs therapy.

4.3 Future perspectives

The current literature supports the use of MSCs for acute brain injury, however there are still a few fundamental issues that need to be understood to proceed safely to clinical trials.

MSCs isolated from different cellular sources have some general characteristics in common that allow their classification as stromal cells, according to the *consensus* set out by the International Society for Cellular Therapy (ISTC)[1]. However MSCs are very heterogeneous populations[2]: both source-dependent[3,4] and intra source donor-dependent[5,6] differences have been shown among MSC populations. They could be related to intrinsic genetic differences or to several factors including sampling bias during marrow aspiration[7,8], age of the donor[9] and methods used to culture and expand MSCs post-harvest[10,11]. The selected criteria for the definition of MSCs are based on surface marker expression and *in vitro* mesengenic differentiation potential, however no parameters have been established to predict *in vivo* potency for specific pathologies and heterogeneous effects have been obtained. Thus to efficiently transfer MSC therapy to the clinical setting, the development and implementation of specific *in vitro* assays able to predict the biological potency of MSCs, is urgently needed. One example has been provided by Rizzo et al. (2011) reporting that IL-10 stimulated expression of HLA-G in MSCs is significantly positively correlated with inhibition of PBMC proliferation stimulated with PHA[12]. In addition François et al. (2012) demonstrated that the MSC immunosuppressive

potential on T cell proliferation inhibition is correlated to the amount of indoleamine 2,3-dioxygenase (IDO) produced[13]. These data suggest that these assays may be used to evaluate and compare the immunoregulatory function of MSC populations. However it should be noted that *in vitro* potency assays may not reliably predict cell function *in vivo*. For example the long-term follow up of patients with steroid-refractory acute graft versus host disease treated with MSCs revealed no correlation between the ability of MSCs to suppress T cell proliferation in mixed lymphocyte assays *in vitro* and their clinical efficacy *in vivo*[14]. So it is of pivotal importance to find *in vitro* tests that predict *in vivo* potency. An useful example has been provided by Deskins et al. (2013) for wound healing repair: the authors characterized ten BM-MSCs cell lines for their *in vitro* growth rate, proliferation and viability and examined MSC ability to *in vivo* engraft and form granulation tissue in a murine wound model. The combination of the *in vitro* test performances accurately predicted which lines functioned better *in vivo*[15]. Such tests for therapeutic potential prediction are missing for CNS acute injury suggesting that efforts should be addressed in order to select the best cell lines to be used for clinical trials. In this way the variability could be confined to patients injury and response to the therapy and not to donor-to-donor and intra-populations heterogeneity of MSCs.

Another important aspect to be solved in order to safely move to clinical trials is the comprehension of the mechanisms of the interaction between infused MSCs and injured tissue.

MSCs contribute to the protection/regeneration modifying the injured environment by multiple mechanisms but still little information is available on the components of the dialogue between resident and transplanted cells, and on the potential of MSC priming to secrete protective molecules and to promptly respond to the need of the injured tissue. On one hand different evidence shows that MSCs primed by inflammatory stimuli like INFγ[16] or IL-1β[17] have improved efficacy in inflammatory conditions like ulcerative colitis, suggesting that the sensing of the inflammatory milieu (either a pre-stimulus before transplant or an active interplay within damaged tissue) is necessary to push MSCs towards the secretion of protective molecules. On the other hand the use of cell-free therapeutic approaches based on conditioned medium (CM) derived from MSCs cultured in basal condition without any injury-activating stimulus has been proved to be effective in different conditions like lung injury[18–20], kidney injury[21], spinal cord injury[22] and traumatic brain injury[23,24]. Those data indicate that MSCs secrete protective molecules in basal condition and that cell-free approaches could be a valid alternative to cell therapy. Again the heterogeneity of source and donor could affect CM composition and efficacy. Thus identification of the protective molecules secreted by MSCs would allow the synthetic reconstruction and the standardized composition of protective cocktails to be tested in different conditions.

In conclusion further steps toward clinical translation include the comprehension of the biological features of each

MSC sub-population to understand the determinants of their intrinsic heterogeneity and to predict their *in vivo* potency and ultimately to identify the “ideal MSC population” for TBI. In parallel the identification of secretome components responsible for the given protection will allow the standardization of a safe and fully characterized cell-free protocol transferable to the clinic.

4.4 References

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Publications

Bone marrow mesenchymal stromal cells drive protective M2 microglia polarization after brain trauma

Zanier ER, **Pischiutta F**, Riganti L, Marchesi F, Turola E, Fumagalli S, Perego C, Parotto E, Vinci P, Veglianese P, D'Amico G, Verderio C, De Simoni MG

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Immunosuppression does not affect human bone marrow mesenchymal stromal cell efficacy after transplantation in traumatized mice brain.

Pischiutta F, D'Amico G, Dander E, Biondi A, Biagi E, Citerio G, De Simoni MG, Zanier ER.

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Six-month ischemic mice show sensorimotor and cognitive deficits associated with brain atrophy and axonal disorganization.

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