Human Neural Stem Cells Ameliorate Autoimmune Encephalomyelitis in Non-human Primates

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Objective: Transplanted neural stem/precursor cells (NPCs) display peculiar therapeutic plasticity in vivo. Although the replacement of cells was first expected as the prime therapeutic mechanism of stem cells in regenerative medicine, it is now clear that transplanted NPCs simultaneously instruct several therapeutic mechanisms, among which replacement of cells might not necessarily prevail. A comprehensive understanding of the mechanism(s) by which NPCs exert their therapeutic plasticity is lacking. This study was designed as a preclinical approach to test the feasibility of human NPC transplantation in an outbreed nonhuman primate experimental autoimmune encephalomyelitis (EAE) model approximating the clinical and complex neuropathological situation of human multiple sclerosis (MS) more closely than EAE in the standard laboratory rodent.

Methods: We examined the safety and efficacy of the intravenous (IV) and intrathecal (IT) administration of human NPCs in common marmosets affected by human myelin oligodendrocyte glycoprotein 1-125–induced EAE. Treatment commenced upon the occurrence of detectable brain lesions on a 4.7T spectrometer.

Results: EAE marmosets injected IV or IT with NPCs accumulated lower disability and displayed increased survival, as compared with sham-treated controls. Transplanted NPCs persisted within the host central nervous system (CNS), but were also found in draining lymph nodes, for up to 3 months after transplantation and exhibited remarkable immune regulatory capacity in vitro.

Interpretation: Herein, we provide the first evidence that human CNS stem cells ameliorate EAE in nonhuman primates without overt side effects. Immune regulation (rather than neural differentiation) is suggested as the major putative mechanism by which NPCs ameliorate EAE in vivo. Our findings represent a critical step toward the clinical use of human NPCs in MS.

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Spontaneous neural tissue repair may occur in patients affected by acute and/or chronic inflammatory and degenerative disorders of the nervous system. However, this process is not robust enough to promote a functional and stable recovery of the nervous system architecture.[1] Thus, the development of cell-based therapies designed to promote functional (direct vs indirect) neural cell replacement is anticipated . However, most (if not all) of the experimental cell therapies with neural lineage-committed progenitors have failed to foster substantial repair in disease models where the anatomical and functional damage is widespread and an inflamed and/or degenerative microenvironment coexists.[2]

Conversely, the systemic injection of somatic as well as embryonic stem (ES) cell-derived neural stem/precursor cells (NPCs) has provided a remarkable amelioration of the clinicopathological features of rodents with experimental autoimmune encephalomyelitis (EAE), the animal model of multiple sclerosis (MS).[3–5]

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This result has been shown to be dependent on the capacity of transplanted NPCs to engage multiple mechanisms of action within specific microenvironments in vivo.[6] Among a wide range of potential therapeutic actions—and in addition to the (expected) cell replacement[3]—this phenomenon may also occur via bystander effect. This effect is likely exerted by undifferentiated NPCs releasing immune regulatory and neuroprotective molecules within specific central nervous system (CNS)[3–5,7] vs non-CNS areas,[8] in response to stimuli elicited by inflammatory cells[7] (therapeutic plasticity). The molecular and cellular mechanism(s) that sustain the therapeutic plasticity of the NPCs remain far from fully characterized.

Thus, cell therapies delivering therapeutically plastic stem cells[3,4] may represent in the near future a plausible alternative strategy in treating inflammatory and degenerative nervous system disorders.[6]

However, prior to developing human applications for nervous system disorders with stem cell-based therapies, we need to address the preliminary and unresolved issues: 1) the ideal stem cell source for transplantation; 2) the most appropriate route of stem cell administration; 3) the best approach to achieve functional and long-lasting integration of transplanted stem cells into the host tissue; and, finally, 4) the clear determination of both the short- and long-term side/toxic effects of the transplantation procedure.

To begin to address these issues, this study was designed as a preclinical study for testing the feasibility (safety and efficacy) of the systemic administration of human NPCs (hNPCs) in common marmosets with myelin oligodendrocyte glycoprotein (MOG)-induced chronic EAE. This animal model is an outbred nonhuman primate disease model that demonstrates a rather unusual yet dynamic demyelination pattern (type I and II)—ranging from enlargement to shrinking to repair[9]—which importantly more closely approximates the neuropathological situation of MS[10] than the standard laboratory EAE model in small rodents.[11–13] The experiments completed in this nonhuman primate model represent a critical aspect of the study, in that numerous mouse studies have reported that both fate and function of transplanted cells are very likely to be determined by the lesion milieu.[3,7,14–16] NPCs injected intravenously (IV) or intratecally (IT) at disease onset attenuated disability and increased survival of EAE marmosets.

Finally, of particular importance in cell and organ transplantation—where allo-/xenoreactivity and/or side effects of immunosuppressive and immunomodulatory treatments might offer major complications—preclinical proof-of-principle in a relevant nonhuman primate model is strongly recommended.[12] Our study validates the feasibility of the systemic administration of clinically transferable human tissue-specific somatic stem cells and represents a critical step toward the clinical use of NPCs in the treatment of MS.

Materials and Methods

Human Neural Stem/Precursor Cell Isolation and Expansion

For all experiments, homogeneous batches of hNPCs at subculturing passages 15–23 were used. Further details are provided in the Supplementary Data.

Vector Production and In Vitro Transduction

To avoid carryover of viral particles, hNPCs were used for transplantation after 3–13 passages post-transduction (up to 30 subculturing passages in total). A total of 8 different batches of hNPCs, corresponding to different dates of transduction, were used in the experiments. Further details are provided in the Supplementary Data.

hNPC Functional Characterization and Chromosome Analysis

Details are provided in the Supplementary Data.

Generation of MOG Peptide-Reactive T-Cell Lines

At necropsy, cell suspensions were prepared from the axillary and inguinal lymph nodes, and stimulated ex vivo with MOG74–96 to establish antigen-specific T-cell lines (TCLs).[17] Cocultures were run by plating purified MOG74–96–specific TCLs in 24-well plates (10^6 cells in a final volume of 800μl) in the continuous presence of hNPCs at 2:1, 4:1, or 8:1 TCL:NPC ratios. After 48 hours, 0.5μCi/well [3H]thymidine was added, and the incorporation of the radiolabel was determined 18 hours later using a Matrix 9600 beta counter (Packard, Meriden, CT).

Peripheral Blood Mononuclear Cell/NPC Cocultures

Peripheral blood mononuclear cells (PBMCs) were isolated as described in the Supplementary Data. Human NPCs were added in the same well at 2:1, 4:1, and 8:1 PBMC (or $CD4^+$ T cell)/NPC ratios directly at the time of cell plating. In some conditions, hNPCs were seeded in the upper chamber of 0.4μm pore size transwell inserts (Nunc, Rochester, NY). Paraformaldheyde (PFA)-fixed NPCs were used as negative control. Carboxyfluorescein succinimidyl Ester (CFSE) histograms were used to calculate the percentages of proliferating cells that had undergone ≥1 cell divisions, and cell proliferation was monitored by CFSE partitioning at flow-activated cell sorting (FACS) 72 hours poststimulation, as previously described.[18]

Dendritic Cell/NPC Cocultures

Monocytes were isolated from PBMCs by CD14 magnetic isolation. Differentiation into dendritic cells (DCs) was induced as described in the Supplementary Data. Cocultures were run by plating purified $CD14^+/CD1a^-$ monocytes in 6-well plates (5×10^6 cells/in a final volume of 4ml) in the continuous presence of hNPCs at 2:1, 5:1, or 10:1 DC:NPC ratios. In some conditions, human NPCs were seeded (2:1 DC:NPC ratio only) in the upper chamber of 0.4μm pore size transwell inserts (Nunc), to avoid cell-to-cell contact.

FACS analysis on either immature DCs (iDCs) or mature DCs (mDCs) from cocultures was performed at day 6 and 8, respectively.

Allogeneic Mixed Leukocyte Reaction

Lipopolysaccharide (LPS)-activated mDCs from cocultures were used as stimulators for allogeneic PBMCs within a classical mixed leukocyte reaction (MLR) as described.[19] Further details are provided in the Supplementary Data.

FACS Analysis

Details are provided in the Supplementary Data.

Migration Assay

Details are provided in the Supplementary Data.

EAE Induction

Common marmosets obtained from the Biomedical Primate Research Center breeding colony (Rijswijk, The Netherlands) were immunized with 100μg recombinant human (RH) MOG dissolved in 300μl phosphate-buffered saline (PBS) emulsified with 300μl complete Freund adjuvant (Difco Laboratories, Detroit, MI) as described.[20] Further details are provided in the Supplementary Data.

hNPC Transplantation

An average of 10^6 cells were obtained from a $75cm^2$ T flask; an average of 10 to 20 flasks were pooled for each transplantation experiment. On the day of transplantation, serially passaged 7-day-old transduced lentiviral vector (LV) human phosphoglycerate kinase promoter (LV.eGFP-T) hNPCs were collected by a first centrifugation (0.1 relative centrifugal force × 15 minutes) and mechanically dissociated to obtain a single cell suspension. hNPC transplantation occurred under ketamine (30 mg/kg intramuscularly, ASP Pharma, Basel, CH) by injection into either the cisterna magna (2×10^6 cells in 300μl PBS) or the tail vein (6×10^6 cells in 1ml PBS). The cell suspension remaining in the tubes after the procedure was rechecked for cell viability, which was usually ≥80%. Further details are provided in the Supplementary Data.

Immunosuppressive Drug Administration

All EAE marmosets received daily cyclosporine A (CsA; Sandimmun, Novartis, Basel, Switzerland) (10mg/kg intramuscular) on randomization into treatment groups—at 48 hours after either the clinical or magnetic resonance imaging (MRI)-based disease onset—through clinical study completion, to prevent the rejection of the transplanted cells.[21]

MRI

Details are provided in the Supplementary Data.

Neuropathology

Details are provided in the Supplementary Data.

Neurophysiology

Marmosets were anesthetized with ketamine and placed under an infrared lamp to maintain body temperature above 34°C. Further information on transcranial electrical stimulation and motor evoked potentials (MEPs) recording is provided in the Supplementary Data.

Statistical Analysis

Data were compared using the unpaired *t* test, the chi-square test, the Mann-Whitney *U* test, or 2-way analysis of variance (with Bonferroni post hoc analysis) for nonparametric data. Statistical significance was accepted with a p value ≤ 0.05.

Results

GFP Expression Without Impairment of hNPC Functional Features

We used nonimmortalized, stable, and self-renewing hNPCs, originally isolated from a single 10.5 postconception week human fetus.[22] hNPCs were tagged with a second generation LV carrying the enhanced green fluorescent protein (eGFP) (Fig 1A)[23] prior to transplantation. Immunofluorescence showed effective labeling in ≥90% of hNPCs (Fig 1B), confirmed by FACS analysis (93.05 ± 3.25%; n = 3 independent hNPC batches). The eGFP expression was stably retained over 10 subculturing passages (Fig 1C) and LV.eGFP-T did not differ from untransduced (UT) NPCs with respect to proliferation, self-renewal, and multilineage differentiation potential (Fig 1C–G). The karyotype of both LV.eGFP-T and UT hNPCs remained stable at 46, XY throughout the experiments (Supplementary Fig 1).[22]

The hNPC Immune Signature

The vast majority (≥90%) of $CD45^-$ hNPCs expressed the hyaluronic acid ligand CD44,[24] integrins (α2, α6, and β1, but not α1, α4, and β7),[25,26] and inflammatory chemokine receptors (CCR3, CCR6, CCR7, CCR9, and CXCR3, but not CCR5) (Fig 2A). Less than 25% of hNPCs expressed prominin 1 (CD133)[25] or the inflammatory chemokine receptors CCR4, CCR5, and CXCR4. Also, hNPCs expressed major histocompatibility complex (MHC) class I but not class II molecules (Fig 2A).[27] Importantly, the lentiviral transduction did not affect expression of mRNAs for the surface cell adhesion molecules (eg, CD11a, CD62L, CD44, and CD162) known to mediate NPC-to-cell interactions with ependymal and endothelial cells during inflammation (Fig 2B).[3,7,28] The receptors for the proinflammatory chemokines CCL5/RANTES (ligand for CCR1, CCR3, and CCR5), CCL20/MIP3α (ligand for CCR6), CCL21/SLC (ligand for CCR7), and CXCL12/SDF1α (ligand for CXCR4) on LV.eGFP-T hNPCs were also functional, as confirmed by in vitro chemotaxis assay (Supplementary Fig 2).

Hence, the hNPCs used in this study did bear a complex array of functional surface molecules that might be critical for sensing (micro) environmental

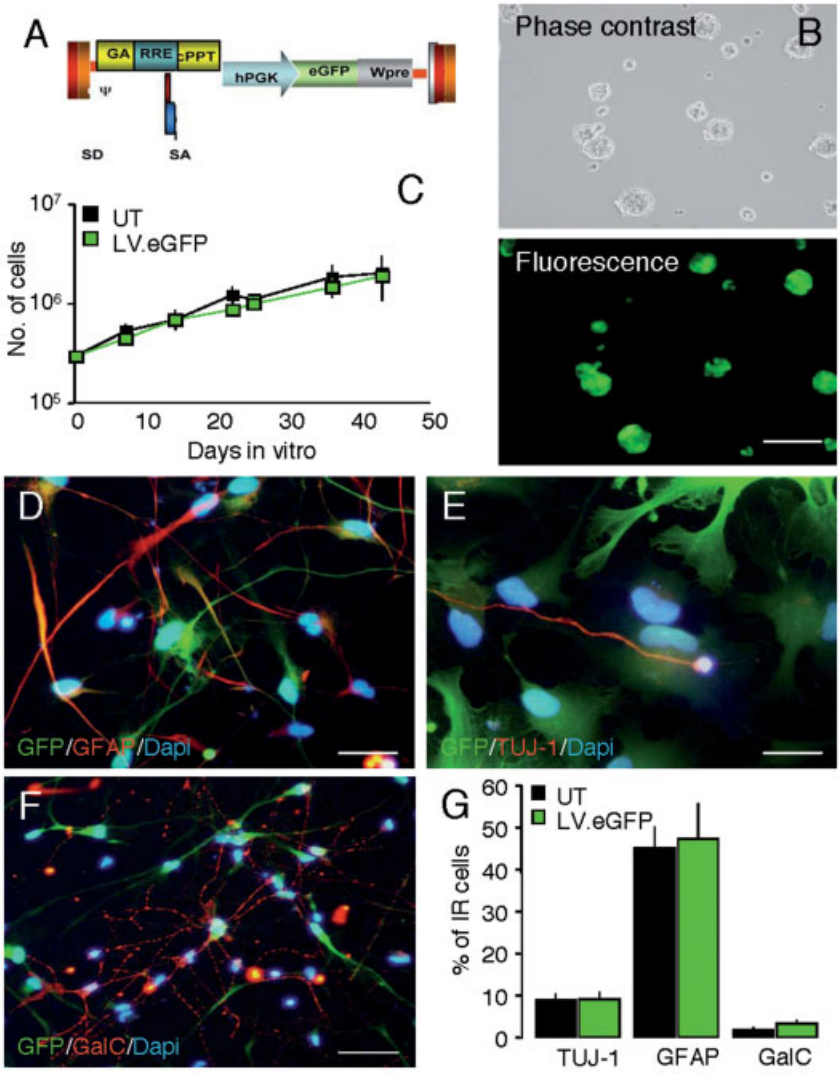

Fig 1. Stable and efficient green fluorescent protein (GFP) expression without impairment of human neural stem/precursor cells (NPC) functional features. (A) Scheme of the second generation lentiviral vector (LV) carrying the enhanced GFP (eGFP) cDNA under the human phosphoglycerate kinase (hPGK) promoter (LV.eGFP). (B) Phase-contrast and direct fluorescence views showing efficient and stable expression of the eGFP in the majority of the transduced hNPCs. (C) Untransduced (UT) (black) and LV.eGFP-T hNPCs (green) show comparable growth rates. Data in C have been generated from ≥3 independent batches of hNPCs (from p21 to p27) and are expressed as mean number of viable cells ± standard error of the mean. The scale bar in B is 150μm. (D–F) eGFP expression is maintained following differentiation on growth factor withdrawal in vitro into astrocytes (D, glial fibrillary acidic protein [GFAP]), neurons (E, TUJ-1), and oligodendrocytes (F, GalC); (G) Quantitative analysis of differentiation shows that LV.eGFP transduction does not interfere with multipotentiality of hNPC. Data in G have been generated from ≥3 independent batches of hNPCs (from p21 to p27) and are expressed as mean percentage of immunoreactive cells (over total nuclei) ± standard error of the mean. Scale bars: D, 30μm; E, 20mgr;m; F, 40mgr;m. Dapi = 4′,6-diamidino-2-phenylindole. Abbreviations: cPPT, central polypurine tract; GA, 5′ portion of the gag *gene; RRE, rev-response element; SA, splice acceptor sites; SD, major splice donor sites; Wpre, post-transcriptional regulatory element of woodchuck hepatitis virus.*

cues and for modulating migratory cell behavior on transplantation into inflammatory settings.

Human NPCs Are Immune Regulatory In Vitro

Recent observations from our own and other laboratories have shown that rodent subventricular zone–derived—and more recently also human ES cell-derived—NPCs exhibit a bystander inhibitory effect on T lymphocytes, both in vitro and in vivo.[4,5,7,8] As such, we investigated whether NPCs from the human fetal CNS might also interfere with specific immune cell functions in an in vitro setting.

We first examined the capacity of hNPCs to inhibit an antigen (myelin)-specific immune response. Interest-

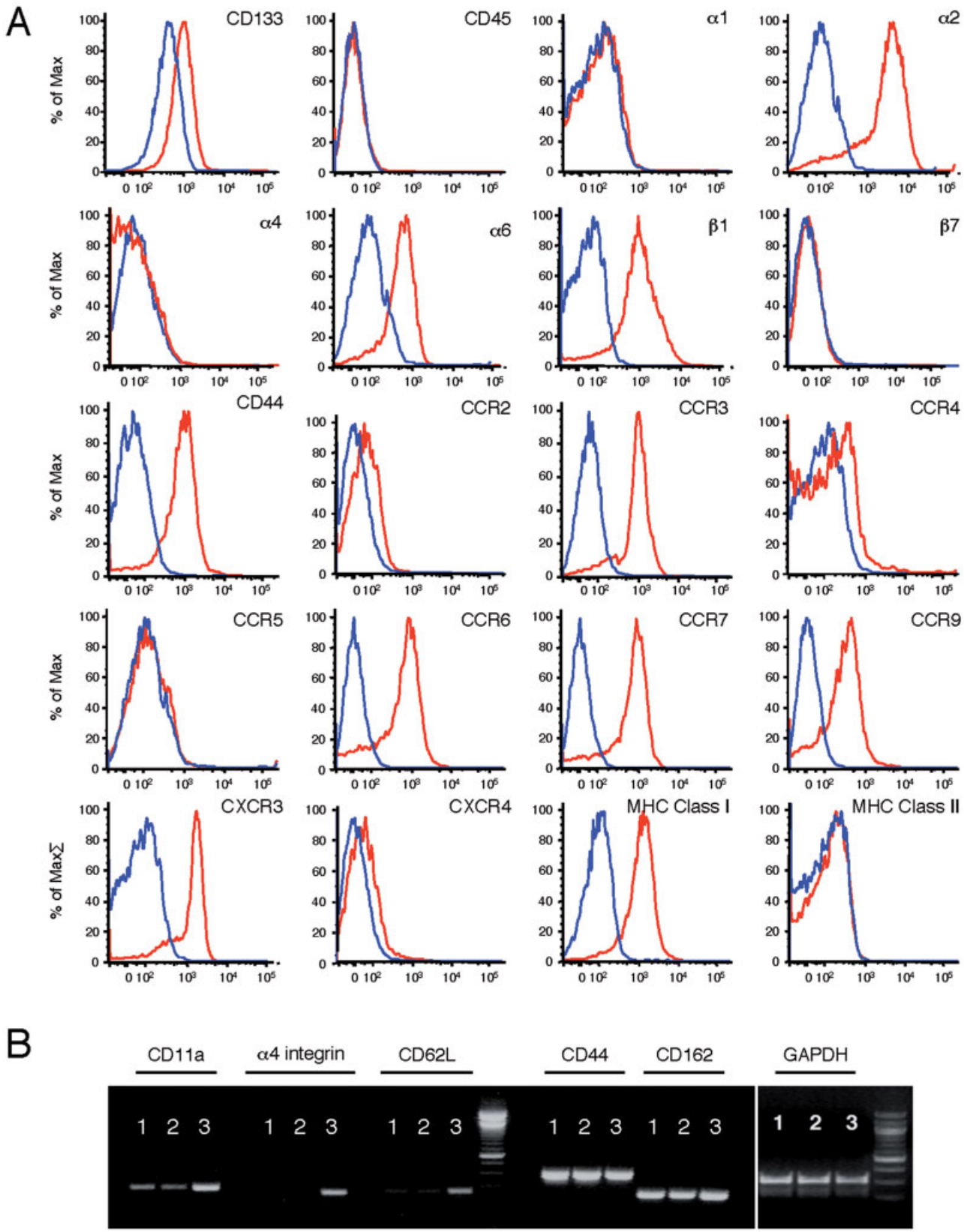

Fig 2. Human neural stem/precursor cells (hNPCs) express adhesion molecules and chemokine receptors. (A) Representative flow-activated cell sorting analyses of untransduced (UT) hNPCs at p25. The majority (≥90%) of CD45⁻ hNPCs express CD44, integrins (α2, α6, and β1, but not α1, α4, and β7) and inflammatory chemokine receptors (CCR3, CCR6, CCR7, CCR9, and CXCR3, but not CCR5). Only a fraction of hNPCs (≤25%) express CD133 and the inflammatory chemokine receptors CCR4, CCR5, and CXCR4. hNPCs express major histocompatibility complex class I but not class II molecules. Blue lines indicate the isotype control, whereas red lines indicate the stained cells. (B) Polymerase chain reaction analysis showing similar levels of CD11a, CD44, and CD162 in UT and lentiviral vector (LV) human phosphoglycerate kinase promoter (LV.eGFP) hNPCs. CD26L and α4 integrin are expressed, although at very low levels, in both cell samples. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is used as internal standard. Lane 1, UT hNPCs (p22); Lane 2, LV.GFP-T hNPCs (p17.5, 5 passages after LV transduction); Lane 3, lymphocytes.

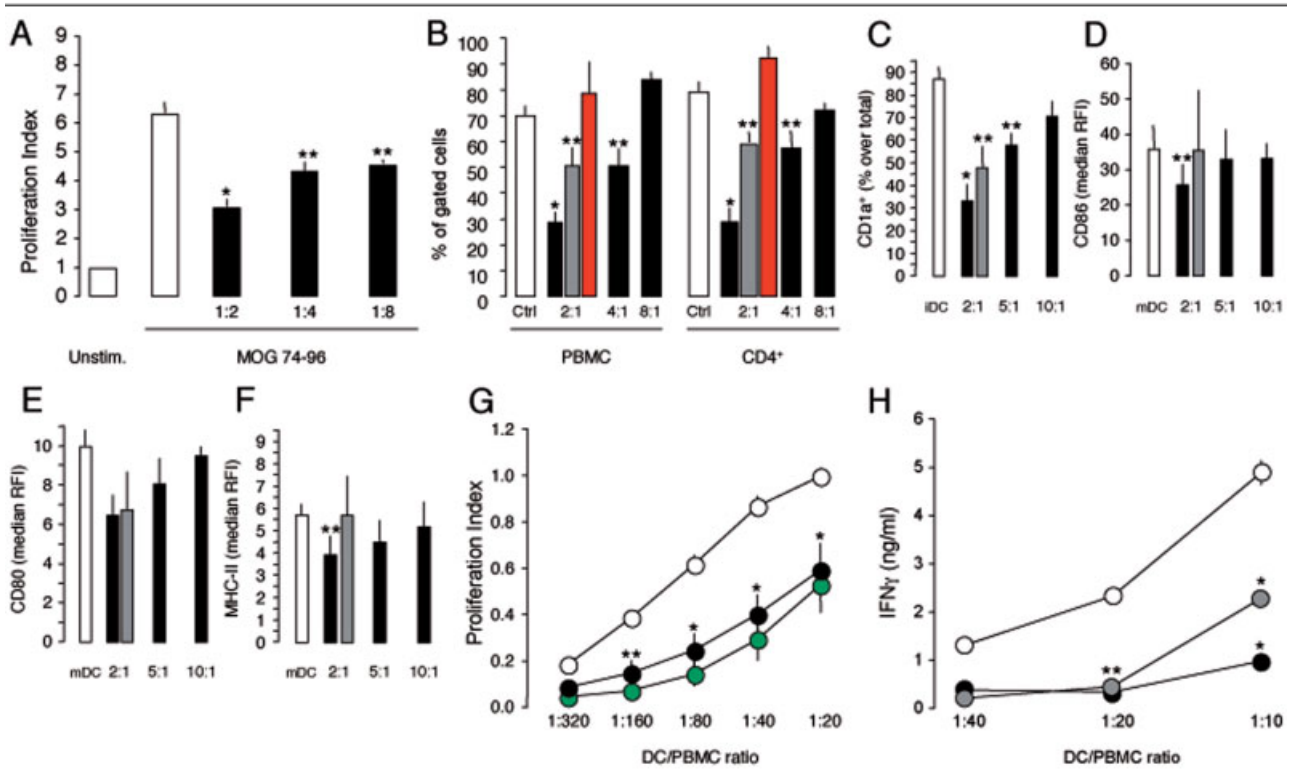

*Fig 3. Human neural stem/precursor cells (hNPCs) are immunoregulatory on T lymphocytes and dendritic cells. (A) Dose-dependent inhibition of the proliferation of myelin oligodendrocyte glycoprotein (MOG)74-96-specific T-cell lines upon coculture with hNPCs. (B) Inhibition of allogeneic immune cell responses upon activation with polyclonal stimuli, such as anti-CD3/CD28. Note the dose-dependent suppression of the cell proliferation, when hNPCs are cocultured with total peripheral blood mononuclear cells (PBMCs) or magnetically sorted $CD4^+$ T cells. Paraformaldheyde fixed hNPCs (red bars) do not interfere with immune cell proliferation. Data are expressed as mean percentage of proliferating (gated) cells (±standard error of the mean) from a total of 3 independent experiments. (C) Impairment of the differentiation of dendritic cells (DCs) from myeloid precursors. Note the dose-dependent inhibition of the up-regulation of the DC marker CD1a, when $CD14^+$ myeloid precursors are forced into differentiation in DCs with recombinant human granulocyte macrophage colony-stimulating factor and recombinant human interleukin-4 in the presence of hNPCs. Data are expressed as percentage of $CD1a^+$ cells (±standard error of the mean) from a total of 3 independent experiments with different donors. (D–F) Impairment of the maturation of DCs. Note the dose-dependent restrain of the upregulation of the costimulatory molecules CD80 (D), CD86 (E), and MHC-II (F) by DCs maturating on lipopolysaccharide activation, while cocultured with hNPCs. Data are expressed as mean relative fluorescence intensity over immature DCs (±standard error of the mean) from a total of 3 independent experiments with different donors. In A–F, white bars refer to non-cocultured immune cells; grey bars refer to trans-well experiments. Ratios in B–E refer to DC:NPC ratios. (G) Impairment of the antigen presentation capacity of DCs. Note the significant reduction of the proliferation of allogeneic peripheral blood mononuclear cells, when DCs derived from DC/NPC cocultures (black circles) were used. White circles are mature DCs (mDCs); green circles are immature DCs (iDCs). Data are expressed as mean proliferation index (±standard error of the mean) from a total of 2 independent experiments. (H) The reduction of allogeneic PBMC proliferation is paralleled by reduction of interferon (IFN)γ production. White circles are mDCs; black and grey are DCs derived from DC/NPC cocultures in the same wells or trans-wells, respectively. *$p \leq 0.0001$; **$p \leq 0.01$. Unstim. = unstimulated.*

ingly, and in support of the data from the mouse model,[8,29] MOG74-96–specific marmoset encephalitogenic TCLs—displaying immunological features consistent with a Th1/Th17 proinflammatory profile[17]—showed a significant and dose-dependent impairment of antigen-specific proliferation, when cocultured with hNPCs (Fig 3A).

Next, we analyzed the capacity of hNPCs to inhibit an allogeneic immune response, such as that occurring on the activation of immune cells with polyclonal stimuli (eg, with anti-CD3/CD28). Dose dependent suppression of cell proliferation was observed when hNPCs were cocultured—both in the same well and in a trans well system avoiding cell-to-cell contact—with total allogeneic PBMCs or magnetically sorted $CD4^+$ T lymphocytes, the suppressive effects being lost on NPC fixation (Fig 3B). In both these sets of results there was no increase of T-cell apoptosis (data not shown).

Moreover, following the recent observation from our

own laboratory that mouse NPCs impair the activation of myeloid DCs,[29] we probed the capacity of hNPCs to interfere with a number of key DC functions, such as the differentiation of myeloid precursor cells (MPCs) into iDCs and the maturation of iDCs to functional (antigen-presenting) mDCs. We found a significant impairment of the differentiation of $CD14^+$ MPCs into $CD1a^+$ iDCs when MPCs were cultured with recombinant human granulocyte macrophage colony-stimulating factor and recombinant human interleukin-4 in the continuous presence of hNPCs (Fig 3C). Additionally, hNPCs were capable of impairing the (up) regulation of the costimulatory molecules CD80, CD86, and MHC-II by LPS-treated maturating DCs (Fig 3D–F), this latter effect having a clear functional relevance in MLRs between allogenic PBMCs and DCs deriving from DC/NPC cocultures (Fig 3G and H).

It would then seem that stem/precursor cells from the human fetal CNS have a very peculiar immune regulatory capacity, whereby the effect of these cells is in part to restrain the proliferation of PBMCs/T cells as well as the differentiation and maturation of professional antigen-presenting cells, such as DCs.

hNPC Transplantation Ameliorates EAE in Marmosets

Now it is critical to study the therapeutic potential of hNPCs in vivo. Immunization of marmosets with an *Escherichia coli*–expressed recombinant protein representing the extracellular domain of the human MOG provides a valid preclinical model for human MS, at both the clinical and the pathological level,[30] where the characteristic MS-like pathological changes within the CNS are reliably visualized with validated brain MRI techniques.[31]

LV.eGFP-T cells were injected into the blood stream (IV; n = 5; 6×10^6 cells/marmoset [15×10^6 cells/kg]) or the cisterna magna (IT; n = 4; 2×10^6 cells/marmoset [5×10^6 cells/kg]) of either clinically symptomatic or subclinical EAE marmosets.[9] To detect subclinical EAE, all immunized monkeys underwent serial (ie, every 3 weeks, from the time of immunization onward) T2-weighted (T2W) and gadopentetate dimeglumine (Gd)-enhanced T1-weighted (T1W-Gd) brain MRI as well as MEP recording. Marmosets receiving PBS (n = 6) were used as controls. To prevent the rejection of hNPCs, all EAE marmosets were treated daily with low-dose (10mg/kg) daily intramuscular CsA, starting at the time of randomization into groups (ie, 48 hours after either clinical or subclinical onset). Experimental details are provided in Supplementary Figure 3. Interestingly, the administration of CsA significantly contributed to modifying the natural rapidly progressing course of EAE,[17] which turned into a long-lasting relapsing-remitting disease. This finding allowed us to study the clinicopathological impact of the hNPC therapy in the long term.

Strikingly, a significant protection from disability-related death was achieved in EAE marmosets over 90 days post-transplantation, when LV.eGFP-T were injected IV (5/5 marmosets protected, 100% survival; $p \leq 0.05$) (Fig 4A). Intermediate protection (2/4 marmosets protected, 50% survival; not significant) was observed in EAE marmosets injected IT with hNPCs.

Significant amelioration of disease was observed upon both IV and IT injection of hNPCs. EAE marmosets transplanted with hNPCs—surviving for at least 55 days postinfection—gathered a significantly lower EAE cumulative score (Fig 4B) and area under the curve (Fig 4C) ($p = 0.01$, for EAE marmosets transplanted IV as compared to sham-treated controls). Also, a clear reduction of the total clinical relapses (sham: 6.7 ± 1.9; hNPCs IV: 2.2 ± 1 [$p = 0.06$]; hNPCs IT: 5 ± 1.1) as well as of the disease progression index (sham: 0.035 ± 0.01; hNPCs IV: 0; hNPCs IT: 0.02 ± 0.01) was observed.

To further prove the clinical efficacy of systemic injection of hNPCs in EAE marmosets, we assessed the central conduction time (CCT) velocities by means of MEPs.[3] At the end of the clinical follow-up, 3 of 4 (75%) sham-treated EAE marmosets showed significant increase of the CCT velocities at the lower limb (6.8 ± 0.1 milliseconds; $p = 0.02$), when compared to preimmunization values, the remainder EAE marmoset having no recordable cortical responses at all. Indeed, the amplitude of cortical MEPs was remarkably reduced after immunization (both 1 and 2 months after immunization), although no statistical significance was observed. At the very same time point(s), the mean CCT values for EAE marmosets injected IV with hNPCs approached baseline levels (5.3 ± 0.4 milliseconds; $p = 0.02$, when compared to sham-treated EAE marmosets) (Fig 4D). The complete list of neurophysiology findings is shown in Supplementary Table.

The clinical and functional recovery of surviving EAE marmosets injected IV with hNPCs was also accompanied by a decrease in the number of inflammatory infiltrates (sham: 0.98 ± 0.4; hNPCs IV: 0.5 ± 0.2; hNPCs IT: 1.1 ± 0.4), the percentage of demyelination (sham: $2.6 \pm 2.3\%$; hNPCs IV: $0.29 \pm 0.1\%$; hNPCs IT: $2.5 \pm 2.2\%$), and the percentage of axonal loss (sham: $2.2 \pm 2.1\%$; hNPCs IV: $0.14 \pm 0.07\%$; hNPCs IT: $2.1 \pm 2\%$) in the spinal cord.

Although additional mechanisms cannot be ruled out, our observations point to a prominent protective effect of immune regulatory hNPCs on not only the anatomical but also the functional integrity of motor axons (eg, the corticospinal tract), in full agreement with previous findings in rodents.[3–5,7,8,29]

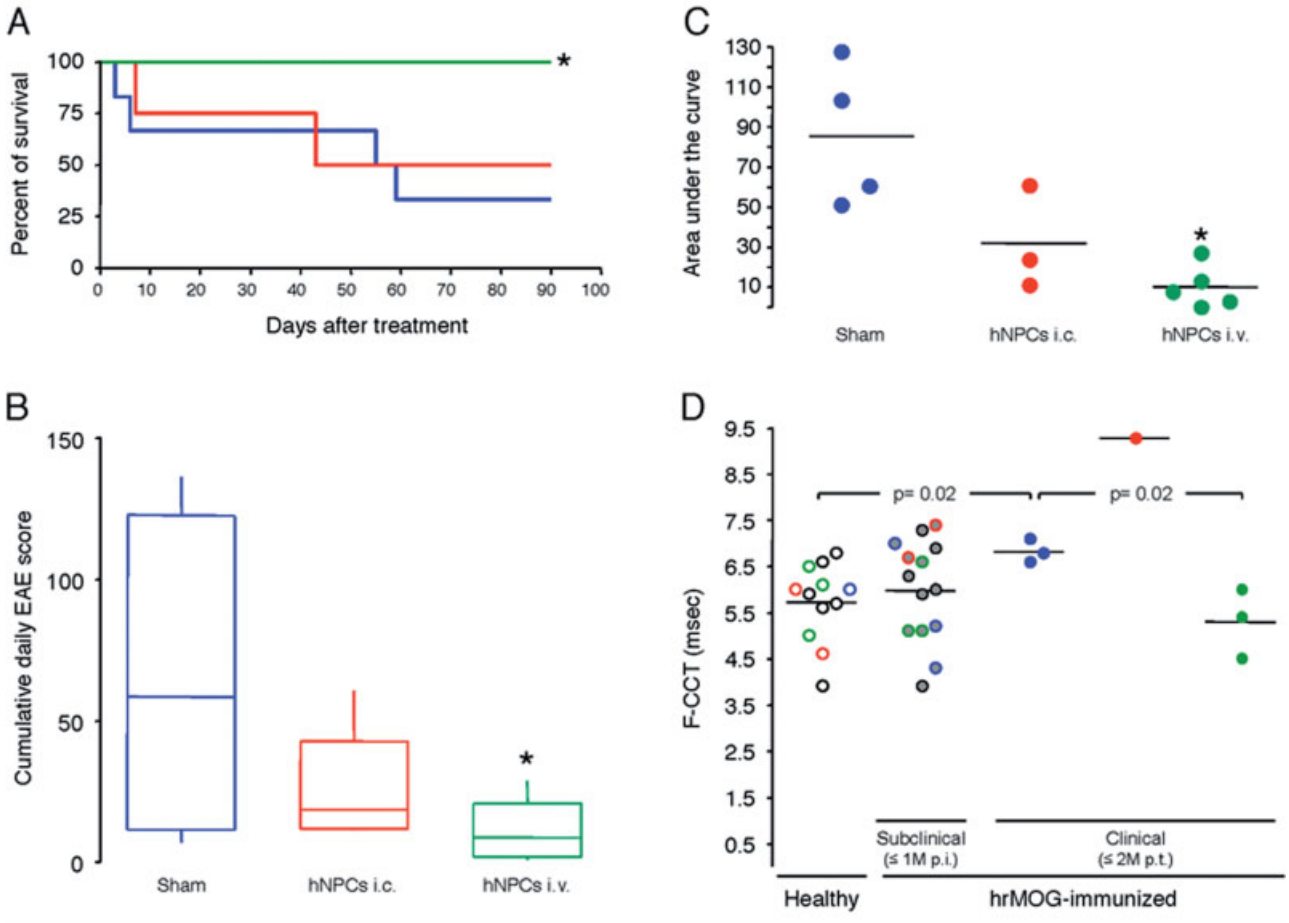

Fig 4. Human neural stem/precursor cell (hNPC) transplantation ameliorates clinical features and protects motor axonal function in experimental autoimmune encephalomyelitis (EAE) marmosets. (A) Complete protection (100% survival) is achieved at 90 days of follow up in EAE marmosets injected intravenously (IV) with hNPCs (green), whereas only intermediate protection (50% survival) is observed in EAE marmosets injected intrathecally (TC) with hNPCs (red). Sham-treated control EAE marmosets (blue) showed 33% survival. (B, C) Significant reduction of both the EAE daily cumulative score (B) and the area under the curve (C) is observed upon ≥55 days of follow-up in EAE marmosets injected intravenously with hNPCs (green). Intermediate lower values are also observed in EAE marmosets injected intrathecally with hNPCs (red). Sham-treated control EAE marmosets are in blue. The EAE daily cumulative score represents the summation of each single score recorded for each marmoset from the day of immunization (day 0) to the day of sacrifice (≥55 days postinfection). (D) Scatter plot diagram of individual Central Conduction Time (lower limb $CCT_{F\text{-}wave}$) values (in milliseconds) recorded at both baseline (Healthy, n = 15)—on subclinical human recombinant myelin oligodendrocyte glycoprotein (hrMOG)-immunized marmoset (≤1 month [1M] postimmunization [p.i.], n = 15)—and on clinical EAE marmoset followed up for at least 2 months (2M) after randomization into the different treatment groups. Clinical EAE marmoset injected intravenously with hNPCs are in green; EAE marmoset injected intrathecally with hNPCs are in red. Sham-treated EAE marmosets are in blue. Individual green, red, and blue symbols in the Healthy and Subclinical human MOG-immunized groups refer to individual EAE marmosets being further followed up after randomization into the hNPC intravenous, intrathecal, and sham treatment groups, respectively. Data in B–D are expressed as mean values (±standard error of the mean) and have been calculated from EAE marmosets completing a minimum of 55 days of follow-up after treatment. p.t. = post-transplant.

Transplanted hNPCs Distribute and Survive in the CNS and Secondary Lymphoid Organs

Our group and other laboratories have shown that, irrespective of the route of cell injection, systemically injected mouse NPCs accumulate and persist within perivascular CNS areas for months after transplantation in EAE rodent models.[3,7,16]

Herein, a similar phenomenon is reported in EAE monkeys transplanted with hNPCs up to 90 days post-transplantation (Fig 5). In addition, we established that the great majority of transplanted $eGFP^+$ hNPCs maintain both the morphology (eg, round shape, mono- or bipolar morphology) and the antigenic features (eg, lack of immunoreactivity for markers of neural differentiation) of undifferentiated neural cells in vivo (Fig 5A–D). Regardless of the delivery route of the hNPCs, $eGFP^+$ was detected in close vicinity to blood-borne CNS-infiltrating inflammatory cells (Fig

5E and F). In all cases, extended immunohistochemical analysis confirmed the in vivo differentiation of transplanted hNPCs was negligible, and that only rare hNPCs were detected inside the parenchyma, which were immunoreactive for the astroglial marker glial fibrillary acidic protein (Fig 5G–I). Nonetheless, we consistently found discrete numbers of hNPCs in the draining lymph nodes in all NPC-injected rodent EAE marmosets, following both IT (Fig 5J and K) and IV (Fig 5L and M) injection routes.

Whole body necropsy was performed in hNPC-transplanted as well as sham-treated EAE marmosets. Non-CNS tissues—such as the heart, liver, and lungs— showed that, similar to rodents, both IV- and IT-injected hNPCs cleared from these organs by day 90 post-transplantation (Fig 5O, Q, and R, respectively).[3] Very few and scattered eGFP$^+$ hNPCs were occasionally found at the level of the gut and the kidney (Fig 5N and P, respectively). There were no side effects observed from either the cell transplantation (eg, from focal or diffuse organ pathology) or the CsA treatment. Furthermore, no pathological signs suggestive of overt toxic effects (eg, inflammation, necrosis, cell degeneration) were found in any of the organs examined.

Discussion

NPCs are broadly advised as an alternative cell source for transplants in brain repair. This directive has arisen from the demonstration that NPC-driven brain repair might be achieved in several preclinical models of neurological disorders.[6] Yet a comprehensive illustration of the different mechanism(s) by which such cells exert their therapeutic potential is lacking. Although the replacement of lost/damaged cells was until few years ago assumed to be the prime therapeutic mechanism of CNS stem cells,[3,16] it has now become clear that transplanted somatic stem cells—including NPCs—may simultaneously instruct several therapeutic mechanisms, among which the sole cell replacement does not prevail. To understand and elucidate the overall therapeutic potential of stem cells in neurological diseases—namely the capacity of stem cells to adapt their fate and function(s) to

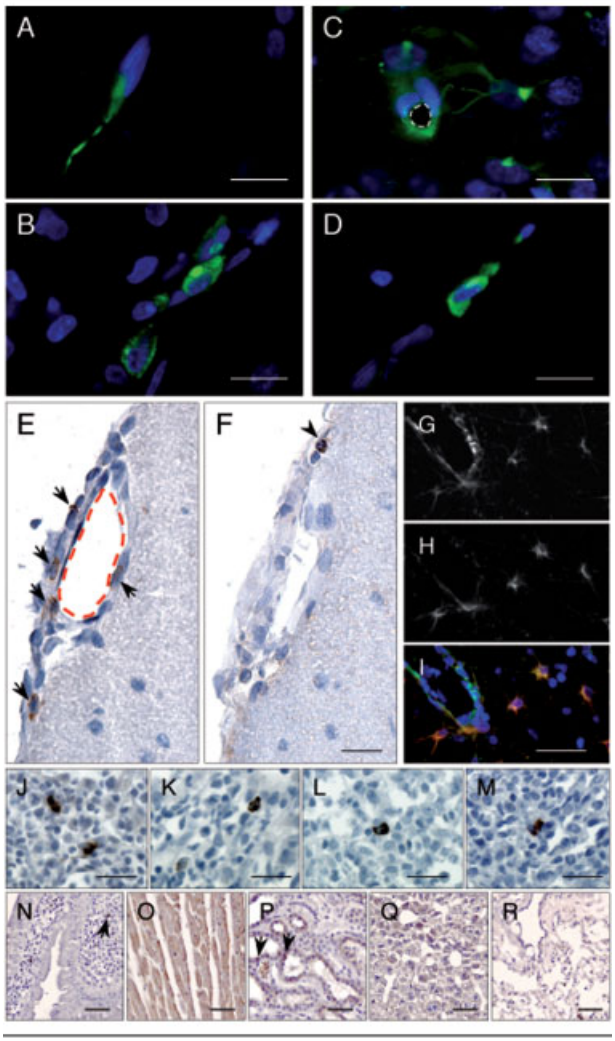

Fig 5. Transplanted human neural stem/precursor cells (hNPCs) accumulate and persist within the central nervous system (CNS) of experimental autoimmune encephalomyelitis marmosets. (A–D) Transduced lentiviral vector human phosphoglycerate kinase promoter (LV.eGFP-T) hNPCs (green), injected either intrathecally (A and B) or intravenously (C and D), accumulate and persist within CNS perivascular areas of the brain (A and C) and the spinal cord (B and D). In most cases, transplanted LV.eGFP-T hNPCs maintained both the morphology (eg, round shape, mono- or bipolar morphology) and the antigenic features (eg, lack of immunoreactivity for markers of neural differentiation) of undifferentiated neural cells. (E, F) Within perivascular areas, LV.eGFP-T hNPCs (arrows in E) were always detected in close vicinity with blood-borne CNS-infiltrating CD3$^+$ inflammatory cells (arrowhead in F). (G–I) In a few cases, intravenously injected GFP$^+$ hNPCs (G and green cells in I) expressing (astro) glial markers, such as glial fibrillary acidic protein (H and red cells in I), were detected in perivascular CNS areas. The panel in I is a merged image of the pictures in G and H. (J-M) hNPCs (brown), injected either intrathecally (J and K) or intravenously (L and M), accumulate and persist at the level of draining inguinal (J and L) and cervical (K and M) lymph nodes. (N–R) A detailed exploration of tissues other than the CNS, such as the heart (K), the liver (M), and the lung (N), showed that intravenously injected hNPCs (brown cells) were cleared from the same organs by day 90 post-transplant. In parallel, very few scattered GFP$^+$ hNPCs (arrows) were found at the level of the gut (J) and the kidney (L). Dashed lines in C and E represent blood vessels. Nuclei in A–D and I have been counterstained with 4′,6-diamidino-2-phenylindole; nuclei in E, F and J–R have been counterstained with hematoxylin. Scale bars: A–D, 10μm; E, F, 30μm; I, 60μm; J–R, 40μm; and N–R, 75μm.

specific different pathological conditions—we have recently proposed the concept of therapeutic plasticity.[6]

As previously shown, the transplantation of different sources of somatic stem cells (eg, NPCs,[3,4,7,16] hematopoietic stem cells,[32,33] mesenchymal stem cells[34,35]), with very little (if any) capability of neural (trans) differentiation, has promoted diffuse CNS repair via intrinsic neuroprotective bystander capacities, mainly exerted by undifferentiated stem cells releasing, in situ, a milieu of neuroprotective and immunomodulatory molecules, whose release is temporally and spatially orchestrated by environmental needs. More recent evidence has suggested that—in addition to these latter major effects on the CNS—NPCs injected through the systemic circulation in rodents with CNS inflammatory demyelination also act as potent immunomodulatory agents by accumulating and persisting at the level of secondary lymphoid organs.[8,29,36]

There are now high expectations for the development of cell therapies based on these encouraging preliminary results with somatic stem cell transplantation in demyelinated rodents. Although somatic stem cells—regardless of their neural or hematopoietic origin—might represent a new and promising route forward, further studies are warranted to assess the safety, the efficacy, and the in vivo therapeutic plasticity of these cells before envisaging future human application in MS and other demyelinating disorders.

To move forward, the primary aim of this study was designed as a preclinical proof-of-principle to test the feasibility of adult human NPC transplantation in an outbreed nonhuman primate EAE model approximating the clinical and complex neuropathological situation of human MS more closely than EAE in the standard laboratory rodent.[37]

Predictably, the fate and function of transplanted hNPCs would depend on the conditions present within the EAE-affected CNS. In the mouse model that we have used thus far, lesion pathology is mainly dominated by Th1 cell-mediated inflammation.[3,7,17] Although lesions in MS involve very complex immunopathogenic mechanisms leading to demyelination and axonal pathology,[38] early in the human recombinant MOG-induced EAE in the common marmoset the majority of lesions have an active (mostly T cells) phenotype (reminiscent of type I and II patterns). However, when individual lesions are followed longitudinally, a dynamic pattern of shrinking and enlargement, and sometimes complete disappearance of the lesion, is observed.[9]

Both IV and IT hNPC transplantation proved safe, and reduced the severity of the disease, as well as the symptoms/related mortality and major clinical and electrophysiological parameters. The IV route of hNPC administration had a better clinicopathological impact, as compared with IT. No overt side effects were recorded.

Ourselves and others have previously highlighted the role of the $\alpha 4$ subunit of very late antigen-4 in the CNS homing capabilities of NPCs injected intravenously in mice with both chronic and relapsing EAE.[7] Herein we provide in vitro and in vivo evidence that this is also the case for hNPCs. As such, we observed that the vast majority ($\geq$90%) of $CD45^-$ hNPCs express CD44 and $\alpha 2$, $\alpha 6$, and $\beta 1$ integrins, but not $\alpha 1$, $\alpha 4$, and $\beta 7$ integrins. This is in line with the recent in vitro evidence that the interactions of hNPCs with activated endothelial cells are mediated by CD44[24] as well as $\alpha 2$ and $\alpha 6$—but not $\alpha 4$ and αv—and $\beta 1$ integrins.[26] Furthermore, we also observe that hNPCs express numerous proinflammatory chemokine receptors, among which CCR5, CCR7, and CXCR4 are also shown to be functional in vitro. This is another key point, as chemokine receptors represent important cofactors for the affinity maturation of integrins at the time of interaction with activated endothelial cells.[39]

These and other observations suggest that the rather peculiar (and dynamic among species) functional immune signature of NPCs is the key requisite allowing the recapitulation of lymphocyte-specific pathways for remarkable extravasations at sites of inflammation.[6]

The administration of the immunosuppressive drug CsA, while not affecting the homing capacity of transplanted cells, had a major impact on the disease course. Low-dose CsA, started as early as 48 hours after randomization into treatment groups, significantly contributed to modifying the disease course, which turned into long-lasting relapsing remitting, when compared with natural disease course data.[17] This drug, although significantly reducing the number of EAE marmosets undergoing ethical sacrifice (eg, due to accumulating disability) very early after disease onset, represented a major advantage of our trial for studying the clinicopathological impact of the hNPC therapy in the long term.

Regardless of the route of cell injection, we consistently did find hNPCs accumulating and surviving up to 3 months after transplant at the level of perivascular inflammatory CNS areas, while establishing close contacts with blood-borne inflammatory cells. This latter observation, together with previous evidence in EAE rodents,[3,7,24,26] prompted us to evaluate the immune regulatory activity of hNPCs also in this xenogeneic experimental setting.

We here demonstrate that adhesion molecule- and chemokine receptor-bearing hNPCs[3,7,24,26] suppress xenogeneic marmoset antigen (MOG74-96)-specific as well as allogeneic polyclonal T-cell proliferation, but also impair the impairment differentiation and functional maturation of DCs in vitro. Although the suppression of T-cell proliferation has been previously shown in rodents with EAE,[5,8] the NPC-mediated impairment of DC function is indeed novel. In addition to this, transplanted hNPCs distributed and survived (on the whole,

undifferentiated) for up to 3 months after transplantation at the level of CNS perivascular areas as well as in secondary lymphoid organs, regardless from the cell delivery route, yet exhibited a stable safety profile.

Interestingly enough, the systemic transplantation of hNPCs reduced the severity of the disease, the symptoms/related mortality, and major clinical and electrophysiological parameters, with the therapeutic impact of the hNPC being much higher when transplantation was performed intravenously. Therefore, we speculate that the reported different therapeutic impact of IT and IV injections might account for either the different cell number used in the 2 treatment groups (higher for IV injection) or the selective capturing of IT-injected hNPCs into cervical lymph nodes, thus showing lesser accumulation at the level of lower regions of the spinal cord, when compared with IV-injected hNPCs.

These latter findings are in good keeping with previous experiments on EAE in mice[3,4,7,8,40] and provide strong evidence that hNPCs represent an efficient tool for the cellular therapy of CNS-compartmentalized chronic inflammatory conditions resulting in widespread tissue damage.[6]

All in all, our findings demonstrate for the first time that IV and IT injected hNPCs ameliorate immune-mediated demyelination in a non-human primate EAE model. We concur that the whole of the observed therapeutic effect is likely to be exerted through an immunomodulatory (rather than tissue trophic) effect exerted by transplanted cells either in the CNS or in the peripheral lymphoid organs.

Finally, although further studies are required to understand an acceptable level of safety for human-grade injectable NPCs,[41] the present findings represent a high-value proof-of-principle efficacy that reinforces the rationale for further development and ultimate application of human somatic CNS stem cell–based therapeutics in clinics.

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