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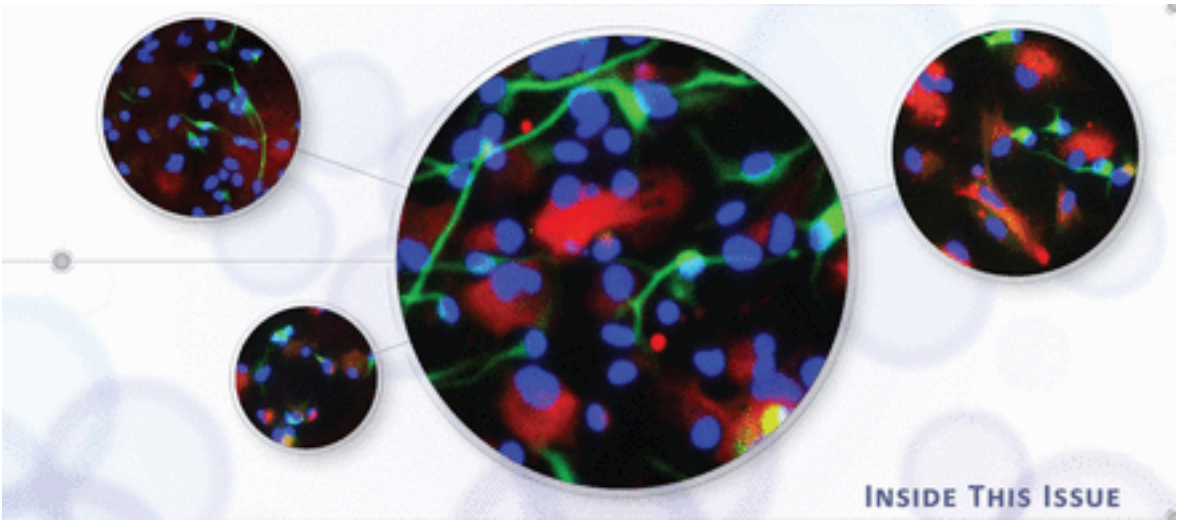
Inside This Issue

STEM CELLS TRANSLATIONAL MEDICINE®

FETAL AND NEONATAL STEM CELLS

Immunosuppressants Affect Human Neural Stem Cells In Vitro but Not in an In Vivo Model of Spinal Cord Injury

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Key Words. Neural stem cell • Immunosuppression • Differentiation • Proliferation • Stem cell transplantation • Spinal cord injury

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ABSTRACT

Clinical immunosuppression protocols use calcineurin inhibitors, such as cyclosporine A (CsA) or tacrolimus (FK506), or mammalian target of rapamycin (mTOR) inhibitors, such as sirolimus (rapamycin). These compounds alter immunophilin ligand signaling pathways, which are known to interact downstream with mediators for human neural stem cell (hNSC) differentiation and proliferation, suggesting that immunosuppressants may directly alter hNSC properties. We investigated whether immunosuppressants can exert direct effects on the differentiation, proliferation, survival, and migration of human central nervous system-derived stem cells propagated as neurospheres (hCNS-SCns) in vitro and in an in vivo model of spinal cord injury. We identified unique, immunosuppressant-dependent effects on hCNS-SCns differentiation and proliferation in vitro. All immunosuppressants tested increased neuronal differentiation, and CsA and rapamycin inhibited proliferation in vitro. No immunosuppressant-mediated effects on hCNS-SCns survival or migration in vitro were detected. These data suggested that immunosuppressant administration could alter hCNS-SCns properties in vivo. We tested this hypothesis by administering immunosuppressants to constitutively immunodeficient spinal cord injured mice and assessed survival, proliferation, differentiation, and migration of hCNS-SCns after 14 weeks. In parallel, we administered immunosuppressants to immunocompetent spinal cord injury (SCI) mice and also evaluated hCNS-SCns engraftment and fate. We identified no effect of immunosuppressants on the overall hCNS-SCns fate profile in either xenotransplantation model. Despite a lower level of human cell engraftment in immunocompetent SCI mice, functional locomotor recovery was observed in animals receiving hCNS-SCns transplantation with no evidence of allodynia. These data suggest that local cues in the microenvironment could exert a stronger influence on hCNS-SCns than circulating levels of immunosuppressants; however, differences between human and rodent metabolism/pharmokinetics and xenograft versus allograft paradigms could be determining factors. STEM CELLS TRANSLATIONAL MEDICINE *2013;2:731–744*

INTRODUCTION

Preclinical data using stem cells in neurodegenerative disorders have led to multiple phase I clinical trials in neural ceroid lipofuscinosis (ClinicalTrials.gov identifier NCT00337636), Pelizaeus-Merzbacher disease (NCT01005004), spinal cord injury (SCI) (NCT01321333), amytrophic lateral sclerosis (NCT01348451), and stroke (NCT01151124), with more predicted to occur soon [1]. One hurdle to effective therapies is ensuring long-term cell survival, leading to a focus on methods of suppressing or tolerizing the immune system [2, 3].

Accordingly, immunosuppressants that block T cell-mediated rejection are commonly used in both preclinical studies and the clinical setting. Cyclosporine A (CsA) [4] and tacrolimus (FK506) [5] act via inhibition of calcineurin, a molecule that has been linked to signaling cascades in many systems, including the central nervous system (CNS) [6]. In contrast, sirolimus (rapamycin) [7] acts via mammalian target of rapamycin (mTOR) inhibition, a signaling pathway implicated in synaptic plasticity, long-term memory, and mental retardation and autism spectrum disorders [8]. Thus, assessing the potential interactions of these agents in preclinical neurotransplantation models is an important element of translation.

Importantly, preclinical neurotransplantation studies can be allogeneic or xenogeneic, both of which require either immunosuppression or immunodeficient animals in order to achieve cell survival and enable assessment of safety/efficacy [9]. Critically, an immune response to transplanted cell populations not only

may result in immunorejection but may also alter cell fate; for example, interleukin-6 can enhance glial fate in embryonic stem cell-derived neural precursors [10].

Using immunodeficient NOD-*scid* mice, which lack T, B, and natural killer cells, we have previously demonstrated robust engraftment, survival, and differentiation of transplanted human central nervous system-derived neural stem cells propagated as neurospheres (hCNS-SCns) [11] following SCI at subacute [12] and chronic [13] time points. Mice receiving hCNS-SCns showed improved locomotor recovery on more than one outcome measure, which was correlated with total hCNS-SCns engraftment [14]. However, although this model permitted assessment of proof of concept in the absence of xenograft rejection, the potential interactions between pharmacological immunosuppressants and transplanted neural stem cells has remained untested.

Accordingly, we hypothesized that immunosuppressants would exert direct effects on hCNS-SCns survival, proliferation, engraftment, migration, or differentiation. These effects could ultimately impact the success of translational therapies in humans. We tested this hypothesis using in vitro and in vivo models. Toward the goal of in vivo evaluation, we again used a NOD-*scid* mouse model, in this case to evaluate the direct effects of immunosuppressants on hCNS-SCns survival, proliferation, differentiation, and migration without the confounding variable of host rejection responses on these parameters. Finally, we tested whether immunosuppression of immunocompetent C57BL/6 mice using an FK506 regimen enabled hCNS-SCns engraftment and functional locomotor recovery without allodynia after hCNS-SCns transplantation.

Materials and Methods

Ethics Statement

All animal housing conditions, surgical procedures, and postoperative care were conducted according to the Institutional Animal Care and Use Committee guidelines at the University of California, Irvine.

hCNS-SCns Differentiation In Vitro

hCNS-SCns derivation, culture, and characterization have been described previously [11]; these methods and the lines used in this study are identical to those in previous publications [12–15]. hCNS-SCns at passage 9 were dissociated and plated at a density of 7,000 cells per well in growth media (X-Vivo 15 media [Lonza, Allendale, NJ, http://www.lonza.com] supplemented with heparin, N2 [10 μg/ml], leukemia inhibitory factor [10 ng/ml], basic fibroblast growth factor [bFGF] [20 ng/ml], epidermal growth factor [20 ng/ml], and *N*-acetylcysteine [NAC] [63 μg/ml]) overnight in glass-bottomed chamber slides coated with polyornithine and laminin. The following day, growth media were removed and replaced with differentiating media, which lack growth factors and induce differentiation (X-Vivo 15 media supplemented with brain-derived neurotrophic factor [10 ng/ml], glial-derived neurotrophic factor [10 ng/ml], N2 [10 μl/ml], B27 [20 μl/ml], heparin [2 ng/ml], NAC [63 μg/ml], bFGF [0.1 ng/ml], and ciprofloxacin [10 μg/ml]). Experimental wells ($n = 4$) were supplemented with a low or high dose of immunosuppressant, based on trough and peak serum levels in human transplant patients [16, 17] (CsA: 350 ng/ml and 3,500 ng/ml; FK506: 10 ng/ml and 100 ng/ml; and rapamycin: 10 ng/ml and 100 ng/ml). Fifty percent of the media was exchanged every other day for 7 days, and cells were fixed in 4% paraformaldehyde.

Immunostaining was performed using anti-rabbit glial fibrillary acidic protein (GFAP) (1:1,000; Dako, Glostrup, Denmark, http://www.dako.com) and anti-mouse β-Tubulin III (1:1,000; Chemicon, Temecula, CA, http://www.chemicon.com). Fluorescent-conjugated secondary antibodies were used at a dilution of 1:500. Nuclei in all wells were detected with 4′,6-diamidino-2-phenylindole (DAPI) (1:1,000; Invitrogen, Carlsbad, CA, http://www.invitrogen.com). Four random images per well were taken at $\times$20 on an Olympus IX71 microscope (Olympus, Tokyo, Japan, http://www.olympus-global.com), for a total of 16 images per condition. Red (GFAP), green (β-Tubulin III), and blue (DAPI) channels were merged in ImageJ. Cells were counted by individuals blinded to treatment conditions using ImageJ with a cell-counting plug-in. Percentages were calculated for each image, averaged, and graphed with SE. Differentiation was assessed with triplicate biological samples and technical replicates of four wells.

hCNS-SCns Cytotoxicity/Apoptosis In Vitro

Cytotoxicity/apoptosis was assessed using the ApoTox-Glo Triplex Assay (Promega, Madison, WI, http://www.promega.com). Briefly, hCNS-SCns at passage 9 were dissociated and plated at a density of 18,000 cells per well in 96-well plates with differentiating media (as described above). Experimental wells ($n = 3$) were supplemented with a low or high dose of immunosuppressant. Three wells were treated with 100 μM ionomycin as a positive control for necrosis, and three wells were treated with 10 μM staurosporine as a positive control for apoptosis. After 6 hours, 24 hours, or 7 days of incubation, viability/toxicity and caspase detection reagents were added to wells and subjected to detection as per the kit instructions. Cytotoxicity/apoptosis was assessed in biological triplicate ($n = 3$) with a technical replicate of three wells.

hCNS-SCns Proliferation In Vitro

Proliferation was assessed using the Click-iT EdU Imaging Kit (Invitrogen). Briefly, glass-bottom chamber slides were coated with polyornithine and laminin. hCNS-SCns at passage 9 were dissociated and plated at a density of 15,000 cells per well in growth media (as described above) overnight. The following day, growth media were removed and replaced with differentiating media (as described above). The media in experimental wells ($n = 4$) were supplemented with a low or high dose of immunosuppressant (as described above). Ten micromolar 5-ethynyl-2′-deoxyuridine (EdU) was added either immediately or after 7 days for 24 hours of incubation. The cells were fixed in 4% paraformaldehyde, and subjected to EdU detection as per the kit instructions. A tiled 5×5 image was captured randomly in each well using an Olympus FSX100 microscope ($\times$20 objective) and quantified using Volocity software (PerkinElmer, Waltham, MA, http://www.perkinelmer.com) [18]. Proliferation was assessed in biological triplicate ($n = 3$) with a technical replicate of four wells.

hCNS-SCns Migration In Vitro

Migration was assessed using the QCM 96-well Chemotaxis Cell Migration Assay (Chemicon). Briefly, hCNS-SCns at passage 9

were dissociated and plated at a density of 30,000 cells per well in 96-well plates with differentiating media (as described above). The media in experimental wells ($n = 4$) were supplemented with a low or high dose of immunosuppressant. Four wells were treated with stromal cell-derived factor 1-α (SDF1α) (200 ng/ml) as a positive control. Following 4 hours of incubation in the Boyden chamber, detection reagents were added to wells and processed as per the kit instructions. Migration was assessed in biological triplicate ($n = 3$) with a technical replicate of four wells.

Contusion Injuries, Cell Transplantation, and Behavior

In NOD-*scid* mice, contusion spinal cord injuries followed by subacute hCNS-SCns transplantation were performed as described previously [12–14]. Briefly, adult female NOD-*scid* mice (Jackson Immunoresearch Laboratories, West Grove, PA, http://www.jacksonimmuno.com) were anesthetized with isoflurane and received a T9 laminectomy using a surgical microscope. All animals received a 50 kilodyne (kD) contusion injury using the Infinite Horizon Impactor (Precision Systems and Instrumentation, Lexington, KY, http://www.presysin.com) ($n = 40$). Five animals were excluded prior to transplantation because of death or spinal bruising during laminectomy procedures, two animals were excluded prior to transplantation because of a contusion injury >50 kD, and two animals were excluded mid-study because of the development of kyphosis. Exclusions were done by staff blind to treatment groups based on exclusion criteria established in advance for all experiments. Mice were randomized to receive subcutaneous injections of either phosphate-buffered saline (PBS) ($n = 7$), 10 mg/kg CsA in PBS ($n = 9$), 5 mg/kg FK506 in PBS ($n = 6$), or 2 mg/kg rapamycin in PBS ($n = 9$) beginning 7 days after injury (2 days prior to hCNS-SCns transplantation) and continuing daily thereafter until sacrifice. This dosing regimen was based on previously published reports [19–21]. Nine days post-SCI, mice were anesthetized and 250 nl of freshly triturated hCNS-SCns (at 75,000 cells per microliter) was injected at four sites, two rostral and two caudal to the site of injury (for a total of 1 μl), via a Nanoinjector system (WPI Instruments, Waltham, MA, http://www.wpiinc.com) and pulled-glass pipettes with a 70-μm inner diameter and 110-μm outer diameter (Sutter Instruments, Novato, CA, http://www.sutter.com). Fifty mg/kg 5-bromo-2′-deoxyuridine (BrdU) in 0.9% NaCl solution was administered intraperitoneally to all animals 2 days post-transplantation and then weekly thereafter until sacrifice.

In C57BL/6 mice, female mice received a T9 laminectomy followed by a 50-kD contusion injury using the Infinite Horizon Impactor ($n = 25$). Four animals were excluded because of either death or spinal bruising during laminectomy procedures, and one animal was excluded because of the development of a toe injury during the study. Mice were tested by two individuals blinded to treatment prior to injury using the Basso Mouse Scale (BMS) [22] and then 14 and 56 days postinjury (dpi). On the basis of BMS scores at 56 dpi, mice were evenly distributed into the following treatment groups: hCNS-SCns with FK506 ($n = 10$) and hCNS-SCns with FK506 + CD4 antibody ($n = 10$). Sixty days postinjury, animals received a total of 75,000 hCNS-SCns injected at four sites as described above. FK506 was administered at 5 mg/kg, subcutaneously, beginning 2 days prior to transplantation and daily thereafter until sacrifice. Anti-mouse CD4 was administered at 10 mg/kg, intraperitoneally, beginning 2 days prior to transplantation, continuing for 5 more days (7 days total) and then weekly thereafter until sacrifice. A final blinded BMS assessment was performed 10 weeks post-transplant, prior to sacrifice for histological assessment.

Mechanical allodynia was assessed as previously described [13, 15] in C57BL/6 mice preinjury; pretransplantation; and at 3, 5, 7, and 9 weeks post-transplantation using a size 3.22 Von Frey filament by blinded observers. Stimulus was applied to all four limbs, 10 times each, and the number of withdraws was recorded. Forelimb and hindlimb withdraws were averaged together.

Histology

NOD-*scid* mice at 14 weeks post-transplantation and C57BL/6 mice at 10 weeks post-transplantation were anesthetized and transcardially perfused with 4% paraformaldehyde. A T6–T12 segment of the spinal cord was dissected based on dorsal spine root counts and postfixed in 20% sucrose/4% paraformaldehyde overnight. Tissue was frozen in isopentane at −65°C and sectioned at 30 μm coronally in a cryostat using a CryoJane tape transfer system (Leica Microsystems Inc., Buffalo Grove, IL, http://www.leicabiosystems.com). Tissue was processed for antigen retrieval using buffer A (pH 6) in an EMS Retriever 2100 system (Electron Microscopy Sciences, Hatfield, PA, http://www.emsdiasum.com). Immunostaining was performed using the anti-mouse human-specific cytoplasmic marker SC121 (1:1,000; StemCells, Inc., Cambridge, U.K., http://www.stemcellsinc.com), anti-mouse human-specific GFAP marker SC123 (1:1,000; StemCells, Inc.), anti-goat doublecortin (DCX) (1:100; Santa Cruz Biotechnology Inc., Santa Cruz, CA, http://www.scbt.com), anti-rat BrdU (1:250; AbD Serotec, Raleigh, NC, http://www.ab-direct.com), anti-goat Olig2 (1:500; Abcam, Cambridge, MA, http://www.abcam.com), and anti-rabbit Ki67 (1:500; Novocastra Ltd., Newcastle upon Tyne, U.K., http://www.novocastra.co.uk). Biotin-conjugated $F(ab')_2$ fragment secondary antibodies were used at a dilution of 1:500 (Jackson Immunoresearch Laboratories), and labeling was visualized using an ABC Kit in conjunction with 3,3′-diaminobenzidine (DAB) (human cells) with hematoxylin or SG Vector Blue (fate and proliferative markers) (Vector Laboratories, Burlingame, CA, http://www.vectorlabs.com). Immunofluorescent staining was performed as described above with Alexa Fluor 488- and 555-conjugated secondary antibodies at a dilution of 1:500 in conjunction with DAPI (1:1,000; Invitrogen).

Stereological Quantification

All quantification was performed using the optical fractionator probe in StereoInvestigator (MicroBrightField Inc., Williston, VT, http://www.mbfbioscience.com) by individuals blinded to the groups on an Olympus IX51 with a ×60 oil objective. Six animals per group were counted for each marker using parameters (supplemental online Table 1) empirically determined to achieve low coefficients of error with a sampling periodicity of 1 in 24 for the NOD-*scid* mice and 1 in 12 for C57BL/6 mice. Fate quantification in C57BL/6 mice was assessed by quantifying SC123, along with double-labeled SC121/Olig2 and SC121/DCX in a series of sections (minimum of 200 total cells counted for each marker in each animal) at ×63.

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Migration Calculations

Stereological quantification data were used to determine migrational distances. The number of markers counted at a given section distance was expressed as a percentage of the total number of markers counted. This allowed for an estimate of the percentage of a given marker at set sampling distances throughout the region of spinal cord analyzed (T6–T12).

Statistical Analyses

All means are expressed ± the SEM. For all in vitro analyses, a one-way analysis of variance (ANOVA) was used with Bonferroni post hoc analysis. For in vivo stereological quantification, a one-way ANOVA was used with Dunnett post hoc analysis. For in vivo migrational analyses, two-way repeated-measures ANOVA with a Bonferroni post hoc analysis was used. Chi square analysis was used to assess the number of animals achieving hCNS-SCns engraftment with a Fisher's exact test. For BMS analyses, a one-way ANOVA was used with Bonferroni post hoc analysis. Chi square analysis was used to assess the number of animals achieving coordination in the open field with a Fisher's exact test. For Von Frey allodynia analyses, a repeated-measures ANOVA was used. Prism (version 5.0a; GraphPad, La Jolla, CA, http://www.graphpad.com) was used for statistical analysis; significance was defined as $p < .05$.

Results

Cyclosporine A, FK506, and Rapamycin Alter the Differentiation of hCNS-SCns In Vitro

Recovery or repair by transplanted neural stem cells in translational models may be dependent on cell differentiation to specific fates [23]. Consequently, altered stem cell differentiation due to the presence of immunosuppressants could prove to be a significant hurdle for regenerative medicine in neuroscience. Therefore, we tested whether the baseline differentiation of hCNS-SCns in growth factor-free media was altered after differentiation in the presence of low and high therapeutic concentrations based on peak and trough levels of immunosuppressants for 7 days. We hypothesized that the peak and trough serum levels established in human clinical subjects would define the theoretical maximal concentration of these immunosuppressive agents to which transplanted stem cell populations could be exposed after in vivo clinical transplantation. We therefore selected low and high doses of immunosuppressant on the basis of these values [16, 17].

Immunocytochemistry for β-Tubulin III βTub a neuronal lineage marker, and GFAP, an astroglial lineage marker, was performed, and the percentage of hCNS-SCns expressing either marker was quantified (Fig. 1). Seven days of exposure to low- and high-dose CsA treatment induced an increase in the percentage of βTub+ cells relative to untreated controls (control, 4.93%; low CsA, 16.83%; high CsA, 26.99%; $p < .001$), with a decrease in the percentage of GFAP+ cells (control, 46.89%; low CsA, 33.18%; high CsA, 27.96%; $p < .001$). The percentage of nonlabeled cells was unchanged. FK506 also resulted in an increase in the percentage of βTub+ cells (control, 12.76%; low FK506, 16.20%; high FK506, 20.48%; $p < .001$) and a decrease in the percentage of GFAP+ cells (control, 43.56%; low FK506, 42.76%; high FK506, 29.92%; $p < .001$). Rapamycin treatment resulted in an increase in the percentage of both βTub+ cells (control, 10.15%; low rapamycin, 17.87%; high rapamycin, 17.33%; $p < .001$) and GFAP+ cells (control, 45.00%; low rapamycin, 56.71%; high rapamycin, 63.37%; $p < .001$), with a decrease in the percentage of nonlabeled cells ($p < .001$). These data suggest that therapeutic concentrations of these immunosuppressive agents may have the potential to alter hCNS-SCns fate in vivo. Specifically, the calcineurin inhibitors, CsA and FK506, could induce neuronal lineage differentiation of hCNS-SCns, as evidenced by an increase in βTub, whereas the mTOR inhibitor, rapamycin, could induce nonspecific differentiation into both neuronal and astrocytic lineages, as evidenced by an increase in both βTub and GFAP. Furthermore, in comparison with control wells, high-dose CsA (Fig. 1C), low-dose rapamycin (Fig. 1H), and high-dose rapamycin (Fig. 1I) all reduced total cell numbers (by 54%, 53%, and 56%, respectively), suggesting either a toxic or an antiproliferative effect. Such an effect could have a negative impact on total cell engraftment in vivo, making this a key issue in understanding the interaction of immunosuppressants with hCNS-SCns. We therefore tested the effects of immunosuppressant treatment on these parameters.

Cyclosporine A, FK506, and Rapamycin Do Not Alter Apoptosis/Necrosis of hCNS-SCns In Vitro

To test the effect of immunosuppressant treatment on cell viability and death, hCNS-SCns were grown for 6 hours, 24 hours, or 7 days in vitro in media containing low and high therapeutic concentrations of immunosuppressants, or with 100 μM ionomycin, a positive control for necrosis, or 10 μM staurosporine, a positive control for apoptosis. No effect of immunosuppressant treatment either on total cell viability (Fig. 2A, 2B) or on cytotoxicity or apoptotic/necrotic cell death (Fig. 2C–2F) was observed at either 6- or 24-hour incubation time points. Furthermore, immunosuppressants continued to not elicit alterations to cytotoxicity or apoptosis/necrosis after 7 days in vitro (supplemental online Fig. 2). Thus, therapeutic doses of immunosuppressants did not alter the short-term survival of hCNS-SCns in vitro.

Cyclosporine A and Rapamycin Decrease Proliferation of hCNS-SCns In Vitro

Since cell survival was unaltered in the presence of immunosuppressants, we tested the effect of immunosuppressant treatment on cell proliferation. hCNS-SCns were grown for 24 hours in vitro in media containing low and high therapeutic concentrations of immunosuppressants and the thymidine analog EdU, a marker for a cell's entry into S phase [24]. EdU incorporation was quantified and expressed as the percentage of hCNS-SCns that proliferated (Fig. 3A–3J). Immunosuppressant treatment did not increase the proliferation of hCNS-SCns. However, the high dose of CsA significantly decreased proliferation of hCNS-SCns (12.6%; $p < .001$; Fig. 3C), as did both low and high doses of rapamycin (9.4% and 13.1%, respectively; $p < .001$; Fig. 3H, 3I). Neither concentration of FK506 altered proliferation. Furthermore, these results were found to persist after 7 days of in vitro exposure to immunosuppressants (supplemental online Fig. 2). This suggests that CsA and rapamycin, but not FK506, could potentially limit the degree of proliferation of hCNS-SCns in a transplant setting, possibly decreasing the overall cellular engraftment and potentially limiting the degree of beneficial recovery.

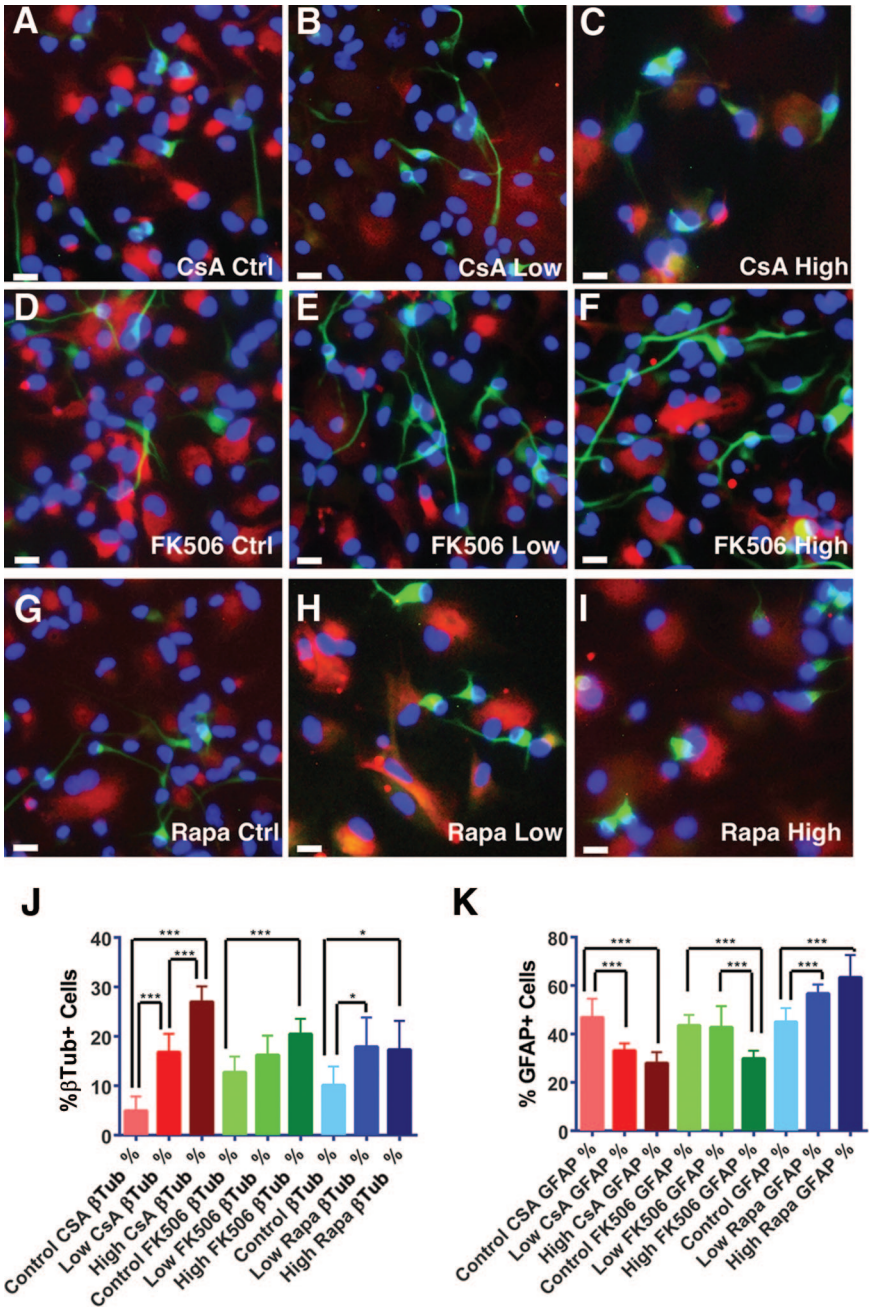

Figure 1. Therapeutic concentrations of cyclosporine A, FK506, and rapamycin alter the differentiation of human central nervous system-derived neural stem cells propagated as neurospheres (hCNS-SCns) in vitro. **(A–I):** Representative images of immunocytochemistry performed on hCNS-SCns after 7 days in vitro in control, low-dose, or high-dose immunosuppressant media. Green, βTub; red, GFAP; blue, DAPI. Scale bars = 80 μm. **(J):** Percentage of βTub+ hCNS-SCns. **(K):** Percentage of GFAP+ hCNS-SCns. All graphs are means ± SEM from three separate experiments. One-way analysis of variance, followed by a Bonferroni's multiple comparison test, was performed. *, $p < .05$; **, $p < .01$; ***, $p < .001$. Abbreviations: βTub, β-Tubulin; CsA, cyclosporine A; Ctrl, control; DAPI, 4′,6-diamidino-2-phenylindole; GFAP, glial fibrillary acidic protein; Rapa, rapamycin.

Cyclosporine A, FK506, and Rapamycin Do Not Alter the Migration of hCNS-SCns In Vitro

Stem cell migration is critical to establishing the appropriate organization of tissues and organs during development, as well as identifying and moving toward the appropriate targets for regeneration in adults [25]. Accordingly, immunosuppressant-mediated inhibition of stem cell migration could diminish therapeutic efficacy. Therefore, we tested whether therapeutic concentrations of immunosuppressants alter the migration of human neural stem cells (hNSCs) in vitro by subjecting hCNS-SCns to a chemotaxis cell migration assay in the presence of low or high therapeutic concentrations of immunosuppressants. Migration data were validated using 200 ng/ml SDF1α as a positive control (Fig. 3K). None of the immunosuppressant treatments altered hCNS-SCns migration in vitro, suggesting that immunosuppressants do not exert chemoattractant/chemorepellent effects on hCNS-SCns in vitro. Thus, in vitro, treatment of hCNS-SCns with immunosuppressants differentially altered fate and proliferation but did not affect survival or migration.

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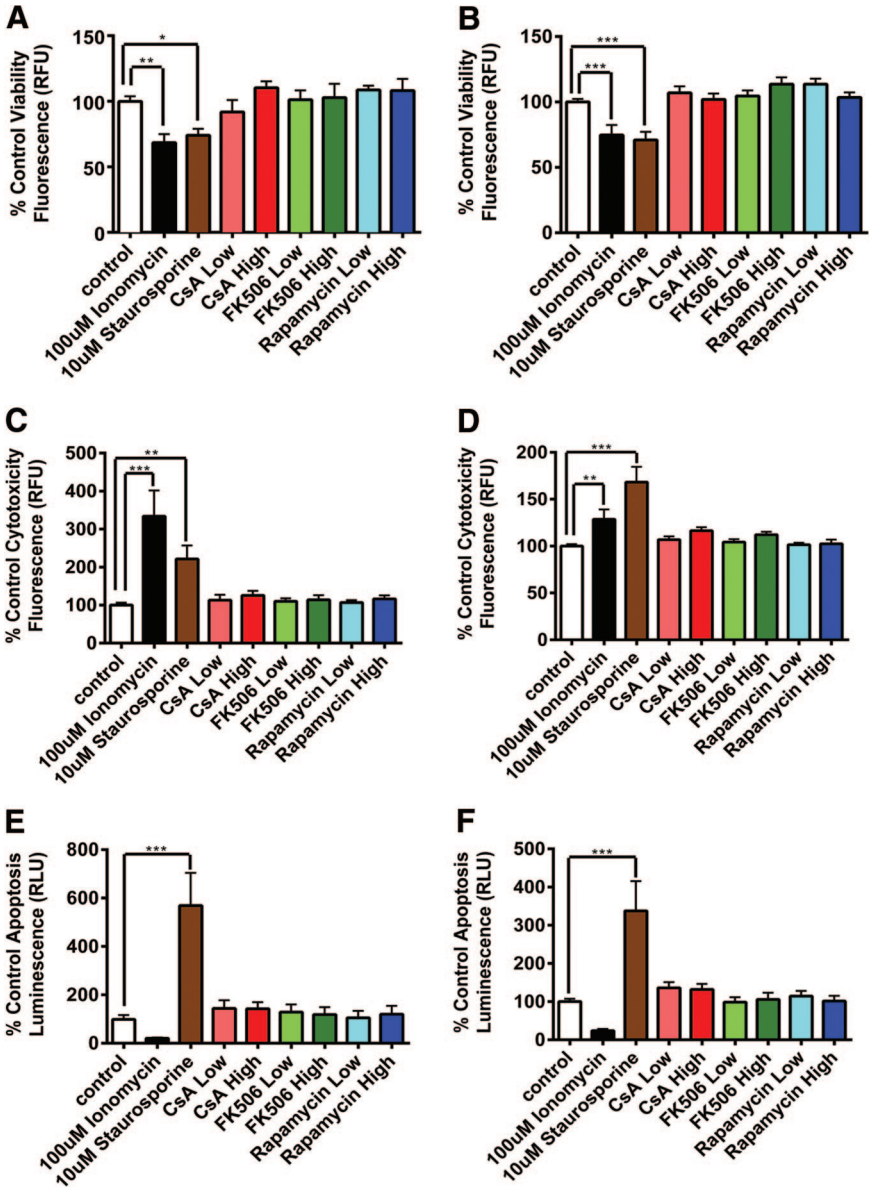

Figure 2. Therapeutic concentrations of cyclosporine A, FK506, and rapamycin do not alter apoptosis/necrosis of hCNS-SCns in vitro. **(A):** Viability of hCNS-SCns after 6 hours in vitro in control, ionomycin, staurosporine, low-dose, or high-dose immunosuppressant media. **(B):** Viability of hCNS-SCns after 24 hours in vitro in control, ionomycin, staurosporine, low-dose, or high-dose immunosuppressant media. **(C):** Cytotoxicity of hCNS-SCns after 6 hours in vitro in control, ionomycin, staurosporine, low-dose, or high-dose immunosuppressant media. **(D):** Cytotoxicity of hCNS-SCns after 24 hours in vitro in control, ionomycin, staurosporine, low-dose, or high-dose immunosuppressant media. **(E):** Apoptosis of hCNS-SCns after 6 hours in vitro in control, ionomycin, staurosporine, low-dose, or high-dose immunosuppressant media. **(F):** Apoptosis of hCNS-SCns after 24 hours in vitro in control, ionomycin, staurosporine, low-dose, or high-dose immunosuppressant media. All data were normalized as percentage of control and graphed as means ± SEM from three separate experiments. One-way analysis of variance, followed by Dunnett's multiple comparison test, was performed. *, $p < .05$; **, $p < .01$; ***, $p < .001$. Abbreviations: CsA, cyclosporine A; hCNS-SCns, human central nervous system-derived neural stem cells propagated as neurospheres; RFU, relative fluorescent units; RLU, relative light units.

Cyclosporine A, FK506, and Rapamycin Do Not Alter the Total Engraftment, Proliferation, or Differentiation of hCNS-SCns in an In Vivo Mouse Model of Spinal Cord Injury

Given the above effects of immunosuppressants in vitro, we next investigated the effect of immunosuppressant treatment on engraftment, proliferation, fate, and/or migration in an in vivo model of SCI. For these studies, our experimental question focused on the relevance of our in vitro proof of concept data to the experimental and preclinical animal model setting, which would have potential implications for the clinical setting as well. We therefore selected immunosuppressant doses that are routinely used for cell transplantation into animal models of disease and injury [21, 26–29]. Additionally, immunodeficient NOD-*scid* mice were used to avoid the confounding variable of a rejection response and allow for the direct comparison of immunosuppressants with the required matching baseline of cell engraftment without exposure to immunosuppressants.

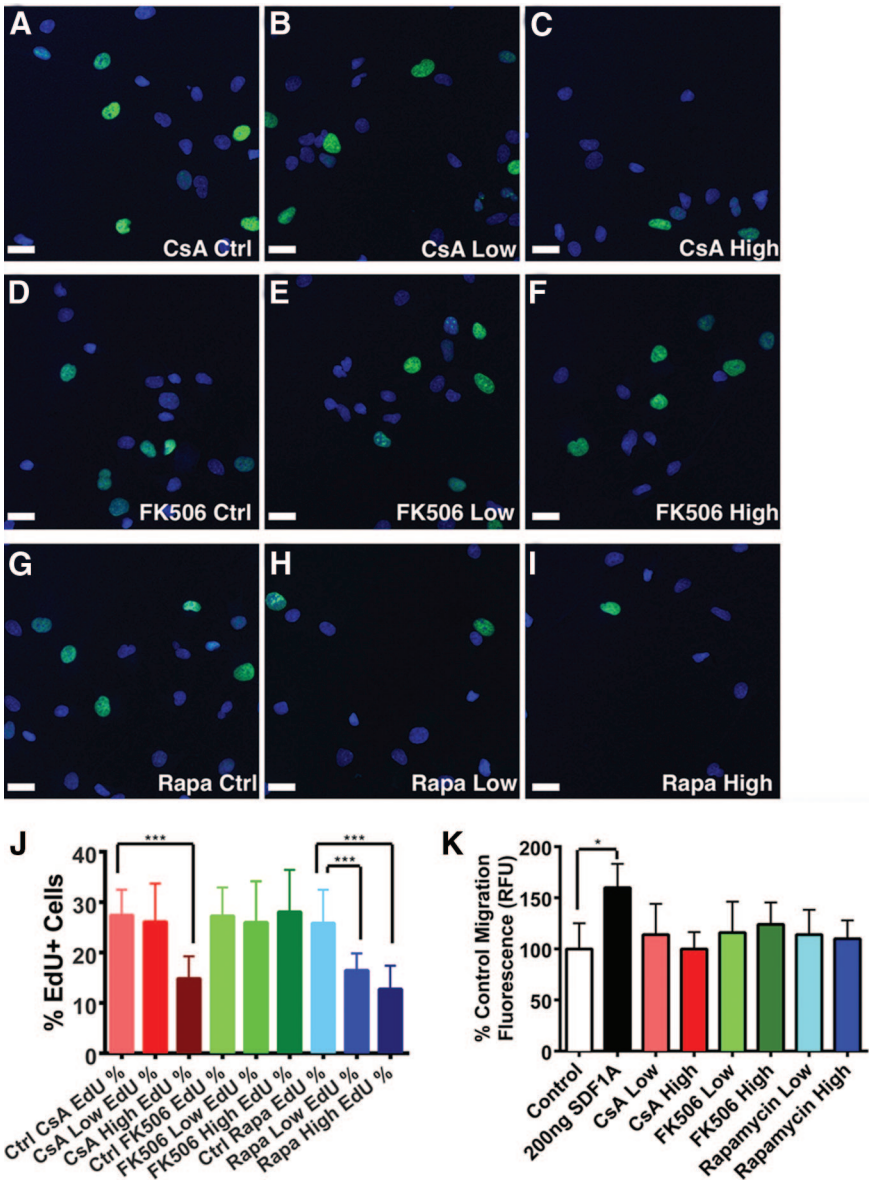

Figure 3. Therapeutic concentrations of cyclosporine A and rapamycin decrease proliferation but do not alter migration of human central nervous system-derived neural stem cells propagated as neurospheres (hCNS-SCns) in vitro. **(A–I):** Representative images of EdU labeling of hCNS-SCns after 24 hours in vitro in control, low-dose, or high-dose immunosuppressant media. Green, EdU; blue, DAPI. Scale bars = 20 μm. **(J):** Percentage of hCNS-SCns incorporating EdU. All graphs are means ± SEM from three separate experiments. **(K):** Percentage of hCNS-SCns migrating after 4 hours in vitro in control, SDF1α, low-dose, or high-dose immunosuppressant media. Data were normalized as percentage of control and graphed as means ± SEM from three separate experiments. One-way analysis of variance, followed by Dunnett's multiple comparison test, was performed. $*$, $p < .05$; $***$, $p < .001$. Abbreviations: CsA, cyclosporine A; Ctrl, control; DAPI, 4′,6-diamidino-2-phenylindole; EdU, 5-ethynyl-2′-deoxyuridine; Rapa, rapamycin; RFU, relative fluorescent units; SDF1A, stromal cell-derived factor 1-α.

Following a moderate contusion SCI, NOD-*scid* mice received a subacute (9 days postinjury) transplantation of hCNS-SCns while receiving daily injections of immunosuppressants or vehicle control. Animals were sacrificed at 14 weeks post-transplantation to assess terminal engraftment, proliferation, differentiation, and migration of hCNS-SCns. Immunofluorescent staining revealed that hCNS-SCns, labeled with the human cytoplasmic marker SC121, had differentiated along neuronal (DCX), oligodendroglial (Olig2), and astrocytic lineages (GFAP) (supplemental online Fig. 1A–1C). Furthermore, hCNS-SCns had proliferated during engraftment, as evidenced by BrdU incorporation, with a small proportion of hCNS-SCns that still exhibited potential mitotic activity at sacrifice, as evidenced by Ki67 expression (supplemental online Fig. 1D, 1E).

Stereological quantification of bright-field immunostaining (Fig. 4) revealed no alteration of the total engraftment of hCNS-SCns by any therapeutic immunosuppressant treatment ($p = .43$) (Fig. 5A). Furthermore, neither the number of hCNS-SCns that had incorporated BrdU nor the number of hCNS-SCns that exhibited Ki67 was altered by immunosuppressant treatment ($p = .89$, $p = .57$, respectively) (Fig. 5E, 5F). Finally, in contrast to the in vitro results, hCNS-SCns fate was also unaffected by immunosuppressant treatment, with no differences observed in astrocytic (SC123, $p = .45$),

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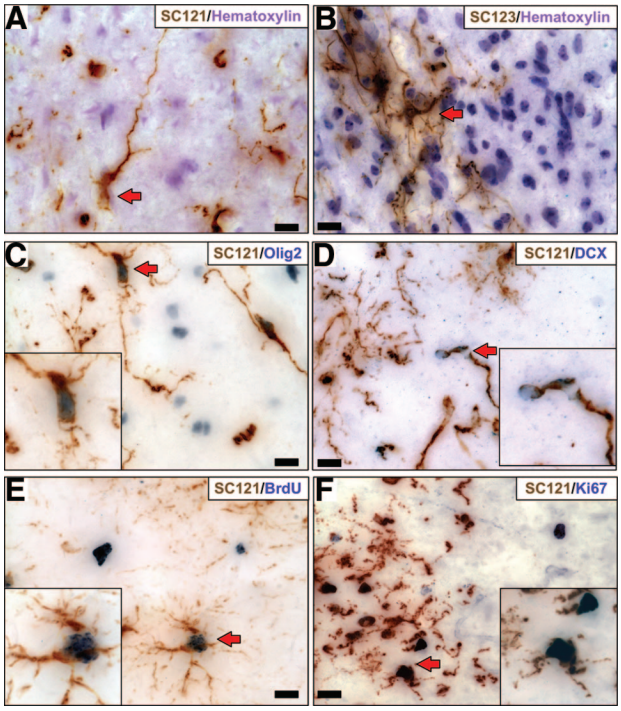

Figure 4. Human central nervous system-derived neural stem cells propagated as neurospheres (hCNS-SCns) engraft, proliferate, and differentiate into the three neural lineages. Representative bright-field images with insets taken at ×100 of hCNS-SCns (red arrows) 14 weeks post-transplantation in the spinal cord of NOD-*scid* mice. **(A):** SC121 (brown) with hematoxylin. **(B):** SC123 (brown) with hematoxylin. **(C):** SC121 (brown)/Olig2 (blue). **(D):** SC121 (brown)/DCX (blue). **(E):** SC121 (brown)/BrdU (blue). **(F):** SC121 (brown)/Ki67 (blue). Scale bars = 20 μm. Abbreviations: BrdU, 5-bromo-2′-deoxyuridine; DCX, doublecortin.

neuronal (DCX, $p = .99$), or oligodendrocytic (Olig2, $p = .93$) lineage differentiation in any of the immunosuppressant-treated groups (Fig. 5B–5D). In sum, these data do not demonstrate in vivo evidence for altered hCNS-SCns engraftment, proliferation, or differentiation in animals receiving therapeutic doses of immunosuppressants.

Therapeutic Doses of Cyclosporine A, FK506, and Rapamycin Do Not Alter the Migration of hCNS-SCns in an In Vivo Mouse Model of Spinal Cord Injury

To investigate any effects of immunosuppressants on hCNS-SCns migration, we plotted the stereological quantification data based on distance from the injury epicenter (Fig. 6). hCNS-SCns migrated extensively from the site of transplantation in both rostral and caudal directions, with some cells being observed at the distal ends of the T6–T12 spinal segment analyzed, as far as 5 mm from the injury epicenter. Two-way repeated-measures ANOVA revealed a significant main effect for distance on cell number ($p < .001$), suggesting that all groups exhibited cell migration. However, no significant main effect ($p = .559$) or interaction effect for group was observed on cell number ($p = .9941$), suggesting that the distribution of hCNS-SCns was not altered by immunosuppressant treatment. Furthermore, the distribution of hCNS-SCns positive for BrdU or Ki67 was unaltered (BrdU interaction effect, $p = .9904$; Ki67 interaction effect, $p = .9552$), as was lineage-directed hCNS-SCns distribution (DCX interaction effect, $p = .36$; Olig2 interaction effect, $p = .25$; and SC123 interaction effect, $p = 1.00$). These data do not suggest any effect of treatment with therapeutic doses of immunosuppressants on hCNS-SCns migration in the injured spinal cord.

Combined Immunosuppressive Therapy Allows for Modest hCNS-SCns Engraftment and Survival in an Immunocompetent Mouse Model of Spinal Cord Injury and Results in Behavioral Recovery

Preclinical testing of therapeutic cell populations for engraftment, fate, and functional recovery in immunodeficient animal models can be criticized for failing to incorporate a clinically relevant pharmacological immunosuppression protocol. However, the in vivo data above suggest that the engraftment parameters of hCNS-SCns remain unaltered by immunosuppressant treatment. Accordingly, we investigated whether a combinatorial immunosuppression protocol could allow for hCNS-SCns engraftment and behavioral recovery in an immunocompetent mouse model of SCI.

Because of the robust nature of the discordant xenograft response [9], a T cell-depleting antibody, CD4, was used in conjunction with FK506 to achieve meaningful graft survival, as has been reported in other cases of neural xenograft transplantations [21]. Following a moderate contusion SCI, C57BL/6 mice received a chronic (60 dpi) transplantation of hCNS-SCns while receiving daily injections of FK506 alone, or FK506 and anti-CD4 antibody. hCNS-SCns transplantation was performed at 60 dpi to further minimize xenorejection due to the acute immune response and to provide a more clinically relevant chronic spinal cord injury model. All animals were sacrificed 10 weeks post-transplantation for histological and stereological assessments.

Data from animals with hCNS-SCns engraftment (defined as the presence of any number of human cells) are summarized in Figure 7A. No mice receiving FK506 alone had engrafted cells, significantly fewer than the cohort receiving FK506 + anti-CD4 antibody, where 50% of total animals engrafted successfully ($p = .0325$). The initial transplant contained 75,000 hCNS-SCns per animal; stereological quantification of hCNS-SCns in FK506 + anti-CD4 engrafted animals revealed an average of 33,954 ± 7,595 cells 10 weeks post-transplantation (Fig. 7B), suggesting that FK506 + anti-CD4 antibody treatment allowed for only modest hCNS-SCns survival/proliferation in immunocompetent animals (~45% of the original transplant dose). The majority of hCNS-SCns had differentiated into the oligodendrocytic lineage (Olig2, 52.3%), with few in the neuronal (DCX, <1%) or astrocytic (SC123, 4.9%) lineages (Fig. 7C).

Locomotor recovery was assessed using open-field testing on the BMS in all animals at 4 days pretransplantation and 10 weeks post-transplantation. hCNS-SCns engraftment was determined based on the presence of any positive immunostaining for SC121; BMS open-field testing scores of animals exhibiting no hCNS-SCns engraftment were compared with those of animals with any degree of hCNS-SCns engraftment based on this blinded histological assessment. BMS scores for animals with no hCNS-SCns engraftment exhibited no change in scores ($p = .17$). In contrast, animals with hCNS-SCns engraftment exhibited significantly improved locomotor function 10 weeks post-transplantation in comparison with pretransplantation ($p < .001$) and in comparison with animals with no hCNS-SCns engraftment ($p < .001$; Fig. 7D). This change reflected a significant increase in the percentage of animals achieving functional coordination, from 20% in nonengrafted animals to 80% in engrafted animals (chi square frequency analysis with Fisher's exact

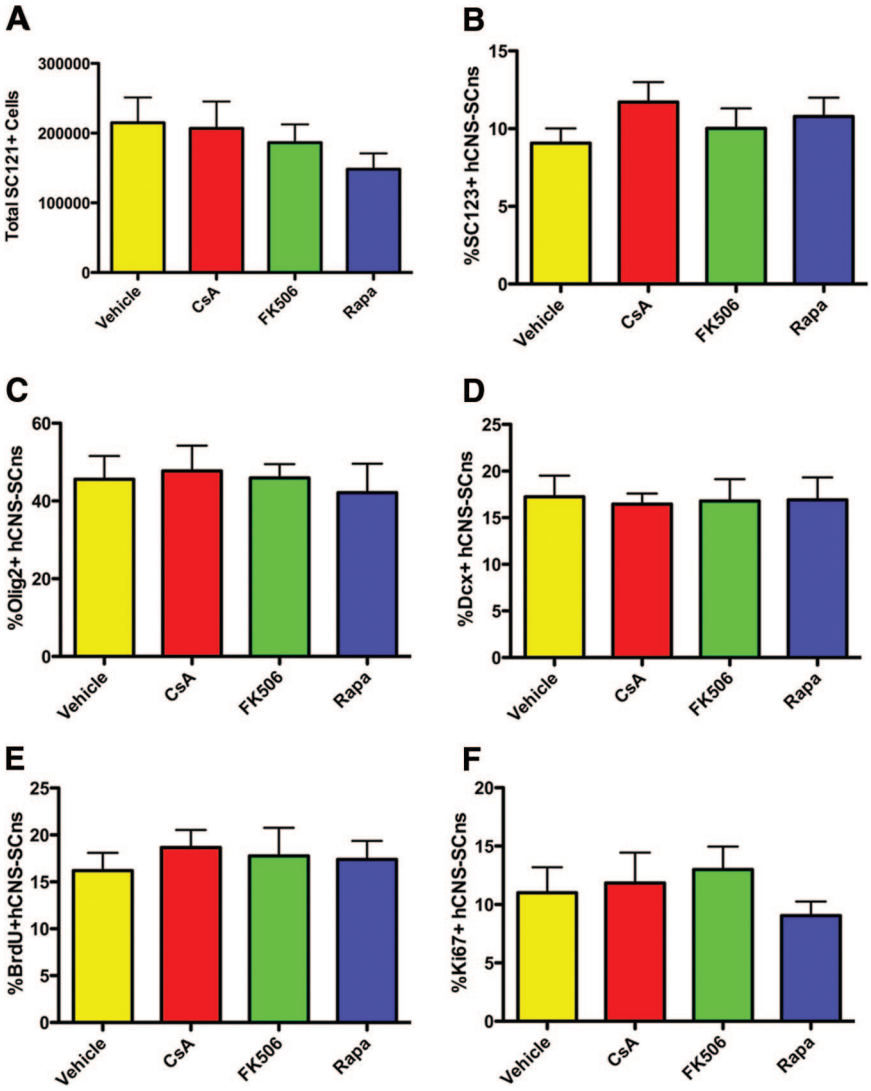

Figure 5. Therapeutic doses of cyclosporine A, FK506, and rapamycin do not alter the total engraftment, proliferation, or differentiation of hCNS-SCns in an in vivo mouse model of spinal cord injury. **(A):** Total number of engrafted hCNS-SCns in the injured spinal cord of NOD-*scid* mice 14 weeks post-transplantation. **(B):** Percentage of glial fibrillary acidic protein-positive hCNS-SCns. **(C):** Percentage of Olig2+ hCNS-SCns. **(D):** Percentage of DCX+ hCNS-SCns. **(E):** Percentage of BrdU+ hCNS-SCns. **(F):** Percentage of Ki67+ hCNS-SCns. All graphs are means ± SEM from $n \geq 6$ animals per group. One-way analysis of variance, followed by a Dunnett's multiple comparison test, was performed. Abbreviations: hCNS-SCns, human central nervous system-derived neural stem cells propagated as neurospheres; CsA, cyclosporine A; DCX, doublecortin; Rapa, rapamycin; BrdU, 5-bromo-2′-deoxyuridine.

test, $p = .031$; Fig. 7E). Recent studies have demonstrated the potential for the development of mechanical allodynia following neural stem cell (NSC) transplantation in animal models of SCI [30, 31], which is an important translational consideration. However, we found no differences in mechanical allodynia of the forepaws or hindpaws between SCI mice with no hCNS-SCns engraftment and SCI mice with hCNS-SCns engraftment ($p = .715$ and $p = .525$, respectively; Fig. 7F). Taken together, these data suggest that combined immunosuppression in immunocompetent mice allows for modest engraftment of hCNS-SCns following SCI, which promotes moderate improvements in behavioral recovery without the development of allodynia.

Discussion

The calcineurin inhibitors CsA and FK506 exhibited similar effects on hNSC differentiation in vitro, suggesting that calcineurin inhibitors selectively drive hCNS-SCns toward a neuronal fate. Inhibition of calcineurin activity could be associated with Notch blockade and increased neuronal fate, as Notch activation has been shown to increase calcineurin activity in keratinocytes [32] and activation of Notch signaling inhibits neuronal specification [33]. However, a recent report exposing primary rat NSCs to higher concentrations of CsA reported more astrocytic differentiation [34], and another group using a lower concentration of CsA with primary mouse NSCs reported no alterations to differentiation [35], suggesting potential dose and/or species-dependent responses that may warrant further investigation.

In contrast, the mTOR inhibitor rapamycin increased differentiation along both a neuronal lineage and an astroglial lineage at the expense of undifferentiated hNSCs, suggesting that mTOR may regulate hNSC progenitor maintenance and that mTOR inhibition by rapamycin may drive differentiation. Consistent with this observation, mTOR inhibition in human glioblastoma cells

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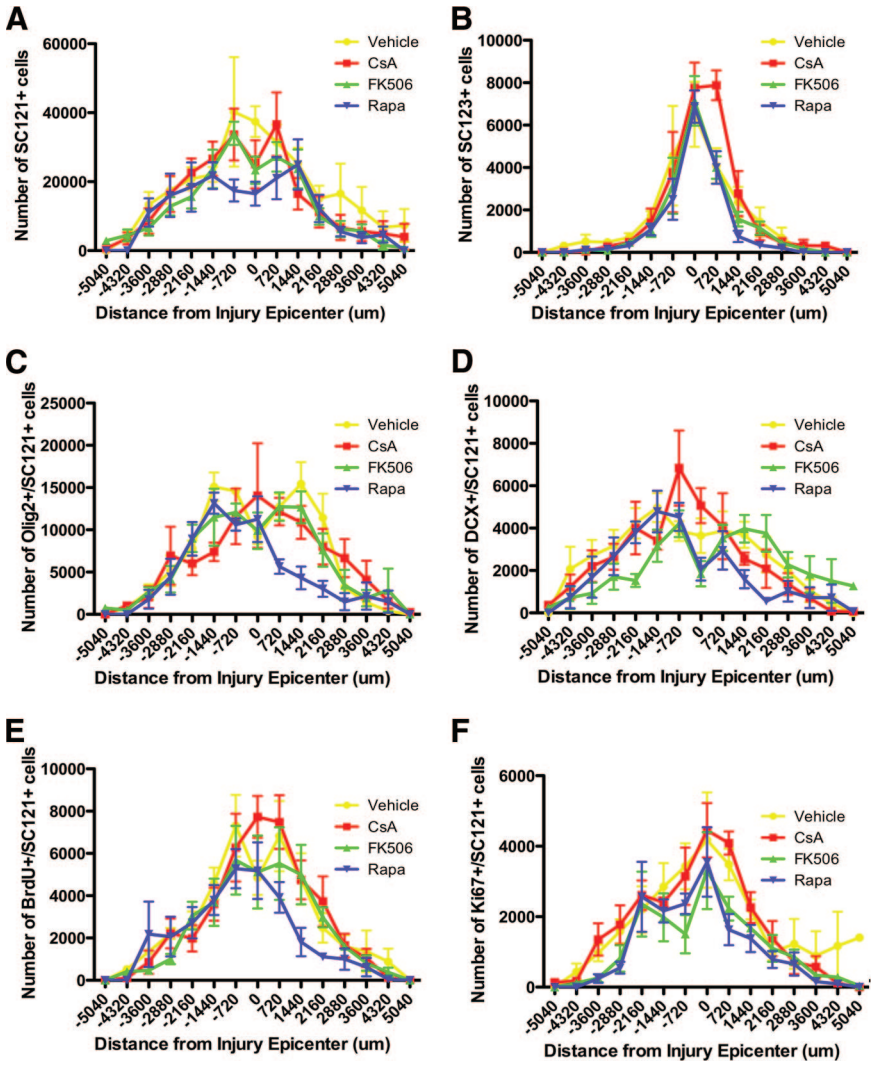

Figure 6. Therapeutic doses of cyclosporine A, FK506, and rapamycin do not alter the migration of human central nervous system-derived neural stem cells propagated as neurospheres (hCNS-SCns) in an in vivo mouse model of spinal cord injury. **(A):** Total number of hCNS-SCns at different distances along the spinal cord 14 weeks post-transplantation into NOD-*scid* mice. Zero denotes injury epicenter, negative numbers are rostral, and positive numbers are caudal. **(B):** Total number of glial fibrillary acidic protein-positive hCNS-SCns at different distances along the spinal cord 14 weeks post-transplantation. **(C):** Total number of Olig2+ hCNS-SCns at different distances along the spinal cord 14 weeks post-transplantation. **(D):** Total number of DCX+ hCNS-SCns at different distances along the spinal cord 14 weeks post-transplantation. **(E):** Total number of BrdU-positive hCNS-SCns at different distances along the spinal cord 14 weeks post-transplantation. **(F):** Total number of Ki67+ hCNS-SCns at different distances along the spinal cord 14 weeks post-transplantation. All graphs are means ± SEM from $n \geq 6$ animals per group. Two-way repeated-measures analysis of variance, followed by a Bonferroni's multiple comparison test, was performed. Abbreviations: BrdU, 5-bromo-2′-deoxyuridine; CsA, cyclosporine A; DCX, doublecortin; Rapa, rapamycin.

has been shown to decrease expression of the NSC/progenitor markers CD133 and Nestin [36], as well as to induce differentiation of these cells [37]. However, genetic hyperactivation of mTOR in mouse NSCs has recently been reported to increase differentiation [38], suggesting a species-dependent response or the potential for a biphasic response of NSCs to mTOR activation levels.

We did not identify any effects on the in vitro survival of hCNS-SCns when grown in the presence of immunosuppressants, via either cytotoxic or apoptotic pathways, even when measured following 7 days of in vitro exposure. In contrast to this observation, it has previously been shown that the calcineurin target, cyclophylin A (CypA), increases survival in mouse embryonic stem cell cultures [39], suggesting that sequestration of this protein by binding to CsA could induce cell death. However, CsA has also been reported to increase the number and size of neurospheres derived from adult mice [35], indicating that the cell source and maturity may be a contributing factor. Furthermore, inhibition of mTOR can downregulate Survivin protein levels in mouse neural progenitors [40]. One possibility is that there is a species-dependent survival mechanism for these factors; alternatively, it may be that therapeutic immunosuppressant concentrations in vitro do not alter CypA or mTOR levels in hCNS-SCns to such a degree as to induce toxicity.

We did identify altered proliferation of hCNS-SCns in vitro when grown in the presence of immunosuppressants. CsA had an antiproliferative effect at peak serum levels; however, FK506 had no effect. Because these agents both act through a calcineurin-dependent downstream signaling pathway, it is likely that the antiproliferative effect of CsA could be mediated through

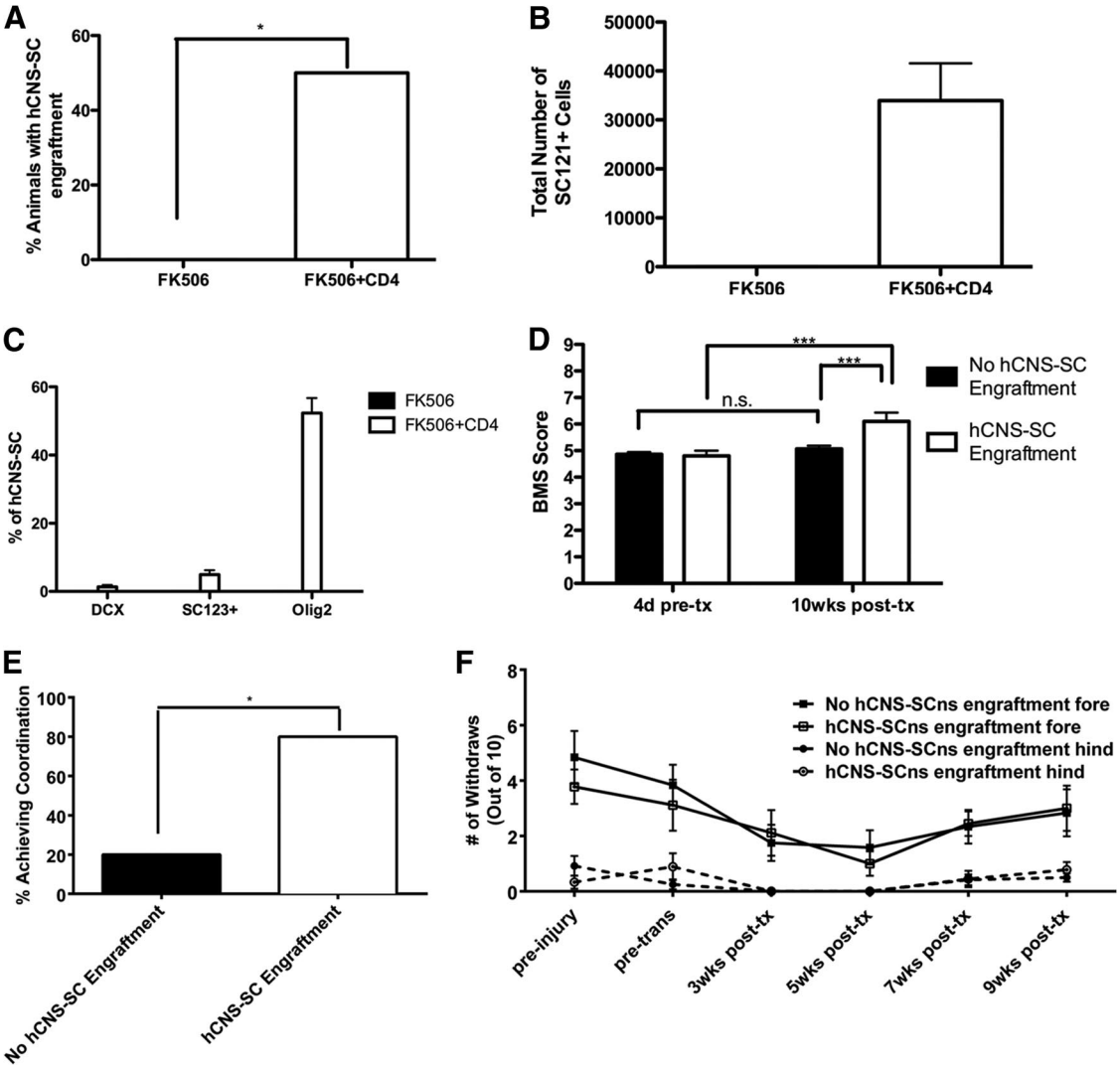

Figure 7. Combined immunosuppressive therapy allows for modest hCNS-SCns engraftment and survival in an immunocompetent mouse model of spinal cord injury and results in behavioral recovery with no allodynia. **(A):** Percentage of C57BL/6 mice receiving either FK506 or FK506 + anti-CD4 antibody with hCNS-SCns 10 weeks post-transplantation. Chi square analysis was performed. $*$, $p < .05$. **(B):** Total number of engrafted hCNS-SCns. All graphs are means $\pm$ SEM from $n \geq 6$ animals per group. **(C):** Percentage of DCX+, SC123+, and Olig2+ hCNS-SCns. All graphs are means $\pm$ SEM from $n \geq 6$ animals per group. **(D):** Open-field BMS scores for mice with and without hCNS-SCns engraftment. All graphs are means $\pm$ SEM from $n \geq 6$ animals per group. One-way analysis of variance (ANOVA), followed by a Bonferroni's multiple comparison test, was performed. $***$, $p < .001$. **(E):** Percentage of mice with and without hCNS-SCns engraftment that achieved coordination. Chi square analysis was performed. $*$, $p < .05$. **(F):** Average number of forepaw and hindpaw withdrawals (out of 10) to a 3.22 Von Frey filament in mice with and without hCNS-SCns engraftment. Repeated-measures ANOVA was performed. Abbreviations: BMS, Basso Mouse Scale; d, day(s); DCX, doublecortin; hCNS-SC, human central nervous system-derived stem cells; hCNS-SCns, human central nervous system-derived neural stem cells propagated as neurospheres; n.s., not significant; tx, transplantation; wks, weeks.

upstream binding partners. Sequestration of CypA by CsA could diminish proliferation, as CypA has been shown to be upregulated in numerous types of cancers and linked to the proliferation of cancer cells [41, 42]. Whereas lower concentrations of CsA have been reported to have no effect on mouse NSC proliferation [35], higher concentrations of CsA have been shown to decrease rat NSC proliferation [34], suggesting that certain concentrations of CsA may decrease proliferation of NSCs, regardless of species.

Both FK506 and rapamycin have been shown to act through FK506 binding protein (FKBP12) as an upstream binding partner. However, only rapamycin exhibited an antiproliferative effect on hCNS-SCns (at both 1 and 7 days in vitro), suggesting a downstream mTOR-dependent mechanism. Aside from being necessary for T-cell proliferation [43, 44], mTOR has also been shown to be important for hemangioma-derived stem cell proliferation [45], supporting this interpretation and suggesting a potentially broad cellular effect. Furthermore, rapamycin administration to mouse embryonic-derived NSCs has also been reported to decrease proliferation [38], suggesting a species-independent effect on proliferation.

It is also important to note that many of the in vitro studies referenced above used high concentrations of immunosuppressants as a method of pharmacological knockdown to allow for the investigation of the role of calcineurin or mTOR in a given model. Our study used immunosuppressants at immunosuppressive concentrations as a method to investigate the translational question of whether clinical immunosuppression could

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influence transplanted hNSCs. Consequently, the concentrations of immunosuppressants in many of the prior studies are more than 100 times that used in the present study. However, taken as a whole, these data suggest that future investigations using multiple concentrations of immunosuppressants could contribute to a better understanding of the pathways involved in neural stem cell development and maturation that may ultimately lead to more effective translational therapies.

Many cell transplantation strategies have been posited on the assumption that the generation of specific cell replacement populations will be required for therapeutic efficacy, for example, oligodendrocytes in multiple sclerosis or dopaminergic neurons in Parkinson's disease. Accordingly, in vivo alteration of fate potential of transplanted cells by pharmacological immunosuppressant regimens, as suggested by our in vitro data, could lead to unforeseen therapeutic limitations. Furthermore, in vivo alteration of proliferation, survival, or migration could similarly limit the capacity for repair. To address these issues in the context of preclinical animal models, we investigated the impact of immunosuppressive doses of these drugs in an in vivo model of spinal cord injury.

In comparison with the in vitro results, we did not observe any effect of immunosuppressant treatment on engraftment, proliferation, differentiation, or migration of hCNS-SCns 14 weeks after transplantation into the injured spinal cord of NOD-*scid* mice. Immunosuppressants, at the doses used here, have been shown to have direct neurological effects in both humans and rodents with either a disrupted or an intact blood-brain-barrier [8, 46–55], suggesting that these agents reach the CNS. Immunosuppressant doses in in vivo studies have generally been based on blood concentrations, as the pharmacokinetics and metabolism of immunosuppressants, especially in animal models [56], make it difficult to determine the exact concentrations of immunosuppressants reaching the spinal cord parenchyma over time. However, concentrations of CsA, FK506, and rapamycin in the intact brain have been reported to be comparable to circulating levels [57–61] and would likely underestimate the concentration in the spinal cord following injury due to long-term blood-brain-barrier disruption. Therefore, the effective concentration of immunosuppressants in the injured spinal cord parenchyma could be lower than in the blood. However, we report increased neuronal differentiation, as well as decreased proliferation, at trough immunosuppressant concentrations in vitro, 1/10th that of the target serum dose. This suggests that immunosuppressant concentrations in the spinal cord parenchyma as low as 1/10th of the circulating serum levels could still be predicted to elicit a physiological response. In this context, it is important to consider the possible contribution of other factors in the in vivo setting. The injured spinal cord is a complex and diverse microenvironment [62], and environmental cues are likely to play a critical role in transplanted cell survival, proliferation, fate, and migration in vivo. Ultimately, it may be that the effects of immunosuppressants are less biologically potent in the context of other environmental cues in a complex in vivo system. Nevertheless, these data suggest that immunosuppression, aside from preventing rejection of the transplanted population, is not likely to alter outcomes in a translational setting.

Conclusion

Taken together, these data demonstrate that immunosuppressants can exert direct effects on hNSC differentiation and proliferation in vitro. Further investigation into the mechanisms underlying these effects could reveal insight into hNSC proliferation and fate specification. In contrast to these in vitro findings, immunosuppressant administration did not alter cell fate, migration, or survival/proliferation in vivo in constitutively immunodeficient SCI animals. Furthermore, both a comparable oligodendrocytic fate profile and the potential to yield recovery of locomotor function were retained when cells were transplanted into immunosuppressed immunocompetent animals 60 days postinjury. Given that this immunosuppression protocol focused on T cells, these findings raise intriguing biological questions about the contributions of other immune cell populations on transplanted neural stem cell fate that warrant future investigation. Lastly, mice receiving hCNS-SCns, regardless of whether cells were ultimately rejected by the host or not, did not develop mechanical allodynia, suggesting potential safety in a translational setting. Ultimately, these data suggest that the proliferation, differentiation, and disease-modifying activity of hCNS-SCns would be retained in the allogeneic translational setting using clinical immunosuppression protocols.

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Author Contributions

C.J.S.: conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing; H.X.N. and N.K.: conception and design, collection and assembly of data; N.U.: conception and design, provision of study materials; A.J.A.: conception and design, financial support, data analysis and interpretation, manuscript writing, final approval of manuscript; B.J.C.: conception and design, financial support, collection and assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript.

Disclosure of Potential Conflicts of Interest

N.U. is a compensated employee of StemCells, Inc., and has compensated stock options. A.J.A. and B.J.C. have uncompensated research funding from StemCells, Inc., unrelated to the immunosuppressant studies reported here, and hold no stock in StemCells, Inc.

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