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Long-term survival and maturation of spinally grafted human fetal and embryonic stem cell-derived neural precursors in implantable tacrolimus pellet-immunosuppressed ALS SOD1-G93A model rat.

A Thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Biology

by

Danielle S. Goldberg

Committee in charge:

Professor Martin Marsala, Chair
Professor Yishi Jin, Co-Chair
Professor Darwin Berg

2012

The Thesis of Danielle Sara Goldberg is approved and it is acceptable in quality and form for publication on microfilm and electronically:

Co-Chair

Chair

University of California, San Diego
2012

DEDICATION

This thesis is dedicated to my family. Thank you for all your support and encouragement. With a special thanks to my son Troy, who has the greatest smile like his dad that lights up the whole world. I Love you all.

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Chapter 2, in part is currently being prepared for submission for publication of the material. Marsala, Martin; Sevc, Juraj; Goldberg, Danielle. “Effective, long-term immunosuppression in SD rat using implantable tacrolimus pellets.” The thesis author was the primary investigator and author of this material.

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ABSTRACT OF THE THESIS

Long-term survival and maturation of spinally grafted human fetal and embryonic stem cell-derived neural precursors in implantable tacrolimus pellet-immunosuppressed ALS SOD1-G93A model rat.

by

Danielle Sara Goldberg

Master of Science in Biology

University of California, San Diego, 2012

Professor Martin Marsala, Chair
Professor Yishi Jin, Co-Chair

Amyotrophic Lateral Sclerosis (ALS) is a fatal neurodegenerative disease characterized by the specific degeneration of upper and lower motor neurons. Current investigative translational therapies focus on neuroprotective and cell replacement benefits demonstrated by transplanted neural precursor cells (NPCs) or fate-restricted NPC derived astrocyte precursors. Long-term survival in relevant animal models is necessary for demonstration of cell differentiation and assessment of therapeutic potential, however aggressive immunosuppression is necessary to achieve such survival. Biodegradable immunosuppressant pellets represent a novel alternative to currently implemented labor intensive and unreliable delivery methods (such as repetitive daily subcutaneous injections). In the present study we demonstrate long-term survival and differentiation of astrocyte precursors grafted spinally in the disease environment of the SOD1-

G93A ALS model rat spinal cord using this alternative Tacrolimus pellet immunosuppression method. We found that the Tacrolimus dose of 5.1 mg/kg/day was associated with optimal graft survival and differentiation of transplanted human ES- derived neural precursor cells for up to three months in ALS model SOD1-G93A rats. T-mediated host immune response and inflammation was near completely blocked in 5.1 mg/kg/day pellet implanted animals up to two months post transplantation. In addition, we found that the long-term survival, differentiation and integration of neural precursor cells resulted in increased alpha motor neurons survival if compared to media-injected SOD+ rats. Our results indicate that transplantable Tacrolimus pellets provide a safe, effective, and and efficient method of immunosuppression that can be used in a long-term xenograft experimental design.

1. INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a lethal disease that lacks effective treatment. It is uniformly characterized by the specific degeneration of upper and lower motor neurons but varies with regards to clinical manifestations, age of onset and disease progression (reviewed in Maragagkis and Rothstein, 2004). Approximately 5-10% of human cases are linked to heritable genetic mutations (familial ALS) while the remaining majority of cases are due to unknown causes (sporadic ALS) (Reviewed in Rowland et al. 2000). Mutations in at least 15 genes have been linked to familial ALS, however the gene encoding Cu/Zn superoxide dismutase 1 (SOD1; 21q22.1) has been linked to 20% of Familial ALS and 5% of sporadic ALS cases (Rosen et al. 1993).

In vitro and in vivo reproductions of human SOD1 mutations suggest that non-cell autonomous interactions play an important role in progressive alpha motor neuron degeneration. Transgenic animals mimicking a substitution mutation of alanine for glycine at position 93 of the SOD1 gene observed in human patients (SOD1-G93A rats) develop visibly progressive motor deficits and a selective alpha motor neuron degeneration (Gertz et al., 2012). These animals also demonstrate altered physiology of non-neuronal astrocytes (reviewed in Maragakis and Rothstein, 2004) including a reduction of GLT1 (EAAT2 in human) astrocytic glutamate transporter expression in regions of motor neuron loss (Howland et al., 2002). In vitro expression of mutant SOD1 in non-neuronal astrocytes was found to induce ubiquitin deposition in co-cultured wild-type motor neurons while the presence of wild-type non-neuronal cells in the vicinity of mutant SOD1 motor neurons was found to prevent neuronal pathology (Clement et al., 2003).

The non-cell autonomous nature of the disease suggests that relevant therapies should target multiple cellular subtypes. Studies indicate the use of transplanted neural precursor cells (NPCs), which have been established in previous in-vivo and in-vitro experiments (Reviewed in Yamanaka et al., 2008; Xu et al., 2009) to be capable of division, self-renewal, and restricted differentiation into astrocytes, neurons and oligodendrocytes (Gage et al., 2000). The differentiation and integration of NPC-derived motor neurons may have the potential of reconstructing motor circuits, creating novel conncections and/or providing trophic factors (reviewed in Maragagkis and Rothstein, 2004). The differentiation and integration of NPC derived astrocytes may provide neuroprotective benefits by delivery of trophic support, filtering of the toxic environment and/or glutamate buffering (Maragakis et al., 2005; Lepore et al., 2011). To establish sufficient differentiation and demonstrate promising therapeutic potential, extended graft survival (4-6+ weeks) is necessary.

Achieving extended graft survival involves the use of immunosuppressants to effectively block the host's acquired immune response to xenografts. Clinical grade immunosuppressants used in transplantation in transplantation studies include but are not limited to: Rapamycin, Tacrolimus and mycophenolic acid. Both Tacrolimus and Rapamycin function differentially to inhibit IL-2 mediated activation of T cells: Tacrolimus effectively inhibits IL-2 transcription while Rapamycin inhibits IL-2 response. Tacrolimus is particularly effective due to its mechanism of action: irreversible binding to a group of calcineuron associated proteins (FKBPs), causing inhibition of calcineurin phosphatase activity necessary for nuclear transportation of transcription factors. Mycophenolic acid (CellCept) is used to inhibit inosine monophosphate dehydrogenase, an enzyme needed for the proliferation of B and T lymphocytes.

Strategic delivery of immunosuppressants is key to establishing adequate and stabilized drug concentration levels. To date, the most effective immunosuppression protocol resulting in long-term NPC graft survival in SOD1 model rodents is daily subcutaneous injections of

combined FK506 and rapamycin immunosuppression (Yan et al., 2006). While this delivery method demonstrated promising results, it is still not optimal due to: i) The invasive nature of daily injections, ii) The labor demand necessary to perform injections iii) The aggressive nature of combined immunosuppression, and iv) The rapid metabolism of subcutaneously delivered drugs that may result in fluctuating plasma drug concentrations. Immunosuppression delivery via implantation of biodegradable pellets represents a novel alternative to subcutaneous injections in rodent transplantation studies. Tacrolimus pellets are commercially available and provide steady drug release for up to 3 months, making delivery labor efficient, minimally invasive, and producing stabilized blood concentration levels.

Accordingly, the goal of the present study was to characterize the potency of implantable tacrolimus (Tac) pellet in inducing a long-term (up to 3 months) immunosuppression as defined by survival of spinally grafted human neural precursors in SOD+ rat. In parallel the effect of spinally grafted cells on the survival of lumbar α-motoneurons was assessed. Our results demonstrate effective immunosuppression achieved with 5.1 mg/kg/day dose Tacrolimus pellets as evidenced by: i) robust grafted cell survival and neuronal and astrocyte differentiation, ii) corresponding near complete block of grafted site T-cell and activated microglia cell infiltration. In addition, a significant increase in number of surviving alpha motor neurons in human cell-grafted regions was identified.

2. MATERIAL AND METHODS

2.1 Experimental design and subjects

In pre-pilot grafting studies in SOD1 G93A rodents we failed to achieve acceptable, consistent pharmacokinetics (Supplementary figure 1) and graft survival with routine, daily injections of FK506 (1.5mg/kg or 3mg/kg doses). The present study was designed to investigate an alternative immunosuppression routine that was less labor intensive, and more effective in maintaining stable drug levels that would permit graft survival. Animals received either 1.9mg/kg/day or 5.1mg/kg/day dosage Tacrolimus pellets and were transplanted with cells 5-7 days after pellet implantation. Animals were divided into three different subject groups according to differential tacrolimus dosages administered and cells transplanted. Group 1 animals received 1.9mg/kg/day immunosuppression dosage and received either Hues7 NPCs (1a, n=2) or Human Spinal Neural Precursors (hSNPs) (1b, n=3); Group 2 animals received 5.1mg/kg/day and either Hues7 NPCs (1a, n=2) or hSNPs (1b, n=3); Group 3 animals received 5.1mg/kg/day immunosuppression and Life Tech NSCs (n=6). Hues7 and hSNPs were used to test optimal pellet concentrations and were selected based on preliminary central nervous system transplantation studies that demonstrated extensive graft survival and therapeutic efficacy. Animals receiving Life Tech NSCs were treated with 5.1mg/kg/day dosage pellets based on Tacrolimus blood concentration levels measured in Hues7 and hSNP transplanted animals. Life Tech NSCs were used to test potential therapeutic efficacy of NSC in rescuing alpha motor neurons in the ALS diseased environment. In addition to the three cell groups, we performed media injections (5.1mg/kg/day immunosuppression, n=5) as a baseline control for host immune response to mechanical injury from injections. Animals received unilateral injections (L2-L6) for the purpose of using the non-injected side as a control to compare alpha motor neuron rescue.

SOD1 G93A rats were utilized in this experiment due to their size that allows transplantation surgeries to be performed at a younger age and with greater ease than in mice. Average weight of ALS rats used was 235.3±32.5g. The SOD1 G93A transgene has been utilized in previous ALS preclinical studies and is generally accepted in the scientific field as a useful preclinical ALS model. All procedures were approved by the University of California, San Diego (UCSD) Intuitional Animal Care and Use Committee. SOD1 (G93A) ALS rats (UCSD colony, Dr. D. W. Cleveland, San Diego, California; 57–75 days old) were used in experiments. Animals were housed in standard cages with free access to food and water.

2.2 Generation of SOD1 transgenic rat

We utilized a transgenic Sprague-Dawley ratline over-expressing ALS-linked familial genetic mutation ($SOD1^{G93A}$) provided by the laboratory of Dr. D. W. Cleveland. The SOD1G93A line was produced by microinjection of a 12-kb EcoR IBamHI restriction fragment of the human SOD1 gene with a G93A point mutation into Sprague–Dawley rat embryos as previously described (Howland, et al., 2002).

2.3 Derivation and culture of transplanted cells

Derivation of the Spinal hSNPs- hSNPs (Neuralstem, Inc., Rockville, Maryland, USA) were derived as we previously described in Hefferan et al. . Briefly, Human SSCs were prepared from the cervical–upper thoracic region of spinal cord tissue obtained from a single 8-week human fetus after an elective abortion. The fetal tissue was donated by the mother in a manner fully compliant with the guidelines of NIH and FDA and approved by an outside independent review board. The spinal cord tissue was removed of meninges and dorsal root ganglia and dissociated into a single cell suspension by mechanical titration in serum-free, modified N2 media. The modified N2 media was composed of: 100 mg/l human plasma apo-transferrin, 25 mg/l recombinant human insulin, 1.56 g/l glucose, 20 nM progesterone, 100 μM putrescine, and 30 nM sodium selenite in DMEM/F12. For growth of the hSSCs, 10 ng/ml basic fibroblast

growth factor (bFGF) as the sole mitogen was added to the modified N2 media. The initial culture was serially expanded as a monolayer culture in pre-coated flasks (T-175) or plates. The growth medium was changed every other day and in the alternate days, 10 ng/ml of bFGF was directly added to the culture. The first passage was conducted 16 days after plating. At this point, the culture was composed mostly of post mitotic neurons and mitotic hSSCs. The mitotic cells were harvested by brief treatment with trypsin (0.05% in 0.53 mM EDTA). Trypsin was stopped by addition of soybean trypsin inhibitor to 0.05% final concentration. The cell suspension was triturated with a pipette to obtain a single cell suspension and centrifuged at 1400 rpm for 5 min. The cell pellet was re-suspended in growth media and the cells were re-plated in new pre-coated plates at 1.2 × 106 cells in 20 ml of growth media per 150-mm plate. The cells were harvested at approximately 75% confluence, which occurred in 5–6 days. This process was repeated for 20 passages. At various passages, the cells were frozen in the growth medium plus 10% dimethyl sulfoxide (DMSO) at $5x10^6$-$10x10^6$ cells/ml using a programmable freezer. The frozen cells were stored in liquid nitrogen. One day prior to each surgery day, one cryo-preserved vial of the previously prepared passage was thawed, washed, concentrated in a hibernation buffer, and shipped from the cell preparation site (Neuralstem, Inc., Rockville, MD, USA) to the surgery site (UCSD, San Diego, CA, USA) at 2–8°C by overnight delivery. Upon receipt the following day, the cells were used directly for implantation without further manipulation. Before and after implantation the viability of cells was measured with trypan blue (0.4%; Sigma). Viability rate of at least 90% was recorded.

Derivation of the HUES7 cell line. Human embryonic stem cells (hESCs; HUES7 line; Melton Laboratory, Harvard University, Massachusetts, USA), were cultured on mitomycin C treated mouse embryonic fibroblast (MEF) feeder layer in HUES hESC medium containing Knockout – DMEM (Life Technologies, Carlsbad, California, USA), 10% plasmanate, 10% KOSR, non-essential amino acids, 20 mM glutamax, penicillin / streptomycin (Invitrogen, Grand

Island, New York, USA), 10 ng/ml bFGF (Invitrogen, Grand Island, New York, USA). Cells were passaged with 0.05 % Trypsin – EDTA. To form embryonic bodies (EBs) hESC were treated with dispase 1mg/ml for 10 minutes. The colonies were lifted off and washed with fresh hESC medium without FGF. Cells were transferred to the low attachment culture dish in the hESC medium without FGF. After 4 – 6 days in culture, EBs were harvested and plated on 20µg/ml poly-L-ornithine and 5µg/ml laminin (PLO/L) coated plates in N2 media (DMEM/F12, N2 supplement, B27 supplement and 10 ng/ml FGF). Columnar rosettes, which typically form after 16 – 25 days, were manually isolated and passaged every 3 days to remove contaminating cells. In this stage NPCs were harvested and FAC-sorted and CD184+, CD44-, CD271-, CD24+ cell population further expanded on PLO/L- coated plates using modified N2 media composed of: 100mg/l human plasma apo-transferrin, 25 mg/l recombinant human insulin, 1.56 g/l glucose, 20 nM progesterone, 100 uM putrscine and 30 nM sodium selenite in DMEM/F12. For cell growth 10 ng/ml bFGF as the sole mitogen was added to the modified N2 media. NPCs were expanded for 10 - 20 passages. Media was changed every other day and cells were split with 0.05% trypsin - EDTA. Cells were periodically frozen (10 - 15 million cells/vial). To confirm differentiation profile a subpopulation of proliferating NPCs were periodically induced by removal of FGF and adding 10ng/ml of BDNF and 10ng/ ml of GDNF and 0.5 mM dibutyryl cyclic AMP for 3 to 4 weeks. On the surgery day, one cryo-preserved vial of the previously prepared passage was thawed, washed, concentrated in a hibernation buffer. Before and after implantation the viability of cells was measured with trypan blue (0.4%; Sigma). Viability rate of at least 90% was recorded.

Derivation of Life Tech NSCs. NPCs were generated by Life Technologies according to their published protocols (https://www.lifetechnologies.com).

2.4 Blood Plasma analysis

Blood was collected 7 days post-pellet implantation (baseline level) from NPC grafted animals to determine if adequate immunosuppressant levels were maintained and to monitor possible drug toxicity. Approximately 200ul of blood was collected from the lateral saphenous vein. Blood was collected in an EDTA tube and plasma was sent for HPLC analysis. Adequate immunosuppressant levels were set to 15-20 ng/ml, while toxic levels were set to >25ng/ml. All animals with plasma levels above 15ng/ml were transplanted with cells. Animals that demonstrated signs of toxicity including: diarrhea, hypersensitivity to touch, excessive body weight loss and anemia were sacrificed. Additional blood draw and analysis was performed every 7-14 days and immediately before perfusion or if signs of possible toxicity were observed.

2.5 High performance liquid chromatography - mass spectrometry (LC/MS/MS)

LC/MS/MS method was also used for determination of concentration of the tacrolimus in whole blood for animal groups 1 and 2. Guard column C18, 4 x 2.0 mm (Phenomenex, Torrance, California, USA) equipped with ABI 4000 QTrap linear ion trap mass spectrometer (AB Sciex, Concord, Canada) were utilized. Whole blood samples were prepared as follows. 40µl of sample (EDTA whole blood) was mixed with 140µl protein precipitation solution (cont. ascomycin and zinc sulfate dissolved in methanol). After centrifugation (17,000g for 5 min.), 40µl of the supernatant was transferred to autosampler vials for injection into the LC/MS/MS system. Guard column C18 was washed for 1 min. (isocratic flow rate, 600µl/min.) with a mixture of methanol containing 2.5mM/l ammonium acetate and distilled water containing 2.5mM/l ammonium acetate (ratio 75:25). Thereafter, the mobile phase was changed to 2.5mM ammonium acetate dissolved in methanol (100% of the volume, flow rate 600µl/min., time 1.50 min.) and tacrolimus/ascomycin were eluted to the detector. The column was reconditioned with methanol cont. 2.5mM/l ammonium acetate (75% of the volume, flow rate 600µl/min., time 1.50 min.). MS/MS analysis was performed in multiple reactions monitoring mode using transactions m/z

821.6>768.5 for tacrolimus and m/z 809.5>756.5 for ascomycin. System control and data acquisition were performed using Analyst 1.2 software (Applied Biosystems/MDS Sciex) for automated data processing.

2.6 Cell grafting

SOD1 G93A rats were subjected to the spinal cord grafting with 1. hSNPs 2. HUES7 hNSCs or 3. Life Technology hNSC cell lines 13 days after Tacrolimus releasable pellet implantation. Rats were anesthetized with isoflurane (2% maintenance in room air), placed into a spinal unit apparatus (Stoelting, Wood Dale, Illinois, USA) and a partial Th12–L1 laminectomy was performed using a dental drill (exposing the dorsal surface of L2–L5 segments). Using a 33 gauge needle connected to micro injector (Kopf Instruments, Tujunga, California, USA), rats were injected with 1µl (app. 15,000 cells per injection) of the hSNP, HUES7 hNSCs, or LT hNSCs in hibernation buffer. The duration of each injection was 60 s followed by a 30 s pause before needle withdrawal. The center of the injection was targeted into the base of the ventral horn. 10 injections (app. 800 µm rostro-caudally apart) were made on unilaterally on the left side of the lumbar spinal cord. After injections, the incision was cleaned with penicillin-streptomycin solution and sutured in two layers. Animals were injected with analgesics and antibiotics and were allowed to recover.

2.7 Post-op IP immunosuppression

Animals received IP delivery of Mycophenolate Mofetil Hydrochloride (dose 30 mg/kg/day) for 10 days post-transplantation. MMF dosages were determined from pre-pilot studies as well as those reported from other laboratories (Wu et al., 2006; Asfari et al., 2005).

2.8 Animal Sacrifice

Animals were surviving until they reach end stage of ALS disease (approx 77 days post cell grafting; 1.9mg/kg/day pellet animals with hSNPs/Hues7 NSCs: group 1a and 2a), until they loss 10% of bodyweight (32-70 days post cell grafting; 5.1mg/kg/day pellet animals with hSNPs /

Hues7 cells; group 1b and 2b), or until they demonstrated first sign of abnormal EMG fibrillation caused by the progress of this neurodegenerative disorder (30-65 days post cell grafting; group 3-Life tech NSCs). Rats were deeply anesthetized with ketamine hydrochloride (Narkamon, 10mg/100 g body weight), followed by xylazine (Rometar, 1.5mg/100g body weight). Both ketamine and Rometar were administered intramuscularly and perfused through the left ventricle with heparinized saline (1 min). 4.0% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS, pH 7.4) was then perfused through the left ventricle.

2.9 Immunohistochemistry

Immunocytochemistry (ICC) studies focused on the survival and differentiation of grafted cells, the structural integration of cells and the characterization of type and intensity of host-versus-graft cellular response.

Spinal cords of perfused rats were dissected and post-fixed in 4% formaldehyde in PBS overnight at 4°C. Cords were then transferred to 30% sucrose in PBS cryoprotection media until 40μm thick transverse sections were cut on a cryostat and stored in PBS. Sections were immunostained overnight at 4°C with primary human-specific (h) or nonspecific antibodies made in 0.2% Triton X-100 in PBS: mouse anti-nuclear matrix protein/h-nuc (hNUMA; 1:100; Millipore, Temecula, California, USA), rabbit anti-nuclear matrix protein/h-nuc (hNUMA 1:1,000; Abcam, Cambridge, MA, USA), rabbit anti-human neuron-specific enolase (hNSE, 1:500; Millipore), rabbit anti-human glial fibrillary acidic protein (hGFAP, 1:500; Origene, Rockville, Maryland, USA), goat anti-doublecortin (DCX; 1:1000; Millipore), chicken anti-Vimentin (1:2000; Millipore), mouse anti-human synaptophysin (hSyn 1:2000; Millipore) goat anti-choline acetyltransferase (CHAT, 1:50; Millipore), mouse anti-CD4 and anti-CD8 antibodies (1:500; AbD Serotec, Raleigh, North Carolina, USA), mouse anti-CD45-APC (1:1,000; ebioscience, San Diego, California, USA), and rabbit anti-Iba1 (1:1000, Wako, Richmond, Virginia, USA) antibodies. In sections stained with two mouse antibodies (CD45-APC+CD8) CD8 staining was performed followed by incubation in

5% mouse serum for 30 minutes before staining with mouse anti CD45-APC with 1% mouse serum in PBS+TX. After incubation with primary antibodies, sections were washed 3x in PBS and incubated with fluorescent-conjugated secondary donkey anti-mouse, donkey anti-rabbit or donkey anti-goat antibodies (Alexa Fluor 488, 546 or 647, 1:1,000, Invitrogen) and DAPI for general nuclear staining. Sections were then mounted on slides, dried at room temperature and covered with Prolong anti-fade kit (Invitrogen, Grand Island, New York, USA).

2.10 Fluorescent Microscopy and Quantification

To determine potential neuroprotective efficacy of transplanted NPCs in SOD+ rat spinal cord, alpha motor neuron quantification was performed. Animals received unilateral cell injections and the non-cell injected side of the cord was used as a control for cell efficacy. Spinal cord sections that demonstrated good graft integration into host tissues were used for quantification. Due to mechanical injury of injection, sections directly adjacent to the site of injection were not used for quantification.

Analysis of fluorescent microscopy sections was performed using Leica DMLB Microscope with a Zeiss Axiocam MRm monochrome camera and Olympus FV1000 confocal deconvolution microscope. Images were captured and analyzed using Stereo Investigator software (mbf Bioscience, Williston, VT) and Olympus Fluoview FV10-ASW (Olympus Corporation, Tokyo, Japan). Z-stacks, three-dimensional, and orthogonal views were generated in Volocity High Performance 3D Imaging Software. All image manipulations were limited to Brightness/Contrast, and were performed in a standardized manner to all images. Images were assembled into figures using Adobe Illustrator (Adobe Systems, Inc., San Jose, CA).

Chapter 2, in part is currently being prepared for submission for publication of the material. Marsala, Martin; Sevc, Juraj; Goldberg, Danielle. “Effective, long-term immunosuppression in SD rat using implantable tacrolimus pellets.” The thesis author was the primary investigator and author of this material.

3. RESULTS

3.1 Pharmacokinetics of slow-releasing Tacrolimus pellets

Drug plasma concentration analysis was performed at 5 separate time points in Hues7 and hSNPs transplanted animals receiving low (1.9mg/kg/day) and high (5.1mg/kg/day) drug dosages to determine pharmacokinetics. In 1.9mg/kg/day treated animals, concentration levels reached a peak value of 11.1±8.6ng/ml at 13 days post pellet transplantation and decayed by 8.5±3.0ng/ml (to 2.6±0.7ng/ml) over a 43-day period (***Fig.1***). 5.1mg/kg/day treated group demonstrated peak values of 46.1±0.7ng/ml at the 27-day time-point, with initially lower values of 20.1±12.3ng/ml measured 10-days post transplantation. Concentration levels in 5.1mg/kg/day pellet animals decreased from peak value by 4.4±3.1ng/ml (to 41.7±9.0ng/ml) over a 26-day period (***Fig.1***).

3.2 Tolerability of SOD+ rats to a long-term tacrolimus pellet-induced immunosuppression

Animals implanted with 1.9mg/kg/day 3 months releasable Tac pellet showed a good tolerability for up to 3 months after pellet implantation and no detectable clinically defined side effects were noted.

In animal group receiving the highest tacrolimus dose (5.1/mg/kg/day) a good tolerability was seen for up to 6-7 weeks after pellet implant. From 54 animals tested in our laboratory with this dose (Some animals not included within this study), 3 animals showed nephrotoxicity at 7-8 weeks. Clinically these animals showed increased agitation and tactile allodynia. In all 3 animals plasma Tac levels were higher than 60ng/ml. Once symptoms were identified, animals usually died within 2-3 days. Postmortem necropsy showed clear kidney atrophy and the presence of blood in the urine (i.e. hematuria). Additional 4 animals were found dead without any pre-clinical

signs of toxicity. In 6 of 44 implanted animals a cyst partially filled with a serous fluid and surrounding the pellet implanted-subcutaneous region was identified.

3.3 Long-term survival and differentiation of transplanted NPCs

To validate the potential effectiveness of tacrolimus pellet-induced immunosuppression in permitting NPC graft survival in transgenic SOD+ (G93A) rats, we accessed cell graft survival in animals receiving high and low dose tacrolimus pellets. We also assessed cell differentiation and graft location to determine potential therapeutic efficacy of different human NPC cell lines. In general, independent on tacrolimus dosage and grafted cell line, comparable cell engraftment and maturation of grafted neural precursors was seen in all transplanted SOD animals.

In animals transplanted with NPCs and receiving 1.9mg/kg/day Tac immunosuppression triple-staining of transverse spinal cord sections with human-specific nuclear marker (hNUMA), early postmitotic neuronal marker DCX and human-specific GFAP antibody (hGFAP) revealed well engrafted hNUMA+ cell populations composed of DCX+ neurons and hGFAP+ astrocytes at 77 days after grafting (***Fig.2 a-d, h***). Co-staining of the adjacent sections with human-specific NSE (hNSE; neuron-specific enolase) antibody showed numerous double DCX/hNSE+ immuno-reactive neurons (***Fig.2 i)***. The majority of grafted cells appeared in the ventral funiculus (***Fig.2 a)*** with extension of the graft into the ventral horn and surrounding NeuN+ alpha motor neurons (***Fig.2 e-g***) In animals grafted with hSNP triple-staining with NeuN, human-specific synaptophysin antibody (hSyn) and DCX showed a dense hSyn-punctuate-like immuno-reactivity on DCX+ processes surrounding NeuN+ alpha motor neurons (***Fig.2 f,g*)**. Confocal analysis of DCX/hSyn/NeuN-stained sections showed a dense population of hSyn+ terminals residing in the vicinity of ventral α-motoneurons and/or associated with DCX+ processes (***Fig.2 g*)**.

In 5.1mg/kg/day Tac group, transplanted with Hues7 or hSNPs, staining with hNUMA and DCX showed a robust cell engraftment with numerous DCX and hNuma+ neurons throughout the grafted region and extending from the dorsal horn to the ventral gray matter (***Fig.3***

e, g-h*).** Numerous solitary DCX+ and hNuma+ neurons were identified as migrated from the graft core into the ventral grey matter (Fig.3 h*).** Staining with hsyn/DCX/Map2 revealed grafted hSYN+ and DCX+ cells in close proximity to Map2+ alpha motor neurons in the ventral horn **(*Fig.3 a-d***). hGFAP+ cells were found dispersed throughout the grey matter surrounded by hSYN positive cells (***Fig.3 f***)

In 5.1mg/kg/day Tac group, transplanted with Life Tech NSCs, tstaining with antibody against human specific cytoplasmic protein SCI-121, early astrocyte marker Vimentin, and DCX revealed SCI-121 positive grafted cells expressing vimentin or DCX immunopositivity (***Fig.4 b-d***). Clusters of SCI-121+ Grafted cells were observed in the ventral grey matter (***Fig.4 b,d***), however the majority of grafted cells (SCI-121+/hNUMA+) cells in all Life Tech NSC transplanted animals appeared dispersed in the white matter (***Fig.4 a,b***). Co-localization of SCI-121 and vimentin was observed in the ventral horn in close proximity to Chat+ alpha motor neurons (***Fig.4 c, yellow arrows***) and in the white matter (Fig.4 b, purple arrows) SCI-121 and DCX double positive cells were observed in discrete populations in the ventral grey matter (***fig.4 b, yellow arrow, d***) and in the white matter (***Fig.4 b, white arrows***). Staining with hGFAP revealed hGFAP positive transplanted cellular processes primarily in the white matter (***Fig.4 a***), with few positive processes observed in the grey matter surrounding Chat+ alpha motor neurons (***Fig.4 a, yellow arrows***). No hNSE+ cells were observed in this animal group.

3.4 Host immune response to grafted NPCs

While comparable graft cell survival and maturation was observed in all experimental groups, remarkable differences in the degree of T-cell and activated microglia infiltration were observed between high and low Tacrolimus dosage groups. The degree of T-cell infiltration was probed by staining with CD45, CD4 and CD8 antibodies. Activated microglia were probed with Iba1.

In animals receiving 1.9mg/kg/day of tacrolimus intense infiltration with CD45+ cells was observed at the core or periphery of cell-grafted regions (***Fig.5 a,c,i***). CD4/8+ lymphocytes surrounded by activated Iba1+ microglia were also observed in these regions (***Fig.5 b***). Cells positive for both CD45 and CD8 were observed at the periphery of hNUMA positive grafted cells (***Fig.5 c, yellow arrows***).

In animals receiving 5.1mg/kg/day of tacrolimus, only occasional presence of CD45+ (red) leukocytes was seen. The majority CD45+ cells were located at the periphery of individual grafts ***(Fig.6 a,c)*** and were CD8 negative ***(Fig.6 c; yellow arrows).*** Only sporadic presence of CD4/8+ cells was observed (***Fig.6 d-f)*** while the majority of CD45+ cells were CD8 negative (***Fig.6 g***; yellow arrows).

3.5 Alpha motor neuron rescue in SOD+ rat by grafted NPCs

In animals receiving Hues7 NPCs or hSNPs, a slight increase in the number of CHAT+ alpha motor neurons was quantified in the cell-injected side of the spinal cord in comparison to the non-cell injected contra-lateral side (***Fig.7 a,b***). This increase was not statistically significant however. In animals receiving Life Tech NPCs a significant increase ($p<0.05$, $=0.0452$ Wilcoxon matched-pairs signed rank test; ***Fig. 7 c***) in the number of CHAT+ alpha motor neurons was quantified in the cell-injected side of the spinal cord compared with the non-cell injected contra-lateral side.

Chapter 3, in part is currently being prepared for submission for publication of the material. Marsala, Martin; Sevc, Juraj; Goldberg, Danielle. “Effective, long-term immunosuppression in SD rat using implantable tacrolimus pellets.” The thesis author was the primary investigator and author of this material.

4. FIGURES

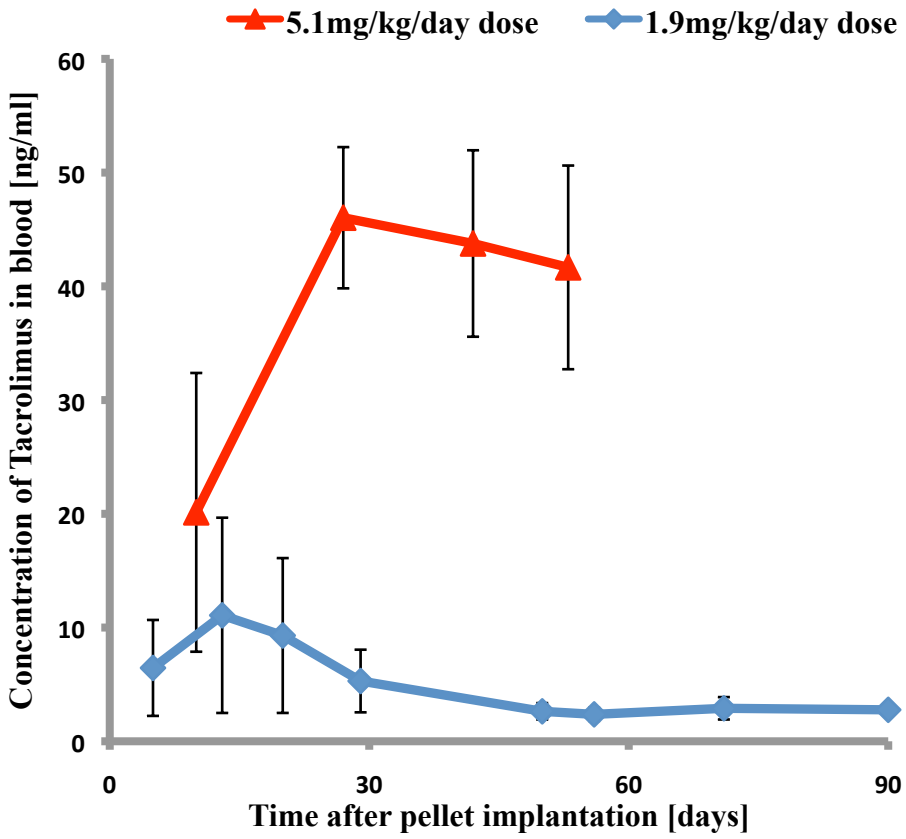

Figure 1: Pharmacokinetics of Tacrolimus administered via 1.9mg/kg/day and 5.1mg/kg/day dosage 3-month releasable pellets. To determine the immunosuppressive effect of Tacrolimus releasable pellets, SOD1 rats were implanted with pellets (dose=1.9mg/kg/day or 5.1mg/kg/day) and were subjected to the human cell grafting. During the survival time, pharmacokinetics of Tacrolimus was also examined (f). Each time point is represented as the mean ± standard deviation of tacrolimus concentrations in whole blood from 4 (1.9mg/kg/day group) or 6 (5.1mg/kg/day group) animals. Tacrolimus pellet implant was performed at day 0. In 1.9mg/kg/day pellet animals, a peak blood concentration level of 11.1±8.6ng/ml was reached at 13 days post-transplantation and gradually decreased to 2.6±0.7ng/ml over a 43-day period. In animals receiving 5.1mg/kg/day immunosuppression, a peak blood Tac concentration of 46.1±0.7ng/ml was measured at 27 days post-implantation. In this animal group an initially lower value of 20.1±0.7ng/ml concentration was measured at 10-days post-transplantation. Drug concentration gradually decreased to 41.7±9.0ng/ml after a 26-day period.

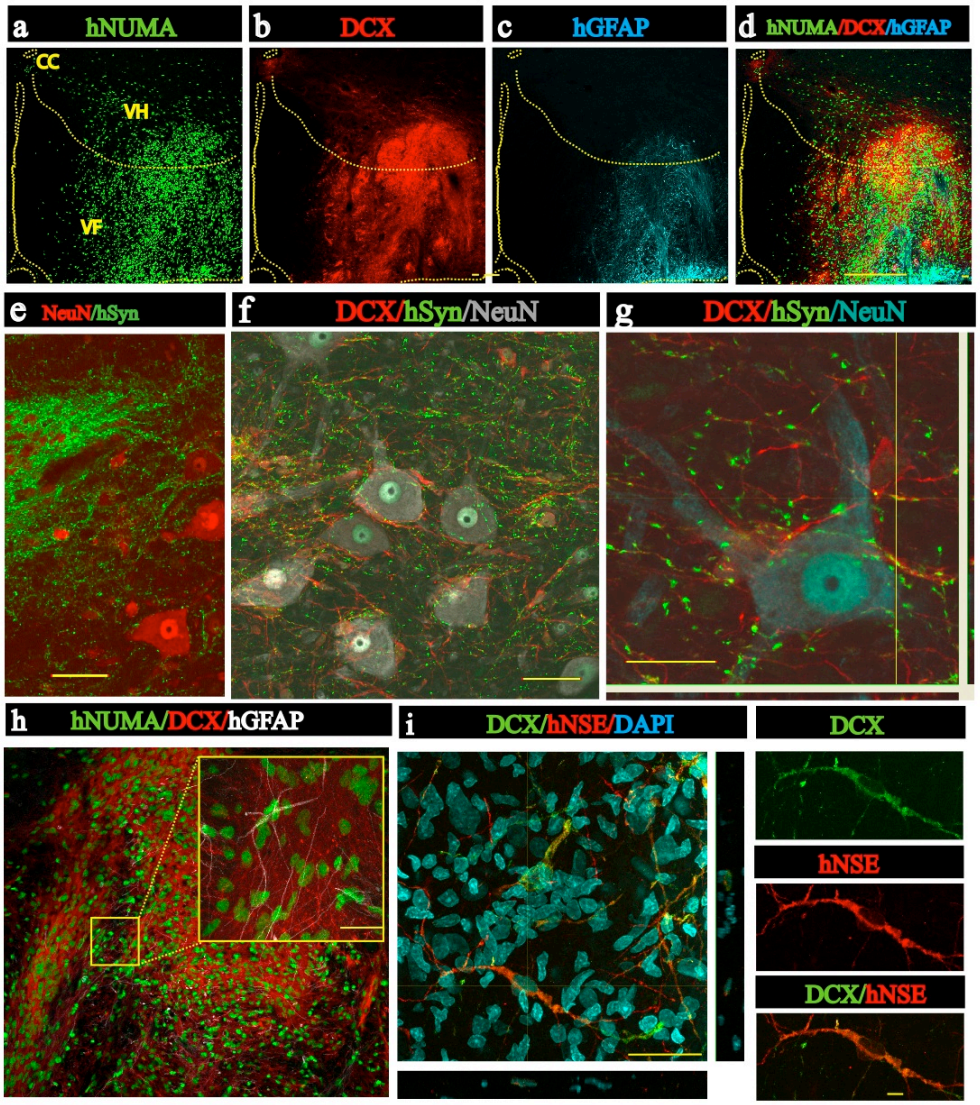

Figure 2: Immunohistochemical examination of graft survival and differentiation of Hues7 NPCs and hNSPs in SOD animals receiving 1.9mg/kg/day Tacrolimus immunosuppression. Tacrolimus releasable pellets (dose=1.9mg/kg/day) were implanted subcutaneously to SOD1 rats (n=4) 13 days prior to grafting with human cell lines HUES7 or hNSPs. Animals were sacrificed at disease end stage (approx. 77 days after cell implantation). Immunohistochemical staining with human specific antibodies (hNUMA-human nuclear protein, green; hGFAP-human astrocytes, cyan) and doublecortin (DCX, red) revealed consistent presence of large grafts in the spinal parenchyma in all animals (**a-d**). Grafted cells in close proximity to alpha motor neurons (NeuN) expressed markers of early neuronal differenation (DCX, red) and fully functional neuronal markers (hSyn, green) (**e-f**). Co localization of doublecortin (green) with human neuron specific enolase (hNSE, red) confirmed the presence of maturating neurons of human origin in the host tissue (**i**). Differentiation of grafted human cells (hNUMA, green) to the neurons (DCX, red) and astrocytes (hGFAP, white) was also demonstrated (**h**).

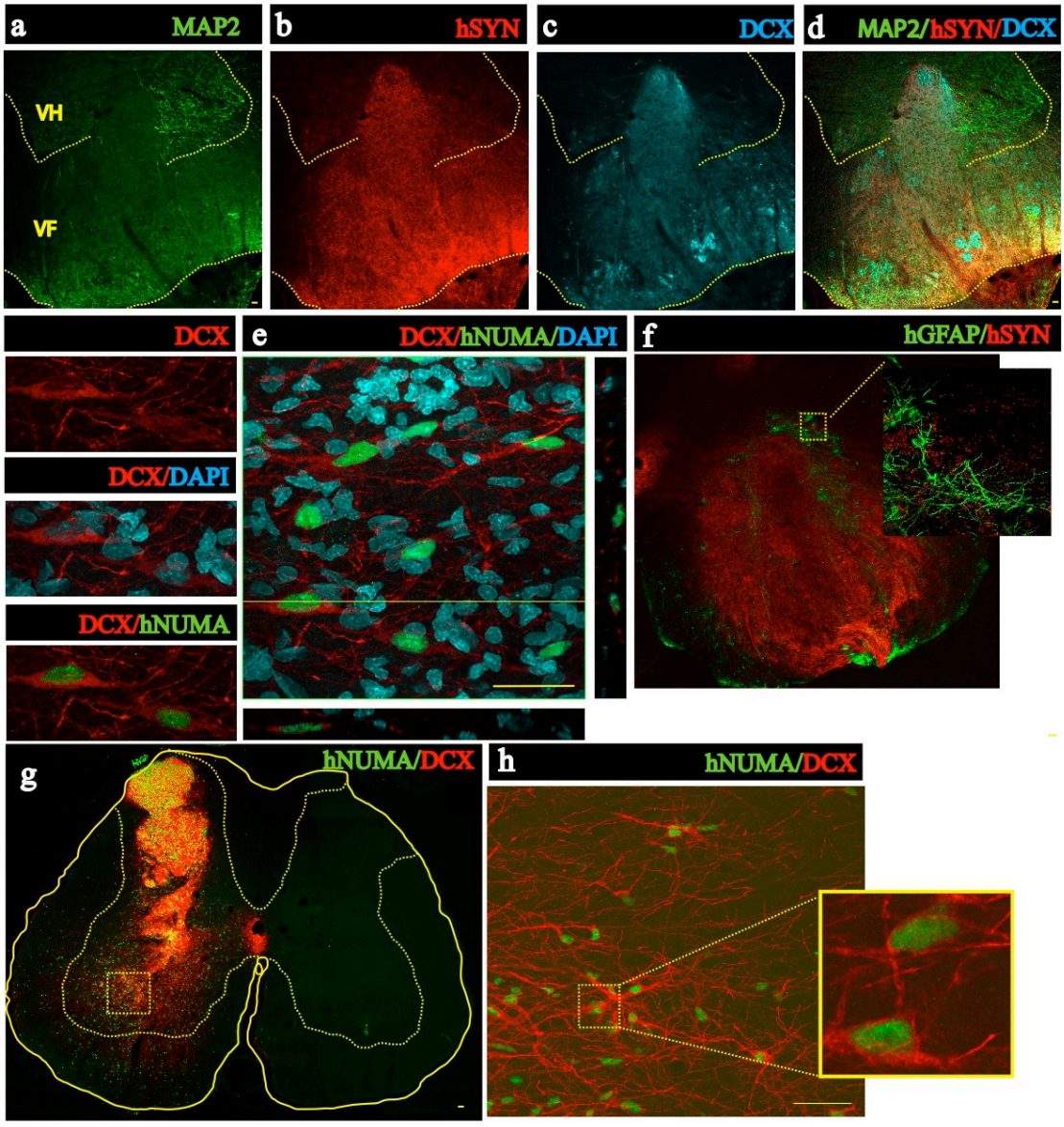

Figure 3: Immunohistochemical examination of Hues7 NPC and hNSP graft survival and differentiation in the spinal cord tissue of SOD1 rats implanted with 5.1mg/kg/day dosage Tacrolimus pellet. Tacrolimus releasable pellets (dose=5.1mg/kg/day) were implanted subcutaneously to the SOD1 rats (n=6) 13 days prior to grafting with human cell lines HUES7 or NSI. Animals were allowed to survive until they lose 10% of bodyweight caused by the progress of the ALS disease (32-70 days after cell implantation). Immunohistochemical staining with human specific antibody (hSyn, human anti-synaptophysin, red) and doublecortin (DCX, red) revealed consistent presence of large grafts in the spinal parenchyma (MAP2-microtubule associated protein 2, green) in all animals (**a-d**). Human origin of the young DCX-positive neurons was confirmed by co localization with hNUMA antigen (human nuclear protein, green) (**e,g,h**). The presence of human neurons (hSyn, red) and human astrocytes (hGFAP, white) was also observed (**f**).

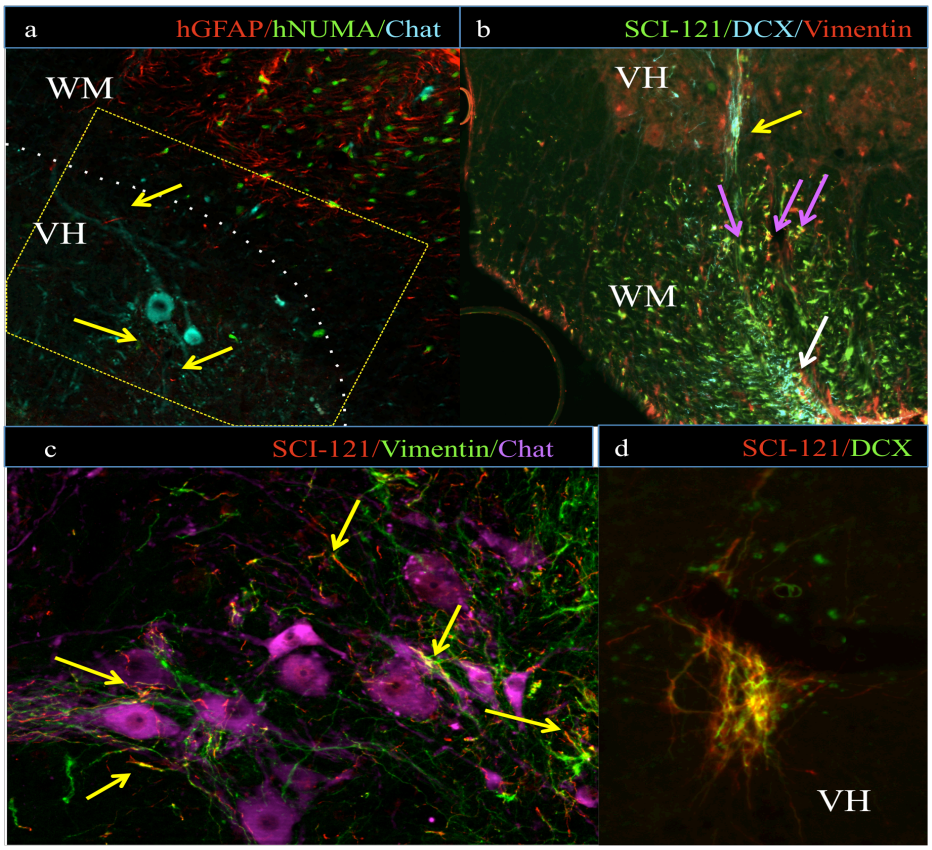

Figure 4: Immunohistochemical examination of Life Tech NPCs graft survival and differentiation in the spinal cord tissue of SOD1 rats implanted with 5.1mg/kg/day dosage Tacrolimus pellet. Grafted cells exhibited dispersed hGFAP positivity in the white matter (WM, **a**) with limited positivity in the ventral horn in the vicinity of Chat+ alpha motor neurons (VH, **a**, yellow arrows). SCI-121/DCX double positive cells were observed in clusters in the ventral horn (**b**, yellow arrow, **d**) and white matter (**b**, white arrow). Numerous SCI-121/Vimentin double positive cells were observed surrounding Chat+ alpha motor neurons in the ventral grey matter (**c**, yellow arrows) and in the white matter (**b**, purple arrows)

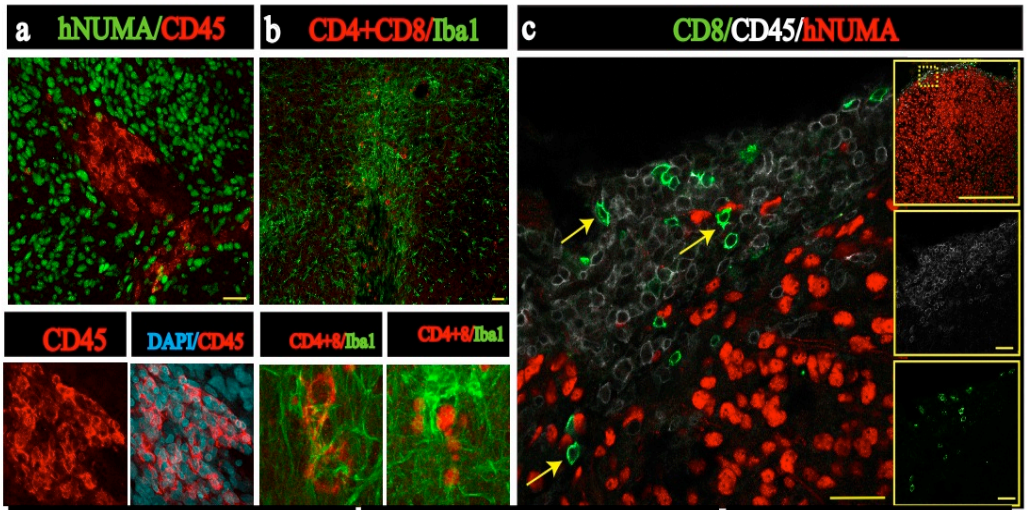

Figure 5: The immunosuppressive effect of 1.9mg/kg/day Tacrolimus pellets. The immunosuppressive effect of the administered dose of Tacrolimus was further tested using antibodies targeting T-lymphocytes. Presence of large amounts of leukocytes (CD45, red) in the vicinity of grafted human cells (hNUMA, green) was observed (**a**). Co-staining with markers of T-lymphocytes (CD4 and CD8, red) and microglia (IB1, green) revealed the presence of T-lymphocytes and activated microglia in the areas of grafted cells (**b**). The presence of cytotoxic subpopulation of T-lymphocytes was confirmed by co localization of marker CD8 (green) with general marker of leukocytes CD45 (white) (**c**, yellow arrows). Scale bar a-p: 30µm.

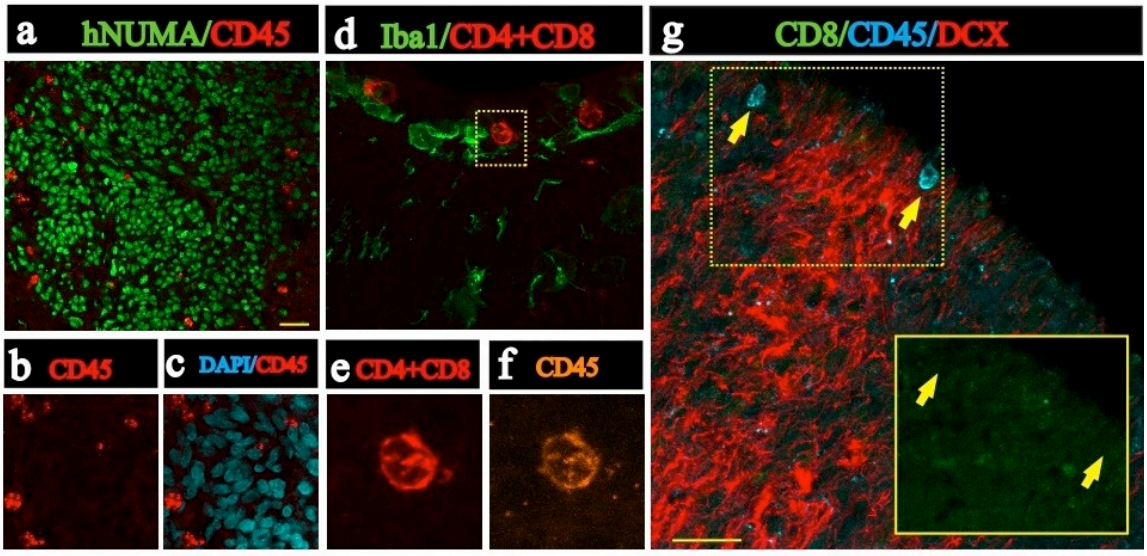

Figure 6: The immunosuppressive effect of 5.1mg/kg/day Tacrolimus pellets. The immunosuppressive effect of the administered dose of Tacrolimus (5.1mg/kg/day) was further validated by identifying the presence of T-lymphocytes in human cell-grafted spinal cord sections. Only occasional presence of CD45+ (red) leucocytes was seen; CD45+ elements were preferentially seen at the periphery of hNUMA+ grafts (**a-c**). Co-staining with CD45 and CD8 antibody showed near complete lack of CD8+ immunoreactivity in CD45+ cells in cell-grafted spinal cord regions and only occasional double stained CD4/CD8/CD45+ cells were seen (**d-f**),while the majority of CD45+ cells were CD8 negative (**g**; yellow arrows). VH – ventral horn, VF – ventral funiculus. Scale bar (a-g): 30µm.

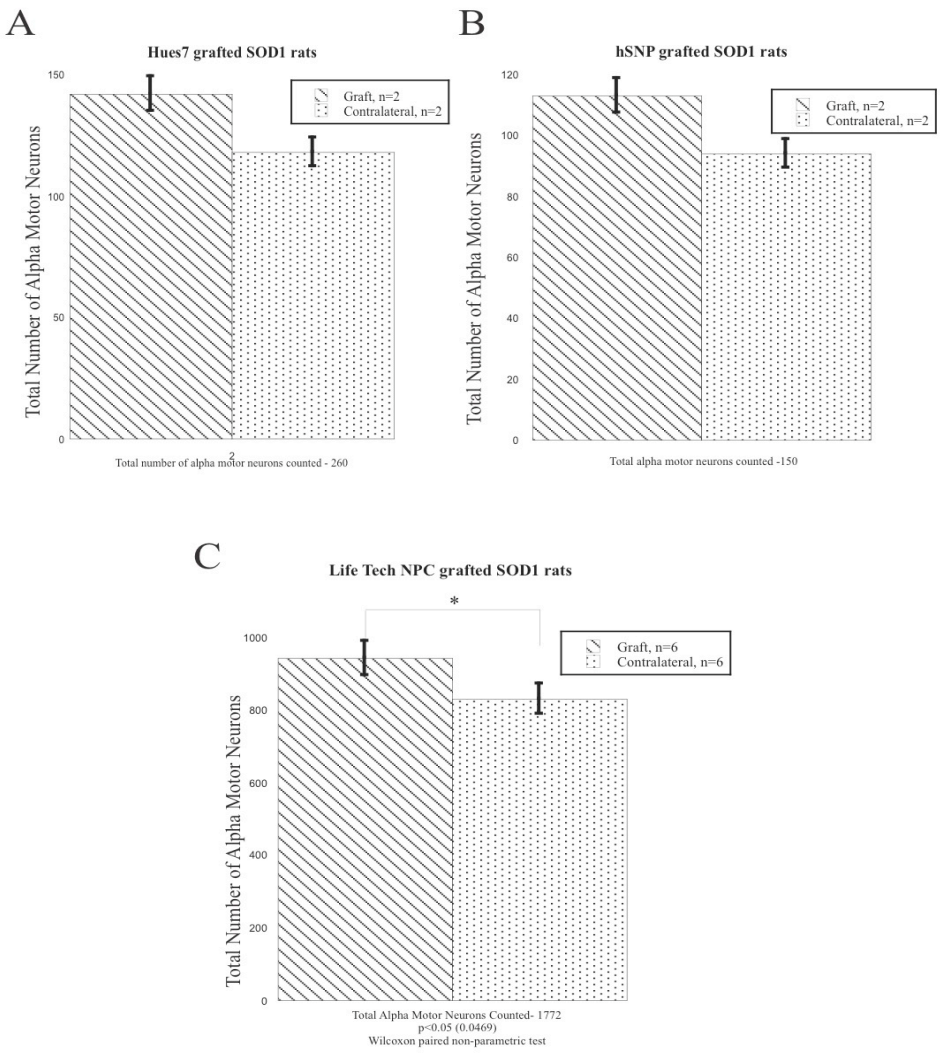

Figure 7: Alpha motor quantification in SOD+ rats transplanted with Hues7, hSNP or Life Tech NPCs. A greater number of CHAT+ alpha motor neurons were observed in injected side compared with the contralateral side in Hues7 (a) and hSNP (b) grafted animals, however this trend was not significant. A significantly greater number of CHAT+ alpha motor neurons were observed in the injected side of the cord compared with the contralateral side in animals receiving Life Tech NPCs (c, $p=0.0469<0.05$, Wilcoxon paired non-parametric test).

Chapter 4, in part is currently being prepared for submission for publication of the material. Marsala, Martin; Sevc, Juraj; Goldberg, Danielle. “Effective, long-term immunosuppression in SD rat using implantable tacrolimus pellets.” The thesis author was the primary investigator and author of this material.

5. DISCUSSION

5.1 Efficacy and optimal dosage of Tacrolimus pellet immunosuppression

Based on drug pharmacokinetics, toxicity, cell survival and host immune response, Tacrolimus pellet immunosuppression represents a novel form of immunosuppression that is safe and effective and may be useful in other xenotransplantation studies. Tacrolimus pellet immunosuppression resulted in relatively stable, non-fluctuating blood concentration levels and pharmacokinetics for up to 3 months post-pellet-implantation. In comparison to commonly used subcutaneous daily administration of Tacrolimus, pellet induced immunosuppression was more reliable and controllable in maintaining targeted blood Tac levels. In preliminary studies we found that precisely timed daily administration of Tacrolimus resulted in a substantial decrease in the blood concentration after 24 hours post injection, with a highly fluctuating suggested pharmacokinetic throughout a 120-hour period post-injection (Marsala et al., submission in progress). In contrast, pellet-induced immunosuppression resulted in a progressive increase in plasma Tacrolimus concentration during the initial 30 days post-pellet implantation that leveled out and decreased only slightly in a 2-3 month period post-pellet implantation. The progressive increase in blood drug concentration observed within the initial 30 days may provide a beneficial adaptation window that reduces drug-induced animal shock and/or toxicity. The steady levels of Tacrolimus maintained during the 2-3 month period after a single pellet implantation suggest this form of immunosuppression is effective in providing a steady release of drug for at least 3 months.

Tac pellet-induced immunosupression was well tolerated by animals, evidenced by the less than 5% toxicity rate in studied animals receiving high dosage (5.1mg/kg/day) pellets and 0% toxicity rate in 1.9mg/kg/day dosage animals. The observed toxicity in high dosage animals is

low compared to the approximately 8-10% toxicity rates observed in studies previously performed by our laboratory using sub-cutaneous Tacrolimus administration (3mg/kg/day; not published data).

In addition we found that animals receiving injectable sub-cutaneous administered Tacrolimus immunosuppression commonly developed extensive tissue fibrosis surrounding the site of injection (not published data) that is not observed in pellet-immunosuppressed animals. This demonstrates that pellet immunosuppression has relatively low toxicity and reduces physical injury and trauma to animals in comparison to currently used injectable immunosuppression techniques. Future studies may aim at testing if sufficient immunosuppression can be achieved with a medial dosage pellet (3.3-3.7mg/kg/day) that may have reduced toxicity rates relative to the high dosage pellets tested in this study.

Extensive long-term (up to 3 months) cell survival (independent of the NPC cell line) was observed in animals receiving both high and low Tacrolimus pellet immunosuppression. This demonstrates the effectiveness of this novel form of immunosuppression in suppressing SOD+ animal immune response sufficiently to permit the survival of three distinct cell lines. Achieving long-term graft survival in SOD+ animals has been previously shown to be particularly challenging (Yan et al., 2006), therefore this novel immunosuppression method may prove to be highly effective in other animal models utilized for cell transplantation studies.

While no significant differences in cell survival were observed between differential pellet dosages, differences in host immune response were substantial. The higher blood drug concentration maintained by 5.1mg/kg/day dosage pellets compared with that achieved by 1.9mg/kg/day dosage pellets appears to be necessary to substantially decrease activation of T lymphocytes. Extensive infiltration of cytotoxic T cells in lower dosage animals suggests the presence of acute host rejection (despite the presence of surviving grafts) and overall insufficient immunosuppression. Minimal cytotoxic T-cell marker staining comparable to non-cell

transplanted SOD+ rats was visible in higher dosage animals, indicating the acquired host immune response to transplanted cells was sufficiently suppressed with this higher dosage. Overall the differential observation of immune response between the two different pellet dosage groups indicate that higher levels of Tacrolimus may provide better conditions for extended cell survival and efficacy.

5.2 Survival and Integration of NPCs

Extensive survival of grafted cells was observed with all three NPC cell lines, demonstrating promising therapeutical strategies for ALS treatments. Graft survival in the toxic environment of the SOD+ ALS rodent model demonstrates that stem cell transplantation is a viable therapeutic approach that can be tested in rodent models prior to clinical use. While previous studies have demonstrated NPC graft survival in SOD+ animals using subcutaneous immunosuppression techniques, our study demonstrates the extensive survival using a less labor intensive and more reliable immunosuppression technique. This immunosuppression technique improves on key clinical parameters of translational research, allowing for increased efficiency and potentially efficacy of this research. Our study also demonstrates extensive survival of a novel human embryonic stem cell-derived neural precursors cell line (Life Tech NPCs) and thereby expands the choice for the selection of an optimal cell line to be used in a potential future human clinical trials.

With regards to xenograft location, it appeared that the grey matter represented a preferential environment for the survival and differentiation of NPCs in SOD+ animals. Cell injections were performed targeting the ventral grey matter, however in all transplanted animals the majority of surviving grafted cells were observed in the spinal white matter. This preferential xenograft survival was also observed in previous studies performed by our laboratory using non-SOD+ animals, suggesting that the environment of the white matter may be more inductive for

the successful integration and survival of xenograft cells and graft location preferences are not likely due to ventral toxicity present in SOD+ animals. This extensive migration and proliferation of grafted cells in the white matter may be attributed to the cellular population and density of the white matter. Glial cells in the white matter may mimic scaffolding radial glia present during neurodevelopment and may promote the migration and proliferation of stem cells. In addition, it is not surprising that transplanted NPC derived astrocytes are found in greater numbers in the white matter, as the majority of cells in the white matter are glia cells. Previous studies have shown that grafted glial restricted precursors preferentially survive and differentiate along glial lineages and migrate along white matter tracts when transplanted into the rodent spinal cord (Han et al., 2003). While the location of the majority of the grafted cells were not in the targeted area, cells survived and integrated nicely into the targeted grey matter. In addition, the cells in the white matter may be capable of some neuroprotective effect despite not being in direct contact with alpha motor neurons by releasing a variety of trophic factors (such as GDNF, BDNF).

5.3 Differentiation of NPCs

The demonstrated ability of transplanted NPCs to differentiate into functional neurons (not observed in animals transplanted with Life Tech NPCs) and astrocytes in the diseased environment of the SOD+ ALS rat supports the therapeutic potential of these cells and the effectiveness of the transplantation protocols implemented in this study. While fully differentiated cells were observed (evidenced by the presence of hGFAP and hSYN), extensive DCX and vimentin positivity indicate that the majority of grafted cells were not fully differentiated and/or mature at 2-3 months post-transplantation. In studies performed by our laboratory using the same NPC lines, we found a substantial decrease in these early differentiation markers and increase in late post-differentiation markers at longer survival (4-5months) time-points (not published data). This indicates that extended survival is optimal for studying the potential therapeutic efficacy of differentiated transplanted cells. Future studies may

focus on 4-5 month survival of NPCs using multiple pellet implantations (an additional pellet after 3 months) to determine if the level neuroprotection is even more pronounced.

5.4 Neuroprotective efficacy of NPCs

Significant increases in the number of alpha motor neurons observed in the region of spinal cord injected with Life Tech NPCs compared with non-injected regions indicates a promising therapeutic effect of transplanted NPCs. This positive effect of grafted cells on alpha motor neuron survival does not appear to be only linked to differentiation of these cells into astrocytes, as the number of differentiated astrocytes were relatively low compared with the overall graft size (qualitative observation). In addition, only a few hGFAP+ cells were observed in the ventral grey matter, indicating that if transplanted astrocytes are providing a neuroprotective effect, it is likely due to long-range mechanisms such as release of trophic factors. Transplanted cells exhibited Vimentin positivity demonstrating the presence of astrocyte precursor cells, however the majority of Vimentin positive grafted cells were observed outside of the ventral grey matter. The majority of grafted cells in the ventral grey matter were DCX positive, indicating these cells were early postmitotic neurons (Brown et al., 2003). Overall, the differentiation and location patterns of transplanted Life Tech NSCs suggest that transplanted NPCs may provide a neuroprotective effect not only by specific differentiation into astrocytes as indicated in previous studies (reviewed in Vargas and Johnson 2010) but also by environmental buffering of toxic extracellular molecules (such as glutamate) and/or by providing necessary trophic factors. Previous studies have demonstrated the presence of toxic molecules in the extracellular space in SOD+ model rodents (reviewed in Vargas and Johnson, 2010). The introduction of exogenous cells may provide additional barriers to the spread and effect of toxic molecules on endogenous cells. Another potential mechanism of NPC induced neuroprotection is NPC release of neurotrophic factors. Previous studies have demonstrated the critical role of neurotrophic factors in promoting the survival of alpha motor neurons in ALS model SOD+

rodents (Lunn et al., 2009). Trophic factors are released during neuronal development and cellular differentiation and play a critical role cell survival regulation and the maintenance of established synaptic connections (Sanes et al., 2012). As transplanted Life Tech NPCs did not express markers of differentiated astrocytes or neurons, it is possible these non-fully differentiated cells are providing neuroprotection through the release of these trophic factors such as GDNF and BDNF. In addition, the observation that the majority of grafted cells were located in the white matter and thereby not directly interacting with alpha motor neurons supports the idea that NPCs may be providing long-range neurotrophic support and/or toxic substances (glutamate) buffering. Future studies may aim to investigate the degree of neuroprotection achieved relative to the proximity of grafted cells to alpha motor neurons. If graft survival and transplantation into the ventral grey matter is not necessary, this may alter the targeted graft location in our future studies.

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