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Effective long-term immunosuppression in rats by subcutaneously implanted sustained-release tacrolimus pellet: Effect on spinally grafted human neural precursor survival

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ABSTRACT

Achievement of effective, safe and long-term immunosuppression represents one of the challenges in experimental allogeneic and xenogeneic cell and organ transplantation. The goal of the present study was to develop a reliable, long-term immunosuppression protocol in Sprague–Dawley (SD) rats by: 1) comparing the pharmacokinetics of four different subcutaneously delivered/implanted tacrolimus (TAC) formulations, including: i) caster oil/saline solution, ii) unilamellar or multilamellar liposomes, iii) biodegradable microspheres, and iv) biodegradable 3-month lasting pellets; and 2) defining the survival and immune response in animals receiving spinal injections of human neural precursors at 6 weeks to 3 months after cell grafting. In animals implanted with TAC pellets (3.4 mg/kg/day), a stable 3-month lasting plasma concentration of TAC averaging 19.1 ± 4.9 ng/ml was measured. Analysis of grafted cell survival in SOD+ or spinal trauma-injured SD rats immunosuppressed with 3-month lasting TAC pellets (3.4–5.1 mg/kg/day) showed the consistent presence of implanted human neurons with minimal or no local T-cell infiltration. These data demonstrate that the use of TAC pellets can represent an effective, long-lasting immunosuppressive drug delivery system that is safe, simple to implement and is associated with a long-term human neural precursor survival after grafting into the spinal cord of SOD+ or spinal trauma-injured SD rats.

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Introduction

One of the essential requirements for successful translation of experimentally-defined cell-based replacement therapies which utilize the allogeneic or xenogeneic cell grafts into clinical practice is the development of safe and effective immunosuppression protocols that will permit long-term survival and maturation of grafted cells. Current clinical and experimental immunosuppression protocols typically use single or combined immunosuppressive drug regimens with drugs delivered orally, intraperitoneally, intravenously or subcutaneously in a single daily dose or divided into multiple daily doses [see reviews (Barraclough et al., 2011; Halloran, 1996; MacGregor and Bradley, 1995; Wente et al., 2006)]. While in human clinical settings a targeted plasma concentration of a variety of immunosuppressant drugs can effectively be achieved by a drug dose titration, accomplishing comparable consistency in targeted plasma levels in animal studies remains a major challenge.

Besides cyclosporines, mycophenolate mofetil (MMF), rapamycin or prednisolone, TAC (FK-506, Prograf) represents an immunosuppressant of choice and is frequently used as a solo therapy or in combination with other immunosuppressive drugs (i.e., MMF) (Hefferan

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et al., 2011; Reis et al., 1998; Xu et al., 2010) [see reviews (Lama et al., 2003; Su et al., 2011)]. TAC couples with immunophilins, proteins termed FK-506 binding proteins (FKBPs) (Siekierka et al., 1989; Thomson et al., 1995). The formation of a pentameric complex comprised of TAC, FKBPs, calcineurins A and B and calmodulin results in the inhibition of the phosphatase activity of calcineurin (Halloran, 1996; McKeon, 1991). The action of transcription factors requiring dephosphorylation for transport to the cell nucleus is inhibited and leads to the suppression of T-cell proliferation and function (Thomson et al., 1995).

In human clinical allogeneic organ transplantation, the recommended concentration of TAC in blood is in the range of 10–20 ng/ml (Pirsch et al., 1997; Przepiorka et al., 1999; Staatz and Tett, 2004) and is effective in maintaining long-term survival of transplanted solid organs (such as kidney, bone marrow or liver) with tolerable side effects typically presented as nephrotoxicity, neurotoxicity, gastrointestinal toxicity or drug-induced diabetes (Vicari-Christensen et al., 2009). In experimental allograft or xenograft animal studies that use rodents (mice, rats) or minipigs as recipients, TAC is typically administered using a chronically implanted intravenous catheter, intraperitoneally or subcutaneously, with doses ranging from 0.05 to 3 mg/kg/24 h (Gold et al., 1995; Hefferan et al., 2011; Saxena et al., 2007; Tze et al., 1992; Usvald et al., 2010). However, despite the use of such aggressive immunosuppressive protocols, experimental xenograft studies are frequently hampered by inconsistent graft survival particularly seen in long-term survival studies. It is believed that the oscillation in plasma drug concentrations and/or insufficient target plasma levels may in part account for inconsistent graft survival. In addition, the requirements of BID (from latin “bis in die”: two times a day) injections in order to achieve satisfactory TAC levels and to minimize toxicity make this approach i) labor intensive, ii) frequently associated with side effects resulting from repetitive animal injections (such as local inflammatory changes and infection), and iii) associated with systemic side effects such as nephrotoxicity and hepatotoxicity [see reviews (Finn, 1999; Gijsen et al., 2010; Teh et al., 2011)].

To extend the half-life of administered drugs in general, several longer-releasing formulations were developed. *First*, the use of TAC-loaded liposomes has been shown to provide moderate prolongation of the TAC half-life in the whole blood of naïve rats in comparison with conventional i.v. injections of TAC diluted in saline (Ko et al., 1994; McAlister, 1998). *Second*, the use of biodegradable microspheres was shown to provide a relatively stable level of TAC in whole blood for up to 10–21 days after single s.c. administration (Miyamoto et al., 2004; Wang et al., 2004). *Third*, the use of implantable biodegradable pellets has been successfully used to deliver a variety of synthetic drugs or hormones in human patients and in animal experimental models and showed up to 3–6 months of stable drug release after a single pellet implantation (Jockenhovel et al., 1996; Packard, 1992; Srinivasan et al., 2002; Studd and Magos, 1987). To our knowledge, no immunosuppressive pellet formulation has been reported to be successfully used in rodent or other animal models of xenogeneic neural precursor transplantation.

Accordingly, the goal of the present study was two-fold. First we characterized the pharmacokinetics of four different subcutaneously delivered/implanted TAC formulations, including: i) caster oil/saline solution, ii) unilamellar or multilamellar liposomes, iii) biodegradable microspheres, and iv) biodegradable 3-month lasting pellets. The optimal TAC formulation, as defined by simplicity of TAC delivery and stable/predictable blood TAC concentration was then selected and used in the second component of our study. The primary goal of the second part of the study was to validate the level of functionally effective immunosuppression in a separate group of $SOD1^{G93A}$ transgenic or spinal trauma-injured SD rats implanted with 3-month lasting TAC pellets and grafted spinally with human fetal spinal stem cells (hSSC) or human ES-derived neural precursors (ES-NPC). The survival of grafted cells was determined at 1–3 months after grafting using human-specific antibodies and confocal microscopy.

In addition, the potency of TAC pellet-induced immunosuppression was validated by the quantitative analysis of the circulating T-cell population (CD45, CD4, CD8) and by the qualitative and quantitative analyses of the infiltrating T-lymphocytes (CD45, CD4, CD8) in cell-grafted spinal cord regions.

Our results indicate that s.c. implanted 3-month lasting biodegradable TAC pellets represent an effective, safe and simple method to achieve long lasting and effective immunosuppression as evidenced by i) consistent xenograft survival and cell maturation, ii) near complete suppression of grafted site T-cell infiltration, and iii) suppression of circulating blood T-cell concentration.

Material and methods

All procedures were approved by the Institutional Animal Care and Use Committees by the University of California, San Diego and by the Czech Academy of Sciences. Adult Sprague–Dawley albino rats (Velaz Praha, Czech Republic and Harlan Industries, Indianapolis) and $SOD1^{G93A}$ ALS rats (SOD+) (UCSD colony, Dr. D. W. Cleveland, San Diego, California; 49–57 days old) were used in experiments. Animals were housed in standard cages with free access to food and water.

Animal experimental groups were divided into 2 principal studies: i) TAC pharmacokinetic study, and ii) spinal grafting of human neural precursors in TAC pellet-immmunosuppressed animals.

TAC pharmacokinetic study

Four different TAC (Prograf®, Astellas Pharma, Deerfield, Illinois, USA) vehicle-delivery systems were used and delivered into the subcutaneous space (see Table 1 for summary).

1) TAC caster oil/saline solution (Group No. 1 and No. 2):
   Because the hydrophobic nature of TAC powder and its poor solubility in water solutions (e.g., saline) (Kino et al., 1987), TAC powder was dissolved in a mixture of 100% ethanol (8% of total volume), caster oil (2% of total volume) and sterile saline for injections (90% of total volume; Fig. 1g). Two dosing designs were studied. In the first dosing design, animals (n = 4; Grp. No. 1) received 3 mg/kg of TAC in 24-h intervals for a total of 5 days. Blood samples for TAC measurement were collected at 2, 9, 24, 72 and 120 h. At 24 and 72 h the blood samples were collected just before subsequent TAC injection. In the second dosing design, animals (n = 4; Grp. No. 2) received 1.5 mg/kg of TAC in 12-h intervals for a total of 5 days. Blood samples for TAC measurements were collected at 2, 12, 14, 24 and 120 h. At 12 and 24 h the blood samples were collected just before subsequent TAC injections.
2) TAC liposomes (Group No. 3 and No. 4):
   Two structurally different liposome designs (unilamellar or multilamellar; Figs. 1I, J) were used (Encapsula NanoSciences LLC, TN). In the first group, TAC-loaded unilamellar liposomes (n = 4; 3 mg/kg; Grp. No. 3) were used. In the second group, TAC-loaded multilamellar liposomes (n = 4; 3 mg/kg; Grp. No. 4) were used. In both groups, TAC liposomes were injected as a single bolus. Blood samples for TAC measurements were collected at 2, 12, 24, 48 and 72 h.
3) TAC microspheres (Group No. 5 and No. 6):
   TAC-containing microspheres were prepared from tacrolimus powder and poly (D,L-lactide-coglycolide) copolymer (Resomer LG 503H, Aldrich) adopting the procedure previously described (Wang et al., 2004). The tacrolimus content in the resulting dry TAC microspheres was 45 mg TAC/g of microspheres as determined by HPLC. Rats were injected with a single bolus of TAC-containing microspheres at a dose of 10 mg/kg (n = 3; Grp. No. 5) or 20 mg/kg (n = 3; Grp. No. 6). Blood samples for TAC measurements were collected at 2, 9, 24 h and at 2, 4, 7, 10, 13, 16, 19, and 22 days.

Table 1
Summary of experimental groups used in tacrolimus PK study.

Group	Route of tacrolimus	Vehicle	Dose	Frequency	n	Time points for detection of Tacrolimus level in blood
No.1	s.c. injection	Powder dissolved in caster oil/saline solution	3 mg/kg	Every 24 h	4	2,9,24,72,120 h
No.2	s.c. injection	Powder dissolved in caster oil/saline solution	1.5 mg/kg	Every 12 h	4	2,12,14,24,120 h
No.3	s.c. injection	Unilamellar liposomes	3 mg/kg	Once, at beginning	4	2,12,24,48,72 h
No.4	s.c. injection	Multilamellar liposomes	3 mg/kg	Once, at beginning	4	2,12,24,48,72 h
No.5	s.c. injection	Microspheres	10 mg/kg	Once, at beginning	3	2,9,24 h,2,4,7,10,13,16,19,22 days
No.6	s.c. injection	Microspheres	20 mg/kg	Once, at beginning	3	2,9,24 h,2,4,7,10,13,16,19,22 days
No.7	s.c. implantation	3 months lasting pellet	1.8 ± 0.15 mg/kg/day	Once, at beginning	4	5,13,20,29,50,56,71,90 days
No.8	s.c. implantation	3 months lasting pellet	3.43 ± 0.21 mg/kg/day	Once, at beginning	3	5,7,10,17,26,38,59,74,90 105, 120, 140 days
No.9	s.c. implantation	3 months lasting pellet	5.1 ± 1 mg/kg/day	Once, at beginning	6	10,27,42,53 days

4) TAC releasable pellets (Group No. 7, No. 8 and No. 9):
TAC-containing 3-month releasable pellets (45, 65 or 90 mg/pellet; Innovative Research of America, FL, USA or Tacropellet, MD, USA; Fig. 1h) were implanted subcutaneously in the interscapular region of the neck in isoflurane (2%)-anesthetized rats. Animals with three different body weight ranges (120–350 g) were employed resulting into 3 different dosing groups releasing 1.8 mg/kg/24 h (n = 4; Gr. No. 7), 3.4 mg/kg/24 h (n = 3; Grp. No. 8) and 5.1 mg/kg/24 h (n = 6; Grp. No. 9) of TAC. Blood samples for TAC measurements were collected periodically between 5 and 140 days after TAC pellet implant (see Table 1 for details).

Blood collection and TAC analysis

In all experimental groups, blood samples were collected from the saphenous vein in fully awake restrained animals. The lateral aspect of the hind leg was shaved, cleaned with 70% EtOH and disinfected with Chlorhexidine. The leg was immobilized in the extended position by applying gentle downward pressure immediately above the knee joint. Petroleum jelly was applied on the site in order to visualize the saphenous vein and to optimize the blood flow. A 20 gauge needle was used to puncture the vein. We first punctured the vein proximal to the ankle and then moved up if additional punctures were necessary (no more than three needle punctures were attempted in any of blood collection sessions). A maximum of 200 μl of blood was collected per time point. Blood was collected into EDTA tubes and kept at −20 °C until analysis using 2 equivalent validated assays: chemiluminescent microparticle immunoassay (CMIA) and liquid chromatography/mass spectrometry (LC/MS/MS).

Chemiluminescent microparticle immunoassay (CMIA)

CMIA — ARCHITECT TAC (Abbot Architect, Chicago, Illinois, USA) assay was used according to the manufacturer's protocol. The detection limits of quantification for this method were 2 ng/ml (min) and 30 ng/ml (max), respectively. If the concentration of TAC in the sample exceeded the 30 ng/ml limit, blood was diluted with saline (in a 1:1 ratio) and the measurement repeated. The CMIA method was used for quantification in groups 1, 2, 3, 4 and 8.

High performance liquid chromatography–mass spectrometry (LC/MS/MS)

A guard column C18, 4 × 2.0 mm (Phenomenex, Torrance, California, USA) equipped with ABI 4000 QTrap linear ion trap mass spectrometer (AB Sciex, Concord, Canada) was used. Whole blood samples were prepared as follows: 40 μl of sample (EDTA whole blood) was mixed with 140 μl protein precipitation solution (conc. ascomycin and zinc sulfate dissolved in methanol). After centrifugation (17,000 ×*g* for 5 min.), 40 μl 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, 600 μl/min.) with a mixture of methanol containing 2.5 mM/l ammonium acetate and distilled water containing 2.5 mM/l ammonium acetate (ratio 75:25). Thereafter, the mobile phase was changed to 2.5 mM ammonium acetate dissolved in methanol (100% of the volume, flow rate 600 μl/min, time 1.50 min) and TAC/ascomycin was eluted to the detector. The column was reconditioned with methanol containing 2.5 mM/l ammonium acetate (75% of the volume, flow rate 600 μl/min, time 1.50 min). MS/MS analysis was performed in multiple reactions monitoring mode using transactions m/z 821.6 > 768.5 for TAC 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. The detection limits of quantification for this method were 2 ng/ml (min) and 50 ng/ml (max), respectively. LC/MS/MS method was also used for determination of concentration of the TAC in whole blood in groups 5, 6, 7 and 9.

Assessment of Tacrolimus pellets — induced side effects

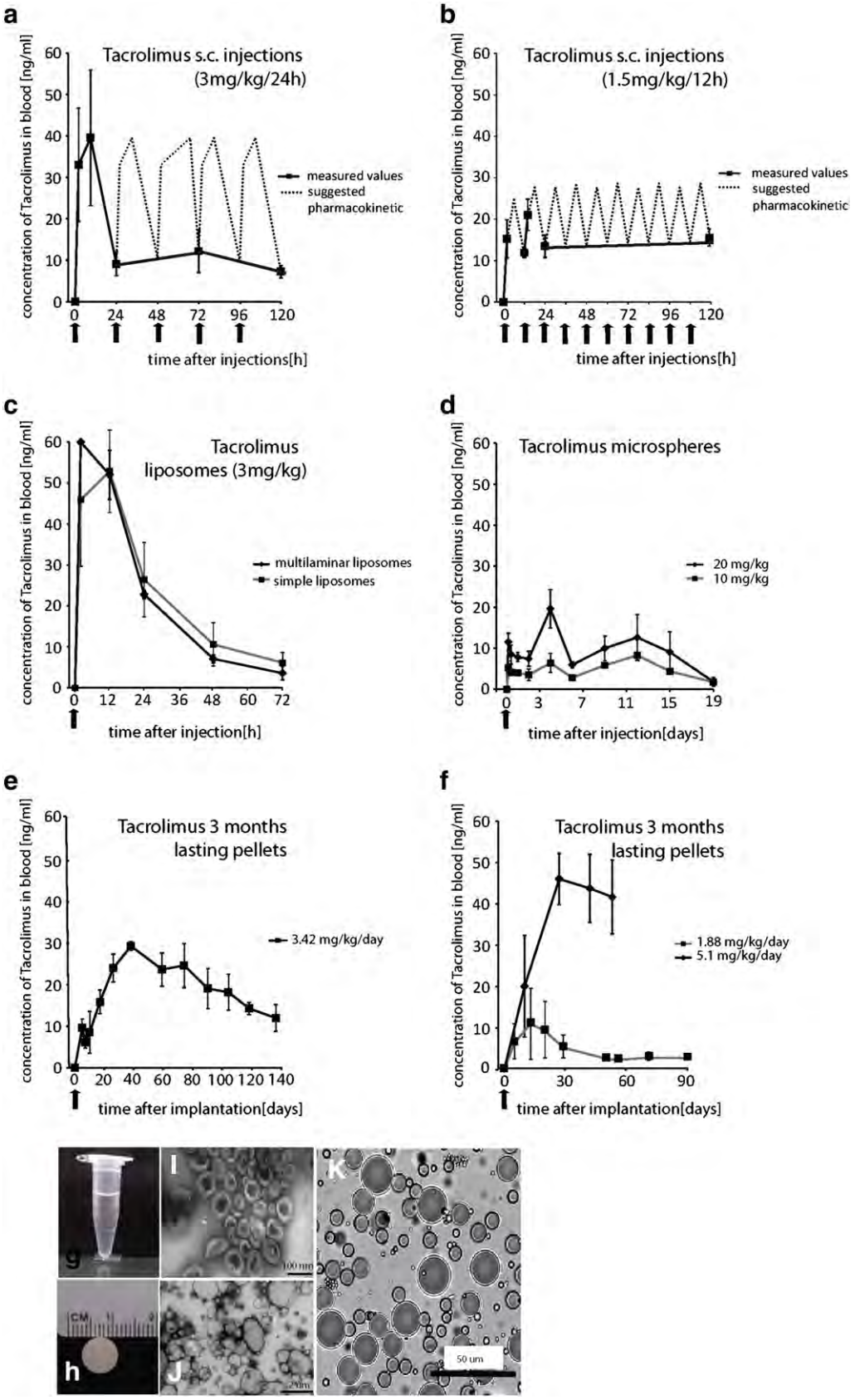
Animals were evaluated daily for the presence of motor dysfunction (upper extremity motor function assessment was used in paraplegic spinal trauma animals), agitation, jumpiness, and tactile allodynia. In addition, animals were monitored for the presence of other potentially TAC-treatment-related signs of toxicity including: scruffy coat, porphyrin (an indicator of stress, known as "red tears", often mistaken as blood), weight loss, hypothermia, pale eyes (a reliable indicator of anemia), soft stool, diarrhea, lethargy, vocalization, and swollenness of the pellet implanted subcutaneous site (potential indicator of a local cyst formation).

Spinal cord grafting of human fetal tissue-derived stem cells or embryonic cell line-derived neural precursors

To assess the effectiveness of immunosuppression in TAC pellet-immunosuppressed rats, human fetal spinal cord derived stem cells (hSSC) or human embryonic cell line (HUES-7)-derived neural precursors (HUES7-NPC) were employed for intraspinal grafting in transgenic SOD+, (n = 10) rats or in SD rats with previous L3 compression injury (n = 4) (see Table 2 for experimental cell grafting groups).

Derivation of the human fetal spinal stem cells and HUES-7-derived neural precursors

Derivation of both cell lines was described in detail in our previous studies (Johe et al., 1996; Kakinohana et al., 2012; Usvald et al., 2010; Yuan et al., 2011). Briefly, human fetal spinal stem cells (Neuralstem, Inc., Rockville, Maryland, USA) were derived from the cervical-upper thoracic region of spinal cord tissue obtained from a single 8-week human fetus after an elective abortion. One day prior to each surgery day, one cryopreserved vial of the previously prepared neural precursors was washed, concentrated in 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). On average, 88–93% viability was seen.

Second, the human embryonic stem cells (hESCs; HUES-7 line; Melton Laboratory, Harvard University, Massachusetts, USA), were cultured on a mitomycin C-treated mouse embryonic fibroblast (MEF) feeder layer in HUES hESC medium. Columnar rosettes were manually isolated from induced embryoid bodies and passaged every 3 days to remove contaminating cells. In this stage, NPC were

harvested and FAC-sorted (*Fluorescence-activated cell sorting-FACS*) and CD184+, CD44−, CD271−, and CD24+ cell populations further expanded on PLO/L-coated plates using modified N2 media. For cell growth, 10 ng/ml bFGF as the sole mitogen was added. NPC were expanded for 10–20 passages and frozen aliquots prepared from passage 15–20. On surgery day, one cryopreserved vial of the previously prepared passage was thawed, washed and concentrated in hibernation buffer. Before and after implantation, the viability of cells was measured with trypan blue (0.4%; Sigma). On average, 85–95% viability was seen.

Spinal cord cell-grafting procedure

SOD + rats: animals weighing 267 ± 20.9 g (Group No. 7, age 57 days; n = 4; Tables 1 and 2) and 203.6 ± 44 g (Group No. 9, age 49–52 days; n = 6; Table 2) received spinal grafts of hSSC or HUES7-NPC at 13 days after TAC pellet implantation. To implant cells, the previously described technique was used (Kakinohana et al., 2004). 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 a microinjector (Kopf Instruments, Tujunga, California, USA), the spinal cord was injected with 1 μl (approx. 15,000 cells per injection) of the hSSC or HUES7-NPC cells 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 (approx. 800 μm rostrocaudally apart) were made 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. For the first 14 days after transplantation, additional immunosuppression was performed as previously described (Hefferan et al., 2011) with the daily injection of mycophenolate mofetil (MMF, Cellcept, Roche Pharmaceutical, Nutley, New Jersey, USA, dose 30 mg/kg). Animals were allowed to survive until they reached the endstage of the disease (Group 7; 33–77 days after cell grafting, for details see Table 2) or until they lost 10% of their bodyweight caused by the progression of the disease (Group 9; 32–70 days after cell grafting, for details see Table 2).

Spinal cord (L3) contused rats: four female SD rats (n = 4; Group No.10; BW: 205 ± 11.9 g) were anesthetized with isoflurane (2% maintenance in room air), placed into a spinal unit apparatus (Stoelting, Wood Dale, Illinois, USA) and a partial Th13 laminectomy was performed using a dental drill (exposing the dorsal surface of L3 spinal segment). Spinal cord compression injury was induced by placing a 3.2 mm acrylic rod (weight = 32 g) for 15 min on the dorsal surface of the exposed L3 segment. After compression, the incision was cleaned with penicillin–streptomycin solution and sutured in two layers. Animals were injected with Depomedrol (methylprednisolone, 10 mg/kg) and antibiotics (Cefazoline, 10 mg/kg) and were allowed to recover. Three days after injury animals were re-anesthetized, previously injured L3 spinal segment exposed and received spinal grafts (total of 5 injections; 1 μl per injection, approx. 400 μm rostrocaudally apart) of HUES7-NPC targeted into the epicenter and just above and below the injury. The following immunosuppression protocol was used: for the initial 14 days after cell grafting, animals received combined immunosuppression composed of daily single s.c. injection of MMF (30 mg/kg) and BID injections of TAC (1.5 mg/kg/12 h). At 14 days, animals were implanted with TAC pellets delivering the TAC dose of 4.9 ± 0.27 mg/kg/day. Because our initial PK study showed that on average 10–15 days is required to achieve a targeted plasma TAC concentration (i.e., above 15 ng/ml), animals continued to receive BID injections of TAC (1.5 mg/kg/12 h) for an additional 12 days. MMF treatment (30 mg/kg/day; s.c.) was only used for the initial 14 days after cell grafting. In this experimental group, animals survived 105 days after cell grafting (for details see Table 2).

Naïve non-immunosuppressed SD rats: four female SD rats (n = 4; Group No.11; BW: 367 ± 14 g) received lumbar spinal grafts of hSSC (total of 5 injections; 1 μl per injection, approx. 400 μm rostrocaudally apart; approx. 15,000 cells per injection) as described. No immunosuppression was performed and animals survived for 14 days.

Perfusion, fixation and immunofluorescence staining of spinal cord sections

Rats were deeply anesthetized with pentobarbital and phenytoin and transcardially perfused with 200 ml of heparinized saline followed by 250 ml of 4% paraformaldehyde in PBS. The spinal cords were dissected and post-fixed in 4% formaldehyde in PBS overnight at 4 °C and then cryoprotected in 30% sucrose PBS until transverse or longitudinal sections (40-μm-thick) were cut on a cryostat and stored in PBS. Sections were immunostained overnight at 4 °C with the following primary human-specific (h) or non-specific antibodies made in PBS with 0.2% Triton X-100: mouse anti-nuclear matrix protein/h-nuc (hNUMA; 1:100; Millipore, Temecula, California, USA), rabbit anti-human neuron-specific enolase (hNSE, 1:500, Chemicon), mouse anti-human synaptophysin (hSYN, Chemicon; 1:2000), rabbit anti-human glial fibrillary acidic protein (hGFAP, 1:500, Origene, Rockville, Maryland, USA), goat anti-doublecortin (DCX; 1:1000; Millipore), goat anti-choline acetyltransferase (CHAT, 1:50, Chemicon), mouse anti neuronal nuclei antigen (NeuN, 1:1000, Chemicon), mouse anti-CD4 and anti-CD8 antibodies (1:500; AbD Serotec, Raleigh, North Carolina, USA), mouse anti-CD3 and anti-CD45 antibodies (1:1000, e-Bioscience, San Diego, California, USA), rabbit anti-Iba1(IB1) (1:1000, Wako, Richmond, Virginia, USA), mouse anti-RT1B (MHC class II) (1:500, BD Biosciences, San Diego, California, USA), mouse anti CD11b (1:200, BD Biosciences, San Diego, California, USA) and donkey anti-rat IgG (1:500, Invitrogen). After incubation with primary antibodies, sections were washed three times 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, 1:250, Invitrogen) and DAPI for general nuclear staining. Sections were then mounted on slides, dried at room temperature and covered with a Prolong anti-fade kit (Invitrogen).

Fluorescent microscopy and leukocyte quantification

Fluorescence-stained sections were analyzed using a 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, Vermont, USA) 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 for all images. Images

Fig. 1. Blood TAC pharmacokinetics after subcutaneous injections of four different TAC formulations. First, TAC powder was diluted in caster-oil/saline solution and injected every 24 h for 5 days (3 mg/kg) (a) or every 12 h for 5 days (1.5 mg/kg/12 h) (b). Blood was collected prior to subsequent TAC injection (arrows). Second, TAC loaded unilamellar or multilamellar (c) liposomes were injected subcutaneously as a single bolus (arrow) (3 mg/kg) and TAC concentration measured periodically for 72 h. Third, another two groups of animals were injected with single bolus of biodegradable TAC microspheres in the dose 10 mg/kg or 20 mg/kg (d). Note the lack of TAC plasma concentration "overshoot" during the first 9 h after delivery and a relatively stable plasma TAC concentration for up to 15 days after a single TAC microsphere bolus injection. Fourth, the biodegradable 3-month releasable TAC pellets delivering 1.88 mg/kg/day, 3.42 mg/kg/day or 5.1 mg/kg/day of TAC were implanted s.c. and the TAC plasma measured for up to 4.5 months (e, f). Note a progressive increase in TAC concentration during the initial 3–4 weeks after TAC pellet implantation and then followed by a relatively stable and dose-dependent long-lasting TAC release. Arrows indicate the TAC pellet implantation. Each time point is represented as the mean ± SD of TAC concentrations. Macroscopical and microscopical images of different TAC formulations including caster oil–saline (g), 90 mg pellet (h), unilamellar (I) or multilamellar (J) liposomes or microspheres (K).

Table 2
Experimental cell grafting groups.

Rat strain	Group	Animal ID number	Grafted cell line	Survival time after cell grafting
SOD1 (ALS)	No.7	4746	HUES7	77
SOD1 (ALS)	No.7	4753	HUES7	33
SOD1 (ALS)	No.7	4750	hSSC	77
SOD1 (ALS)	No.7	4752	hSSC	77
SOD1 (ALS)	No.9	5038	HUES7	40
SOD1 (ALS)	No.9	5081	HUES7	32
SOD1 (ALS)	No.9	5041	hSSC	38
SOD1 (ALS)	No.9	5076	HUES7	32
SOD1 (ALS)	No.9	5095	hSSC	70
SOD1 (ALS)	No.9	5104	hSSC	37
SD (SCCI)	No.10	149	HUES7	105
SD (SCCI)	No.10	153	HUES7	105
SD (SCCI)	No.10	155	HUES7	105
SD (SCCI)	No.10	160	HUES7	105
SD (Naïve)	No.11	1	hSSC	14
SD (Naïve)	No.11	2	hSSC	14
SD (Naïve)	No.11	3	hSSC	14
SD (Naïve)	No.11	4	hSSC	14

SCCI — spinal cord contusion injury.

were assembled into figures using Adobe Illustrator (Adobe Systems, Inc., San Jose, CA).

Semi-quantitative evaluation of grafted spinal cord tissue infiltration with different types of leukocytes in animals immunosuppressed using TAC biodegradable 3-month lasting pellets was performed using 40-μm-thick coronal sections. Quantification was performed in 5 sections per animal in TAC dose groups of 1.8 mg/kg/day and 5.1 mg/kg/day (see Table 1, Group No. 7 and No. 9; n = 3 for each group).

The number of different classes of leukocytes positive for CD45, CD8/CD45, CD4/CD45, RT1B/CD45 and CD11B was counted and analyzed separately with respect to the location of positive cells in: i) the core of the graft; ii) the pial surface of the graft, and iii) the spinal cord parenchyma outside of the graft. The degree of leukocyte infiltration was graded as follows: 0—absence of immune cells, *—1–10 cells, **—11–50 cells, *** >51 cells per section.

Quantitative analysis of grafted cell survival

Quantitative analysis of grafted cell survival was performed in animals immunosuppressed with 1.8 mg/kg/day of TAC or 5.1 mg/kg/day of TAC (TAC pellet Group No. 7 and 9; see Tables 1 and 2) and grafted with HUES7-NSCs or hSCC (n = 2 for each TAC dose and cell line).

For quantification, 5 transverse spinal cord sections were selected (minimum 500 μm apart) from the cell-grafted segments from each rat. An image of virtual tissue of the whole hNUMA-stained section was captured at 10× using Olympus Imager M2 microscope equipped with MBF Stereo Investigator System. The total number of NUMA+ nuclei was then counted using ImageJ "Analyze Particles" plug-in function after setting an identical detection threshold for all analyzed sections. The number of counted cells was then averaged and expressed as number of cells counted per section for each TAC dose and grafted cell line analyzed.

All images used for quantification (i.e., leukocyte-stained and hNUMA+ stained sections) were examined by a blinded observer.

Flow cytometry

Peripheral blood from rats (saphenous vein) was collected into microtainer EDTA tubes (BD Biosciences, San Jose, CA, USA). Red bloods cells were lysed using RBC lysis buffer (Sigma-Aldrich, St. Louis, MO, USA). The lysed blood was stained with antibodies directed to rat CD4, CD8, CD45, and CD3 (Biosciences, San Diego, CA, USA). A minimum of 10,000 live events (defined by FSC and 7-AAD) was acquired on a six-color BD FACSC. Analysis was done using FACSDiva software. CD4+ and CD8+ cell percentages was measured using a T cell lineage gate defined by 7AAD-CD45+ CD3+ small lymphocytic cells.

Results

Pharmacokinetic profile after subcutaneous TAC delivery (Fig. 1)

Four different TAC vehicle-delivery systems were used for subcutaneous delivery (see Table 1 for details). 1) TAC-containing caster oil/saline solution: the whole blood TAC concentration in animals receiving a single bolus of TAC (3 mg/kg) was on average 39.5 ng/ml at 9 h after injection and decreased to 9 ng/ml at 24 h. With continuing daily injections at the same dose (i.e., 3 mg/kg), the plasma concentration of TAC was 12.2 and 7.2 ng/ml at 72 and 120 h, respectively (i.e., at 24 h after the previous injection) (Fig. 1a); the whole blood TAC concentration in animals receiving a single bolus of TAC (1.5 mg/kg/BID) was 11.9 ng/ml at 12 h after injection. With continuing injections every 12 h at the same dose (i.e., 1.5 mg/kg), the blood concentration of TAC was on average 13.5 and 14.9 ng/ml at 24 and 120 h, respectively (i.e., at 12 h after the previous injection delivered at 12 h and 108 h, respectively) (Fig. 1b). 2) TAC-containing unilamellar or multilamellar liposomes: the blood TAC concentration in animals receiving a single bolus of unilamellar or multilamellar liposomes-containing TAC (3 mg/kg) was on average 52.9 (unilamellar liposomes) and 52 ng/ml (multilamellar liposomes) at 12 h and decreased to 6.1 (unilamellar) and 3.6 ng/ml (multilamellar) at 72 h, respectively (Fig. 1c). 3) TAC-containing microspheres: the whole blood TAC concentration in animals receiving a single bolus of TAC-containing microspheres (20 mg/kg or 10 mg/kg) showed a biphasic TAC release profile. The first peak was measured at 4 days after delivery and was 19.6 (20 mg/kg dose) and 6.4 ng/ml (10 mg/kg dose), respectively. The second peak was measured at 12 days after delivery and was 12.6 (20 mg/kg dose) and 8.3 ng/ml (10 mg/kg dose), respectively. The TAC concentration at 19 days after delivery was on average 1.5 and 1.7 ng/ml in 20 mg/kg and 10 mg/kg group, respectively (Fig. 1d). 4) TAC-containing pellets: groups of animals were implanted with 3-month releasable TAC pellets releasing TAC at 1.9, 3.4 or 5.1 mg/kg/day. In the 1.9 mg/kg group (n = 4) the peak value of 11.1 ng/ml was measured 13 days after pellet implantation and then gradually decreased to 2.8 ng/ml at 90 days. In the 3.4 mg/kg group (n = 3) the peak TAC concentration was measured at 38 days after pellet implantation and was on average 29.4 ng/ml. At 3 months

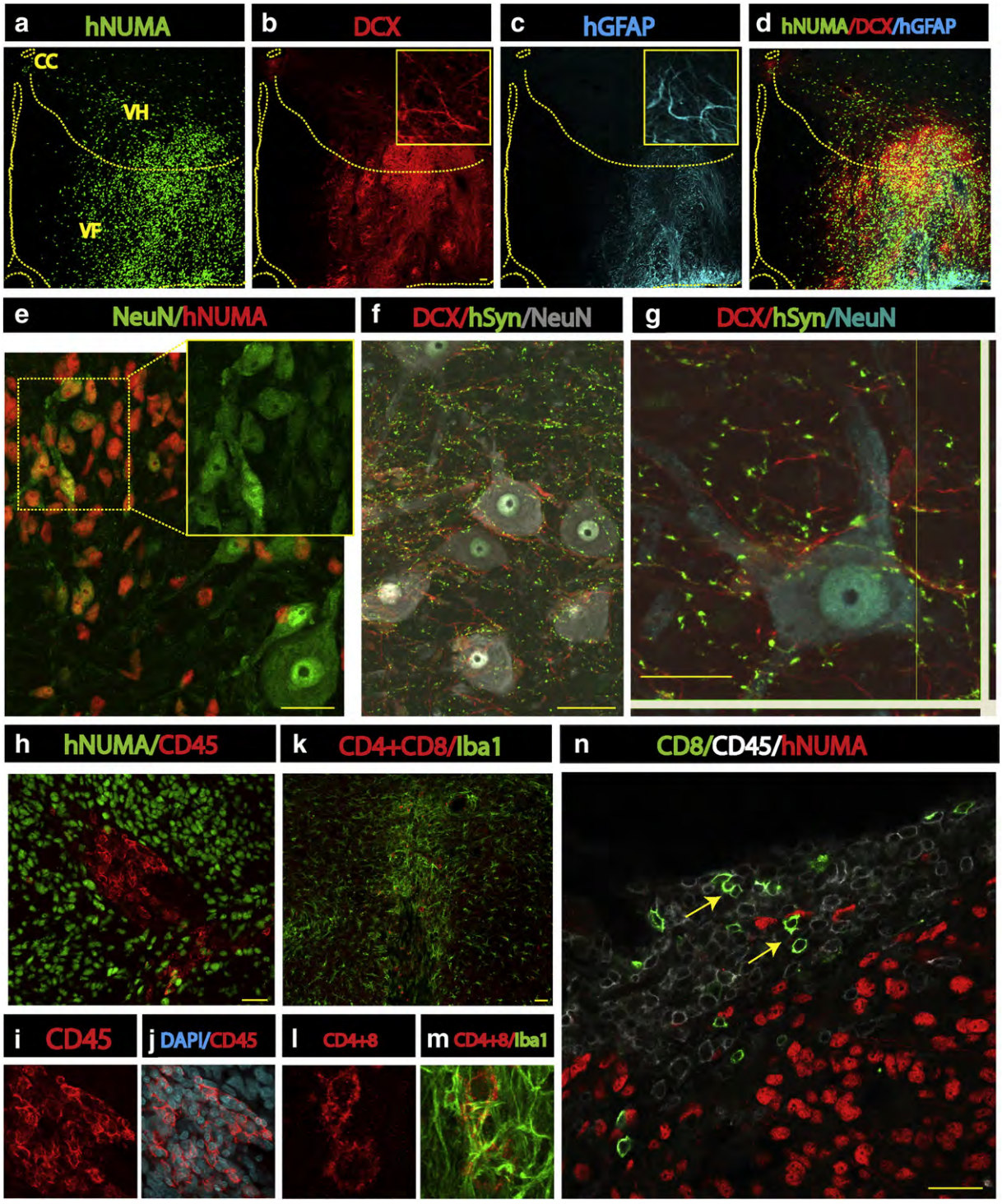
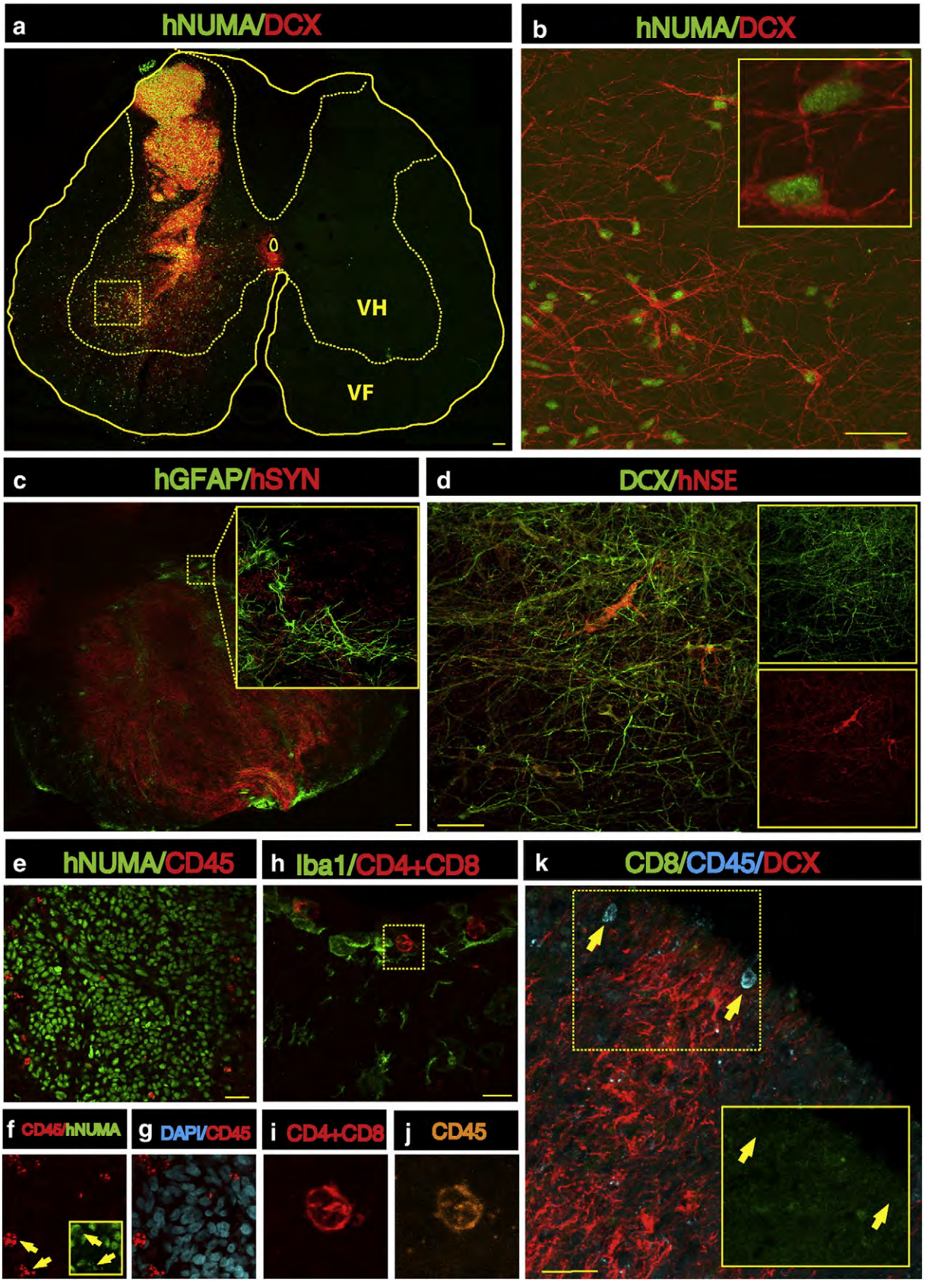


Fig. 2. Immunofluorescence examination of the presence of grafted human ES-derived neural precursors (HUES7-NPC) and immunological response in the spinal cord tissue of SOD + rats implanted with 3-month lasting TAC pellet (1.9 mg/kg/day). TAC releasable pellets were implanted subcutaneously in SOD + rats (n = 4; 60–65 days of age) 13 days prior to grafting with HUES7-NPC. After cell grafting, animals were allowed to survive until they reached the endstage of the ALS disease (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 well-engrafted cell populations in the spinal parenchyma (a–d). Colocalization of hNUMA (red) with NeuN (green) can be seen in a dense population of hNUMA + grafted cells (e). Triple labeling with DCX, hSYN and NeuN antibody showed numerous DCX + processes projecting towards large α-motoneurons and co-expressing hSYN punctate-like immunoreactivity (f, g). The immunosuppressive effect of a given TAC dose (1.9 mg/kg/day) was further tested by identifying the presence of infiltrating T-lymphocytes. A high density leukocyte population (CD45, red) in the vicinity of grafted human cells (hNUMA, green) was observed (h–j). 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 (k–m). The presence of cytotoxic subpopulation of T-lymphocytes in the regions containing hNUMA + grafted cells (red) was confirmed by colocalization of marker CD8 (green) with general leukocyte marker CD45 (white), (n). VH — ventral horn, VF — ventral funiculus. Scale bar a–n: 30 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the TAC levels were 19.1 ng/ml. In the 5.1 mg/kg group (n = 6) the peak of TAC concentration was detected at 27 days and was 46 ng/ml. At 53 days the TAC concentration was 41.7 ng/ml (Figs. 1e, f). Macroscopical and microscopical images of all TAC formulations used in this study including caster oil–saline, 90 mg pellet, unilamellar or multilamellar liposomes and microspheres are shown in Figs. 1g–K.



Tolerability of SD rats to long-term TAC pellet-induced immunosuppression

Animals implanted with 3-month releasable pellets (dose 1.9 mg/kg/day) showed good tolerability for up to 3 months after pellet implantation and no detectable clinically-defined side effects were noted.

In the animal group implanted with 3.4 mg/kg/day TAC pellets, good tolerability was seen for up to 3 months without any prophylactic

antibiotic treatment. Three animals were allowed to survive for an additional 2 months while a progressively decreasing TAC blood concentration was monitored. At the end of 5 months, 2 animals succumbed to infection, which was determined to be of gastrointestinal origin.

In the animal group receiving the highest TAC dose (5.1 mg/kg/day), good tolerability was seen for up to 6–7 weeks after pellet implantation. From the total number of 60 animals so far tested in our laboratory with this dose, 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 60 ng/ml. Once these symptoms were identified, the 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 between 3 and 8 weeks after pellet implantation. In 6 of 44 implanted animals, a cyst partially filled with serous fluid and surrounding the pellet implanted-subcutaneous region was identified.

Survival and maturation of spinally grafted human fetal spinal cord stem cells (hSSC) or human ES-derived neural precursors (HUES7-NPC) in SOD + rats and TAC-dose dependent suppression of T-cell infiltration (Figs. 2, 3)

To validate the effectiveness of TAC pellet-induced immunosuppression in an experimental xenograft design, transgenic SOD + rats were implanted with TAC pellets and then received spinal grafts of hSSC or HUES7-NPC 13 days after TAC pellet implantation. Based on the initial pharmacokinetic study, 3 groups of transgenic SOD + rats previously implanted with TAC pellets delivering either 1.9 mg/kg/day, 3.4 mg/kg/day or 5.1 mg/kg/day of TAC were used for cell grafting. In addition, a group of SD rats with previous L3-4 compression injury were used. Spinal trauma animals were grafted with HUES7-NPC. Animals in all experimental groups survived between 32 and 105 days after cell grafting (see Table 2 for experimental groups). In general, independent of the TAC dose group and grafted cell line, comparable long-term engraftment and maturation of grafted cells was seen in SOD + animals.

In the 1.9 mg/kg/day TAC group (SOD + rats) grafted with HUES7-NPC, triple-staining with human-specific nuclear marker (hNUMA), early postmitotic neuronal marker DCX and human-specific GFAP antibody (hGFAP) of transverse spinal cord sections taken from grafted spinal segments showed well engrafted hNUMA + cell populations composed of DCX + neurons and hGFAP astrocytes at 77 days after grafting (Figs. 2a–d). Double-staining with NeuN (neuronal nuclei antigen) and hNUMA showed that the majority of hNUMA + cells in the gray matter acquired NeuN immunoreactivity (Fig. 2e; inset). Confocal analysis of DCX/hSYN (synaptophysin)/NeuN-stained sections showed a dense population of hSyn + terminals residing in the vicinity of ventral α-motoneurons and were associated with DCX + processes (Figs. 2f, g).

The degree of T-cell infiltration was probed by staining with CD45, CD4 and CD8 antibodies (see Table 3 for quantitative analysis). High-density CD45 + cell populations were identified typically at the core or at the periphery of cell-grafted regions (Figs. 2h–j). In the same areas CD4/8 + lymphocytes surrounded by activated IB1 immunoreactive microglial cells were seen (Figs. 2k–m). Double-staining with CD45 and CD8 antibody showed a clear population of CD45/CD8 + cells in the vicinity of hNUMA + grafted cells (Fig. 2n; yellow arrows).

In the 5.1 mg/kg/day TAC group (SOD + rats) grafted with hSSC, double-staining with hNUMA and DCX antibody showed a robust cell engraftment with high population of hNUMA/DCX + grafted neurons throughout the grafted region and extending from the dorsal horn to ventral gray matter at 70 days after cell grafting (Fig. 3a). Numerous solitary hNUMA/DCX + neurons which migrated from the core of the graft were also identified (Fig. 3b; insets). Staining with human specific synaptophysin antibody showed a wide-spread hSYN punctate-like staining pattern throughout the grafted region (Fig. 3c). In the same areas a high density of hGFAP + astrocytes were also seen (Fig. 3c—inset). Double staining with DCX and human-specific NSE antibody (hNSE) showed that in areas with high density of DCX + processes solitary hNSE + neurons were also present (Fig. 3d).

Quantitative analysis of hNUMA + cells in 1.9 mg/kg/day TAC group showed on average 2712 ± 320 cells in HUES7-NPC-grafted animals (n = 2) and 1772 ± 629 cells in hSSC-grafted group (n = 2). In the 5.1 mg/kg/day TAC group on average 2750 ± 527 cells were counted in HUES7-NPC-grafted animals (n = 2) and 2164 ± 638 cells were counted in the hSSC-grafted group (n = 2).

In both TAC dose groups (3.4 and 5.1 mg/kg/day), staining with CD45, CD4 and CD8 markers showed a similar pattern. The number of CD45 + stained cells was relatively low (if compared to 1.9 mg/kg/day TAC group; see Table 3 for quantitative analysis) with the majority of CD45 + cells typically found at the periphery of individual grafts (Figs. 3e–g). Staining with CD4/8 antibody showed only sporadic presence of CD4/8 + cells (Figs. 3h–j) and the majority of CD45 + cells found in grafted DCX + regions were CD8 negative (Fig. 3k; yellow arrows).

Survival and maturation of spinally grafted HUES7-NPC in adult SD rats with previous lumbar (L3) spinal contusion injury (Fig. 4)

To characterize the survival of spinally grafted human ES-derived neural precursors, adult SD rats with previous L3 spinal contusion injury received spinal grafts of HUES7-NPC at 3 days after injury and survived for 3 months. The dose of TAC delivered by implanted TAC pellet was 4.9 mg/kg/day. Immunofluorescence analysis of hNSE/hNUMA/DCX triple-stained sections showed an advanced stage of neuronal maturation as evidenced by an intense hNSE immunoreactivity (Fig. 4a; insets). Numerous double stained hNSE/DCX solitary neurons which migrated outside of the grafts were also identified (Figs. 4b–d). Similarly as in SOD + rats immunosuppressed with a high TAC dose, staining with CD45 antibody revealed only occasional presence of CD45 + cells at the periphery of individual grafts (Figs. 4e–g). Co-staining with CD45 and CD8 antibody showed only occasional presence of CD8 + cells (not shown).

Effect of TAC pellet-induced immunosuppression on circulating blood T-cell population and IgG immunoreactivity in spinal human cell-grafted regions (Fig. 5)

To further probe the effect of TAC pellet-induced immunosuppression on T-cell activity, we quantified the density of circulating T-cell

Fig. 3. Immunohistochemical examination of the presence of grafted human fetal spinal stem cells (hSSC) and immunological response in the spinal cord tissue of SOD + rats immmunosuppressed with 3-month lasting TAC pellet (5.1 mg/kg/day). TAC releasable pellets were implanted subcutaneously in the SOD + rats (n = 6; 60–65 days of age) 13 days prior to grafting with hSSC. Animals were allowed to survive until 10% of bodyweight loss caused by the progression of the ALS disease (i.e., 32–70 days after cell implantation). Immunohistochemical staining with human specific antibody hNUMA (human nuclear protein, green) and doublecortin (DCX, red) revealed the consistent presence of high density grafts in the targeted spinal parenchyma in all animals (a, b). Double staining with human-specific synaptophysin (hSYN) and GFAP antibody (hGFAP) revealed a dense hSYN punctuate-like immunoreactivity and numerous hGFAP + grafted astrocytes (c). In addition to a dense population of DCX + grafted neurons the presence of human-specific enolase + neurons (hNSE; late neuronal marker) can be seen (d). The immunosuppressive effect of the administered dose of TAC (5.1 mg/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) leukocytes was seen; CD45 + elements can preferentially be seen at the periphery of hNUMA + grafts (e–g). Co-staining with CD45 and CD8 antibody shows near complete lack of CD8 + immunoreactivity in CD45 + cells in cell-grafted spinal cord regions and only occasional double stained CD4/CD8/CD45 + cells can be identified (h–j),while the majority of CD45 + cells are CD8 negative (k; yellow arrows). VH — ventral horn, VF — ventral funiculus. Scale bar a,c: 100 μm, b,d,e,k: 30 μm, h:10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 3
Quantitative/qualitative analysis of leukocyte infiltration in transverse spinal cord sections with identified human cell grafts at 77–90 days after cell grafting.

Immunological profile	Dose of Tacrolimus	1.8 mg/kg/day			5.1 mg/kg/day (&)		
	Cell type	G-core.	G-pial	SC-par.	G-core.	G-pial	SC-par.
CD45 +	Leukocytes	***	***	***	*	**	*
CD8 + CD45 +	Cytotoxic T-cells	**	***	*	0	*	0
CD4 + CD45 +	Helper T-cells	***	***	**	*	*	0
RT1B + CD45 +	B-lymphocytes	**	***	**	*	*	0
CD11b +	Granulocytes + monocytes	**	***	*	*	*	0

0—absence of immune cells, *—1–10 cells, **—11–50 cells, ***>51 cells per section; G-core—core of the graft, G-pial surface of the graft, SC-par. parenchyma of the spinal cord outside of the graft, &—in animals injected with 3.4 mg/kg/day TAC dose a comparable suppression of leukocyte infiltration as seen in 5.1 mg/kg/day group was identified (not shown).

population in blood in naïve, SOD + TAC non-treated and SOD + TAC-treated (5.1 mg/kg/day) animals. In comparison to TAC non-treated animals, immunosuppressed rats had a significantly decreased number of circulating CD45, CD4 and CD8 cells (Figs. 5a–c), ($p < 0.05$; t-test).

To identify the presence of IgG-secreting cells or soluble IgG in cell-grafted spinal cord regions, sections taken from all 3 TAC dosing groups were stained with anti-rat IgG (fluorescence-tagged) and then further stained to detect MHC class II (R1TB) and CD45 to identify B cells. In 1.9 mg/kg/day TAC group numerous IgG + cells were identified in grafted regions. Similarly as for the CD8 population, the IgG + cells were typically localized at the periphery of individual grafts. Double staining with anti-rat MHC class II antibody and anti-rat IgG showed the B-lymphocyte phenotype in small subpopulation of IgG + cells (Fig. 5d; yellow arrows). In addition to IgG presence on B-lymphocytes, an overall increase in the density of IgG staining in cell-grafted regions was seen (Fig. 5d; blue dashed area). Since the area had minimal R1TB staining, it is possible that deposited IgG is detected in this area. Similarly, double staining with RT1B and CD45 antibody showed only sporadic presence of RT1B/CD45-double-stained cells in cell-grafted areas (Fig. 5e).

Triple staining with macrophage and monocyte marker CD11b, anti-rat IgG and CD45 showed a clear colocalization of CD11b and anti-rat IgG immunoreactivity but with relatively weak CD45 staining pattern in the same CD11b/anti-rat IgG + cells (Fig. 5f; yellow arrows).

In 3.4 and 5.1 mg/kg/day TAC groups, only sporadic IgG, RT1B or CD45-stained cells were identified as well as the density of the IgG staining in cell-grafted regions was below detection threshold (Figs. 5g, h) and was similar to that seen in control cell-non-grafted animals (not shown).

Rejection of spinally grafted human fetal spinal cord stem cells (hSSC) in the absence of immunosuppression (Fig. 6)

To validate the importance of immunosuppression in providing grafted human cell survival, we next grafted hSSC into lumbar spinal cord in adult SD rats without immunosuppression. Analysis of spinal cord sections at 14 days after cell grafting showed complete grafted cell rejection and intense infiltration of CD4/8 cells at the injection site (Figs. 6a, b, c, d).

Discussion

Experimental and clinical evidence for need of continuing immunosuppression in xenogeneic CNS grafting design

Extensive previous experimental animal but also clinical data show that xenogeneic neural grafts are rapidly rejected in non-immunosuppressed animals and/or human patients. For example, it was demonstrated that grafted porcine dopaminergic neuroblasts (derived from embryonic days 26–27) are rejected within days to weeks after intracerebral grafting in rats in the absence of immunosuppression (Brevig et al., 2000). It was also demonstrated that the porcine xenografts undergoing rejections are infiltrated with CD8 lymphocytes and are stained positive for IgM and complement component (C3) (Barker et al., 2000). Similarly, in clinical Parkinson's disease trial, rafting of porcine embryonic dopaminergic neurons showed very poor grafted cell survival at 7 months (one postmortem patient analyzed) after grafting despite continuing immunosuppression with cyclosporine (5 mg/kg) (Deacon et al., 1997). In the same study, lymphocyte infiltration was also seen in the cell-grafted region. These data are consistent with our previous reports which demonstrate no or poor graft survival after spinal grafting of human spinal stem cells in SOD + rats or SD rats with previous spinal ischemic injury if animals were immunosuppressed with TAC as a monotherapy (1.5 mg/kg, q12h or 1 mg/kg, qd, respectively) (Hefferan et al., 2011; Kakinohana et al., 2012). Similarly, grafted cell rejection was associated with an intense CD4/8T-lymphocyte infiltration in previously cell-grafted regions. Jointly, these data show that in order to achieve satisfactory and long-term grafted cell survival using xenogeneic grafting design, continuing and aggressive immunosuppression which is effective in suppressing T-cell activity is needed.

Stable and controllable blood TAC concentration and tolerability of SD rats to long-term TAC pellet-induced immunosuppression

In our initial phase of the study, we have compared the plasma kinetics of four different TAC formulations after subcutaneous delivery. Three primary characteristics of the PK data and associated animal manipulations (such as repetitive animal injections) were considered in defining the optimal formulation to be used in the subsequent long-term spinal cell grafting studies and included: i) the stability of plasma TAC concentration over a 24-h period, ii) required frequency of injections to achieve a targeted plasma TAC concentration, and iii) potential side effects associated with a high plasma TAC concentration or repetitive animal injections.

The rationale for the selection of specific time points for TAC measurements (i.e., more frequent measurements in caster oil/saline, liposomes and microspheres formulations-injected animals and less frequent in TAC pellet-implanted animals; see Table 1 for summary) was based on expected differences in TAC kinetics among all formulation and by the need to identify potential blood toxic TAC levels.

First, in caster oil–saline as a vehicle or liposome (unilamellar or multilamellar) TAC-injected animals, a comparable kinetics profile was seen during the initial 12 h after 3 mg/kg TAC injections with the peak concentrations (40–60 ng/ml) measured during 2–12 h after injections. In contrast to the caster-oil–saline TAC (3 mg/kg) group in which the TAC levels dropped to 10 ng/ml at 24 h after single injection, in liposome-TAC injected animals (3 mg/kg) on average 27 ng/ml TAC was measured at 24 h and was still around 11 ng/ml at 48 h after injection. In animals injected BID with the caster-oil–saline formulation (1.5 mg/kg/12 h) for 5 days, the measured peak TAC concentration was seen at 2 h (around 23 ng/ml) and stable levels around 13 ng/ml were then measured between 1 and 5 days if measured at the end of 12 h (i.e., at 12 h after previous TAC injection). These TAC pharmacokinetic data are similar as reported from other laboratories using mice or rats (Ko et al., 1994; McAlister, 1998; Yamauchi et al., 2004).

From the perspective of conducting long-term immunosuppressive therapy, these data demonstrate that, should a caster-oil vehicle system be used for TAC delivery, the BID delivery regimen has the most favorable safety profile (as defined by the peak concentration) and is relatively stable through TAC levels. While more labor intensive, a stable targeted through TAC levels can be achieved and readily adjusted if needed. The use liposome-TAC formulation provides an extended half life, however, a relatively high peak concentration (i.e., above 50 ng/ml) and the cost (approx. $1,600/6 animals) to prepare these formulations appears to limit its routine use at present.

Second, TAC microspheres were used and animals received a single subcutaneous injection of either 10 or 20 mg/kg TAC-containing microspheres. In both doses, a biphasic release profile was measured with the first peak seen at 4 days and the second peak at 12 days after injection. In the 20 mg/kg group, the peak measured at 4 days was around 20 ng/ml and the levels were still around 10 ng/ml at 15 days after a single injection. This TAC kinetics profile is similar if not identical as reported in other studies which employed the same TAC microsphere formulation. In contrast to the caster-oil and liposome formulation, no initial TAC plasma concentration "overshoot" was seen at 2 h after injection and levels were around 12 ng/ml at 9 h after injection (in the 20 mg/kg group). The use of TAC microspheres appears to provide a clear advantage over caster-oil and liposome formulation, permitting stable TAC plasma levels for up to 15 days after a single subcutaneous injection. Moreover, additional injections can readily be added should a rapid and continuing stable increase in TAC concentration be desired.

Third, TAC pellets containing different concentration of TAC and delivering 1.8 mg/kg, 3.4 mg/kg or 5.1 mg/kg of TAC/day for up to 3 months were used for subcutaneous implantation. In higher concentration groups, a progressive increase in plasma TAC concentration was seen during the initial 30 days after pellet implantation and then remained relatively stable for an additional 1–2 months. While the pellets are designed for 3-month continuing TAC release, we have observed continuing TAC release exceeding 4 months ($n = 3$) with the TAC levels around 13 ng/ml measured at 5 months after pellet implantation.

With respect to toxicity, no detectable side effects were seen in animals receiving 1.9 and 3.4 mg/kg/day doses and surviving between 1 and 3 months. From a total of 60 animals receiving the

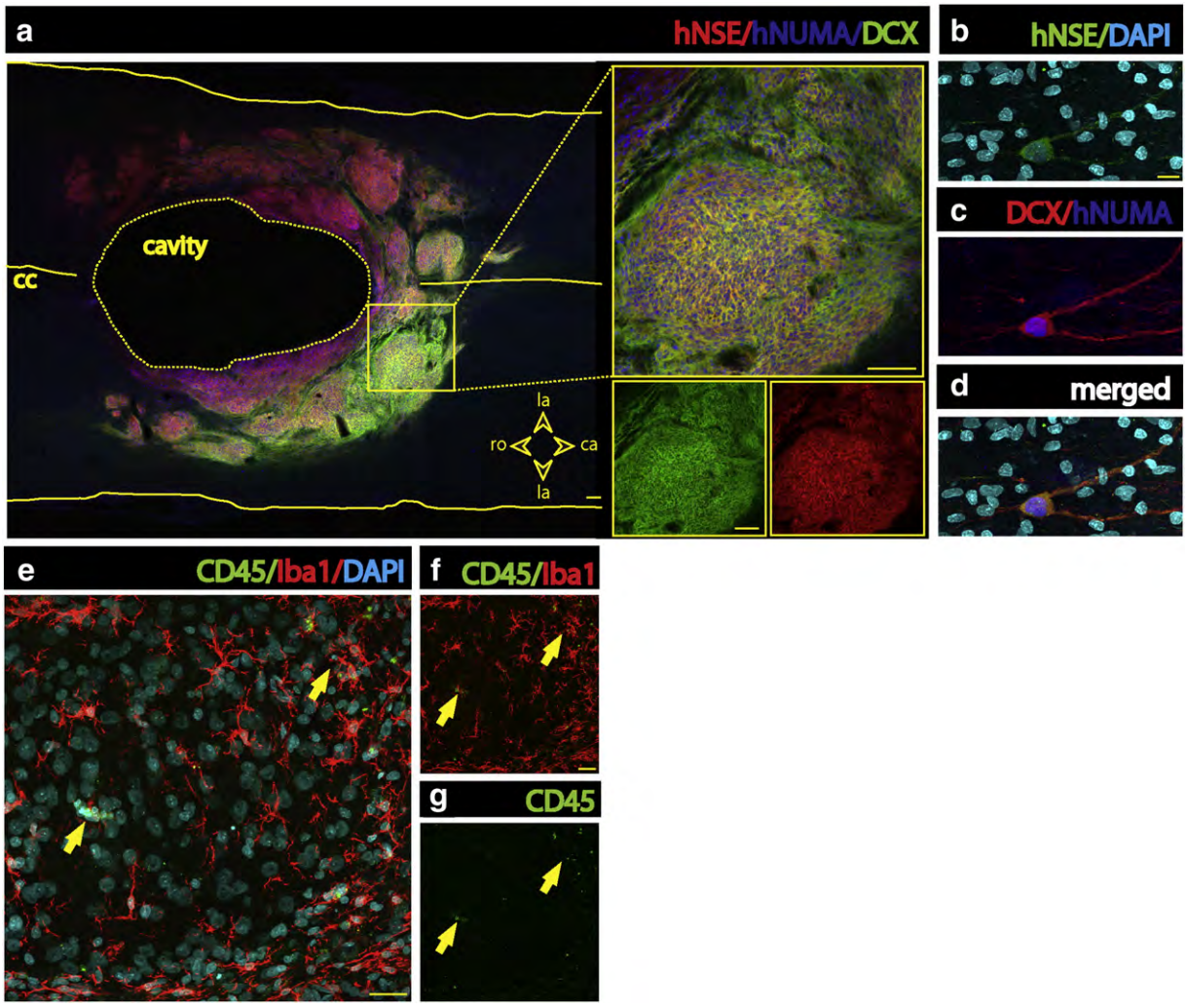


Fig. 4. Immunofluorescence examination of the presence of grafted human ES-derived neural precursors (HUES7-NPC) in TAC pellet (4.9 mg/kg/day)-immunosuppressed SD rats with previous L3 contusion injury. Animals ($n = 4$) received spinal grafts of HUES7-NPC at 3 days after spinal injury and were allowed to survive 105 days after the cell grafting. Immunohistochemical staining with antibodies against human neuronal specific enolase (hNSE, red), human cell nuclei (hNUMA, blue) and doublecortin (DCX, red) on the longitudinally-cut spinal sections revealed continuous graft survival and extensive neuronal differentiation in cell-grafted peri-injury regions (a–d). Staining against the general leukocyte marker (CD45, green) and microglia (IB1, red) revealed the absence of leukocytes and the presence of necrotic bodies (non-specific staining, green, yellow arrows) associated with the cytoplasm of microglial cells (e–g). cc — central canal, la-lateral, ro-rostral, ca-caudal. Scale bar a: 100 μm; b–d: 10 μm; e, f: 30 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

5.1 mg/kg/day TAC dose, 4 animals died from apparent kidney toxicity at 1.5–2 months. These animals showed signs of agitation, tactile hypersensitivity as well as scratching behavior 3–4 days before death. We speculate that these behavioral signs could be the result of progressive kidney failure and increased creatine levels. Increased creatine levels have been reported in patients receiving higher dose of TAC after solid organ transplants (Finn, 1999; Teh et al., 2011).

Defining the optimal TAC immunosuppressive regimen to permit long-term survival and maturation of spinally grafted human neural precursors in rats

As demonstrated in our current study independent of the daily TAC dose delivered (i.e., 1.9, 3.4 or 5.4 mg/kg/day) in the form of a TAC pellet, consistent survival of grafted ES-derived NPC or human

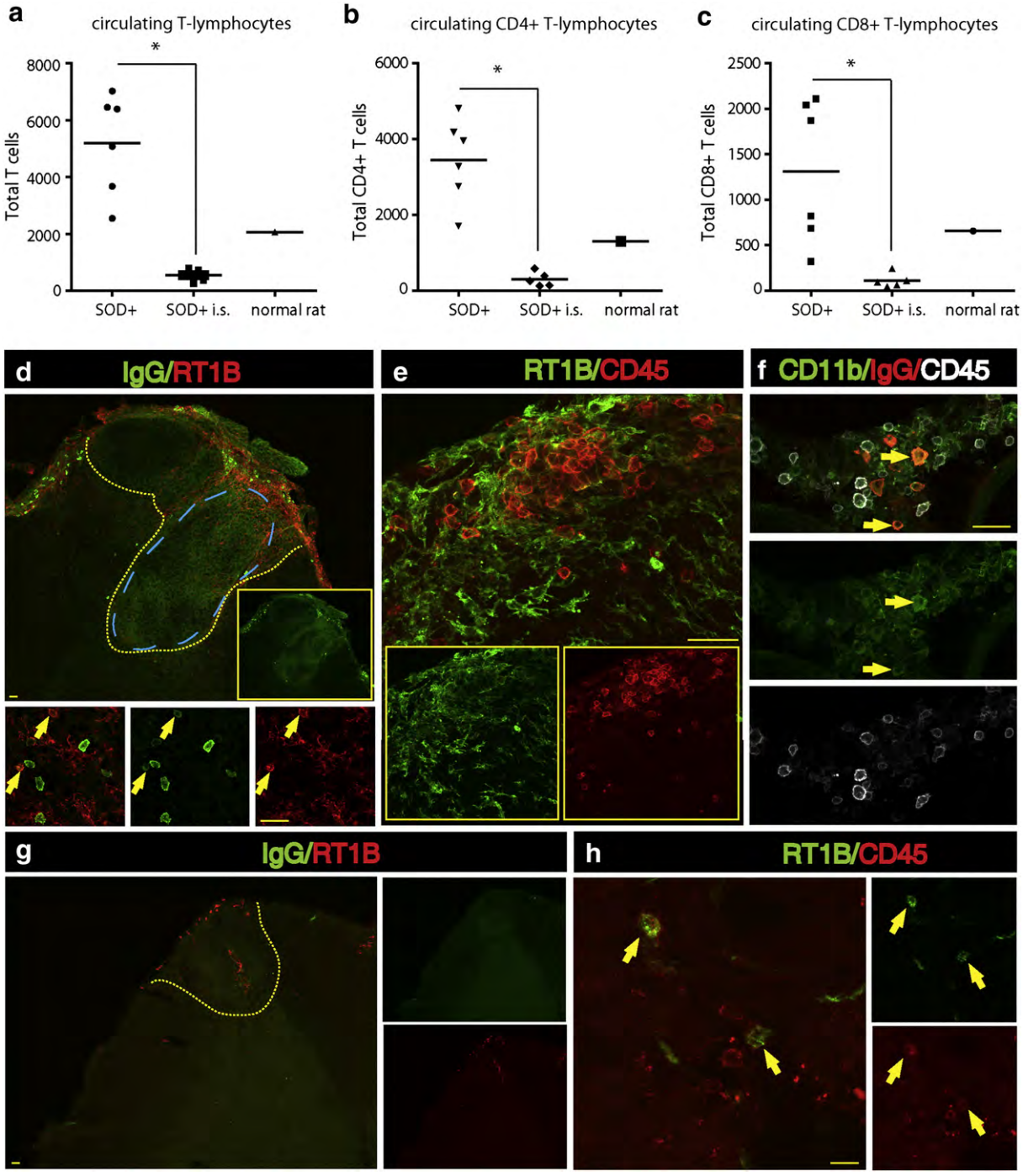


Fig. 5. Effect of TAC pellet-induced immunosuppression of circulating T-cells and the spinal infiltration with IgG + cells, B-cells and granulocyte/monocyte in cell-grafted animals. In TAC pellet-immunosuppressed animals (5.1 mg/kg/day), a significant suppression in the number of circulating blood CD4 and CD8 T-lymphocytes was measured using flow cytometry (a–c), (*$p < 0.05$; T-test). Staining with anti-rat IgG (fluorescence-tagged), anti MHC class II (RT1B) and with anti-CD45 antibody in the 1.9 mg/kg/day TAC group showed a high density of IgG+ cells at the periphery of grafted regions (d). In addition, an overall increase in the density of IgG staining in the same areas was seen (d; blue dashed area). Triple staining with CD11b, anti-rat IgG and CD45 antibody showed co-expression of CD11b in anti-rat IgG+ cells (f; yellow arrows) but no clear colocalization with CD45 marker was detected (f; lower panel). In 5.1 mg/kg/day TAC group a near complete lack of anti-rat IgG, RT1B and CD45+ cell was seen (g) and only sporadic occurrence of RT1B/CD45+ cells was seen at the periphery of grafted regions (h; yellow arrows). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

fetal spinal cord stem cells was seen at intervals 30–90 days after grafting. This was expressed as the presence of high density DCX or NSE immunoreactive-grafted neurons in targeted spinal cord regions. In addition, high density neuronal processes derived from grafted human neurons expressing human-specific synaptophysin and projecting toward host interneurons and α-motoneurons were identified. Comparable grafted cell survival and maturation was seen in both SOD+ rats and in SD rats with previous L3 contusion injury. In our previous experiment, we demonstrated consistent xenograft survival three weeks after the transplantation of hSSC into the spinal cord in SOD+ rats treated with daily i.p. injection of TAC (3 mg/kg/day) combined with daily i.p. injection of Mycophenolate mofetil (30 mg/kg) (Hefferan et al., 2011).

As shown in the present study, the SOD+ mutant rats show a significant increase in circulating CD4/8 cells if compared to naïve SD rats and this increase in CD4/8T-lymphocytes was completely blocked by high dose (5.4 mg/kg/day) TAC treatment (Figs. 5; a–c). In addition, several previous studies have demonstrated significant spinal inflammatory changes in symptomatic SOD+ rats characterized by intense activation of microglia and astrocytes in areas of previous α-motoneuron loss (Hall et al., 1998; Hefferan et al., 2012). The characteristics of this model and requirement of continuous high dose TAC immunosuppression in order to provide long-term grafted cell survival further emphasize the importance of aggressive immunosuppressive therapy to achieve consistent cell survival in this neurodegenerative model.

Similarly, in our recent study, we demonstrated consistent cell survival for up to 2 months after spinal grafting of HUES7-NPC after using 1 mg TAC/day dose delivered s.c. as a bolus in caster-oil preparation in SD rats with previous spinal ischemic injury. However, no cell survival was seen if animals were allowed to survive for total of 4 months (Kakinohana et al., 2012). In another study, on average 28-day survival of allogeneic islet grafts in mice after single injection of TAC loaded microspheres (dose 20 mg/kg) or continuing graft survival (>100 days) after repeated injections of TAC loaded microspheres (dose 10 mg/kg; 7-day intervals) was described (Wang et al., 2004). Jointly, these data show that the TAC dose of around 3 mg/kg/day and divided into two 12-h doses (if delivered as a bolus s.c injection) or in a form of continuously TAC releasing pellet is required for long-term effective immunosuppression to permit xenograft survival in rats.

Translated to plasma TAC concentration, TAC levels of ≤15 ng/ml were measured in 1.5 mg/kg/12 h (caster-oil formulation) or in 3.42 or 5.1 mg/kg/day (pellet groups), i.e., the TAC dosing regimen which was associated with the most potent suppression of T-cell infiltration in the cell-grafted regions. Comparable TAC plasma levels were shown to be required to permit long-term survival of grafted pancreatic islet cells in mice (Wang et al., 2004) or to lead to life-long survival of transplanted solid organs in human patients (Pirsch et al., 1997; Przepiorka et al., 1999; Staatz and Tett, 2004; Vicari-Christensen et al., 2009).

TAC dose-dependent suppression of T- and B-lymphocyte activation and proliferation

Quantitative analysis of T-cells (CD8, CD4) in grafted spinal cord regions showed a near complete absence of this cell population in 3.4 and 5.1 mg/kg/day TAC group if analyzed at 77–90 days after

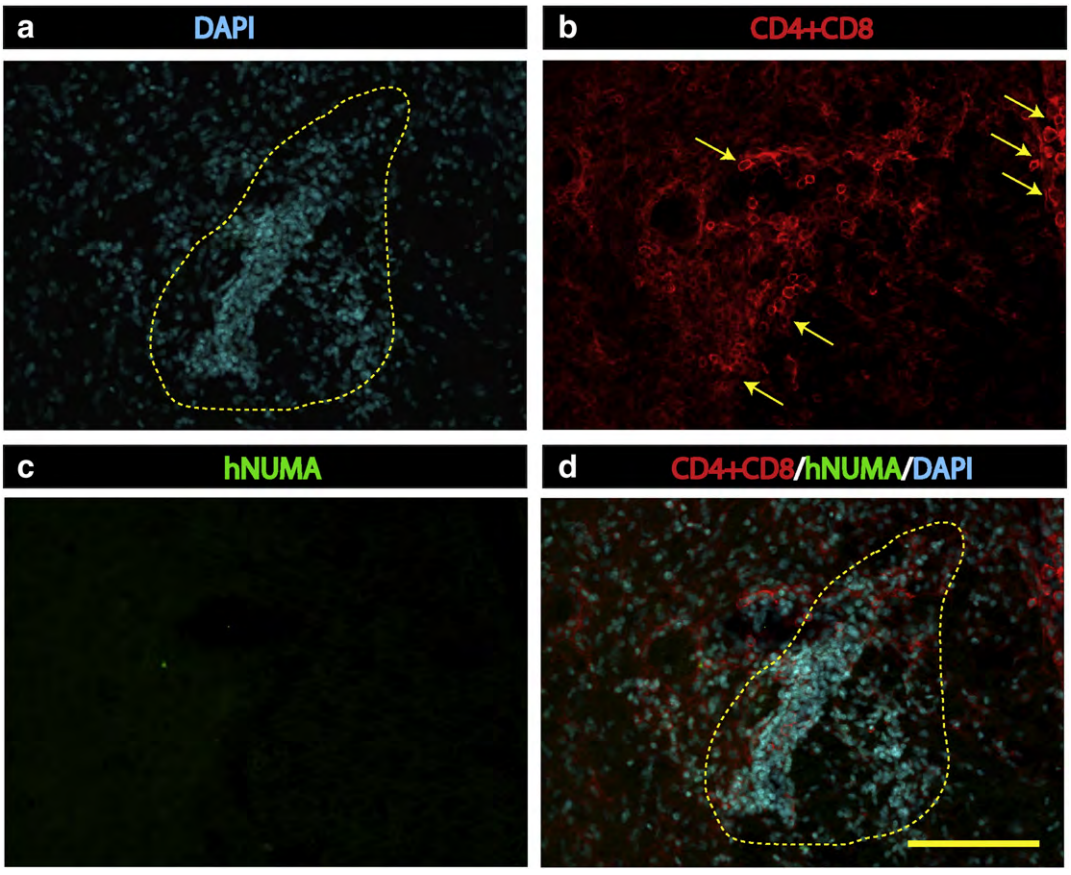


Fig. 6. Rejection of spinally grafted hSSC in SD rats in the absence of immunosuppression. Adult SD rats received 10 bilateral injections of hSSC into lumbar spinal cord and survived for 14 days. No immunosuppression treatment was used in any animal. Triple staining of transverse spinal cord sections with DAPI and hNUMA and CD4/8 antibody showed increased cellularity in cell grafted region (a; yellow dashed circle) but with no grafted cell survival as evidence by the lack of hNUMA+ cells (c). Instead an intense accumulation of CD4/8 cells in previously cell-grafted regions can be seen (b, d; yellow arrows). Scale bar a: 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

cell grafting. Similarly, the analysis of CD8 and CD4 cells in circulating blood showed a significant decrease in the 5.1 mg/kg/day TAC group if compared to non-immunosuppressed SOD + rats. In contrast to the 3.4 and 5.1 mg/kg/day TAC groups, a clear population of CD4/8 cells was seen in 1.9 mg/kg/day TAC-treated and cell grafted animals. Interestingly, a substantially higher density of CD8 population was seen on the dorsal surface of individual grafts facing the pial membrane. We speculate that the presence of grafted cells in heavily vascularized but BBB-lacking pial arterial system is more susceptible to extravasation of the circulating T-cell population and the resulting T-cell mediated response. We also hypothesize that even if a satisfactory cell survival was seen in the 1.9 mg/kg/day group at 77 days after cell grafting, a progressive grafted cell rejection will likely develop should the same level of low level immunosuppression continue over an extended period of time. We also saw small lymphocytic IgG + MHC class II + CD45 + B cells in the graft surface. We speculate that these cells are releasing graft-specific antibodies that are bound the graft or to CD11b + CD45-microglia cells.

Limitation of subcutaneous TAC pellet-induced immunosuppression

Several technical issues need to be considered once a pellet immunosuppression regimen is going to be implemented into any rat xenograft-immunosuppression study. 1) Based on our PK study, it takes on average 7–15 days after pellet implant before targeted (≤15 ng/ml TAC) plasma levels are achieved in 200–350 g rats. Thus, the TAC pellet needs to be implanted before cells are transplanted and the plasma level of TAC validated. However, because consistent and predictable plasma TAC concentrations were measured in our initial PK study in all 3 TAC pellet dosing groups, we have currently implemented less vigorous TAC plasma monitoring and the TAC concentration is only measured 1–2 times during the course of the 2–3 month post-pellet implantation period. Consistent with our initial PK TAC data, expected TAC concentrations were measured in more than 30 animals. 2) Should there be a desire to remove already implanted TAC pellets to achieve an abrupt termination of immunosuppression, the tissue surrounding the pellet implanted region needs to be excised in block. From our experience, the pellet identification is substantially obscured as soon as 7–14 days after implantation because of its structural disintegration. 3) Similarly, should there be a need to increase the dose of delivered TAC, an additional pellet(s) can be implanted. Should this be the case, TAC plasma monitoring is recommended to be performed in 5–7 day intervals for at least 2–3 weeks after additional pellet implant to identify potential unwanted toxic TAC concentrations. However, as shown in our current study, the plasma TAC concentrations up to 45–50 ng/ml were well tolerated for up to 3 months and only a fraction of animals displayed systemic side effects. We are currently testing the tolerability of repetitive TAC pellet implant (3.5 mg/kg/day dose group) and have not seen any detectable side effects at 4.5 months of continuous immunosuppression at this dose (unpublished observation).

Summary

We demonstrated that by using an implantable 3-month lasting TAC-releasing pellet it is possible to achieve functionally effective immunosuppression in SD rats as defined by long-term survival and maturation of spinally grafted human neural precursors derived from human fetal spinal cord or from embryonic stem cell. Plasma TAC concentration of ≤15 ng/ml was found to be required to lead to a near complete suppression of T-cell activity in the human cell-grafted spinal cord region and in circulation blood and was readily achieved by 7–10 days after pellet implantation. Jointly, these data demonstrate that the use of implantable TAC pellets can represent an effective long-lasting immunosuppressive drug delivery system which is technically simple to implement, is safe for an extended period of time (4.5 months) and is associated with consistent and long-term human neural precursor survival after grafting into the spinal cord of SOD + or spinal trauma-injured SD rats.

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