Published in final edited form as:

Exp Neurol. 2008 August ; 212(2): 324–336. doi:10.1016/j.expneurol.2008.04.018.

Treatment Modality Affects Allograft-Derived Schwann Cell Phenotype and Myelinating Capacity

Ayato Hayashi, MD, PhD^{*}, Arash Moradzadeh, MD, Alice Tong, MS^{*}, Cindy Wei, MD^{*}, Sami H. Tuffaha, BA^{*}, Daniel A. Hunter, RA^{*}, Thomas H. Tung, MD^{*}, Alexander Parsadanian, PhD^{**}, Susan E. Mackinnon, MD^{*}, and Terence M. Myckatyn, MD^{*}

^{**} Department of Neurology and Hope Center for Neurological Disorders, Box 8518, Washington University School of Medicine, 660 South Euclid Avenue, St. Louis, MO 63110, USA

Abstract

We used peripheral nerve allografts, already employed clinically to reconstruct devastating peripheral nerve injuries, to study Schwann cell (SC) plasticity in adult mice. By modulating the allograft treatment modality we were able to study migratory, denervated, rejecting, and reinnervated phenotypes in transgenic mice whose SCs expressed GFP under regulatory elements of either the S100 β (S100-GFP) or nestin (Nestin-GFP) promoters. Well-differentiated SCs strongly expressed S100-GFP, while Nestin-GFP expression was stimulated by denervation, and in some cases, axons were constitutively labeled with CFP to enable *in vivo* imaging. Serial imaging of these mice demonstrated that untreated allografts were rejected within 20 days. Cold preserved (CP) allografts required an initial phase of SC migration that preceded axonal regeneration thus delaying myelination and maturation of the SC phenotype. Mice immunosuppressed with FK506 demonstrated mild subacute rejection, but the most robust regeneration of myelinated and unmyelinated axons and motor endplate reinnervation. While characterized by fewer regenerating axons, mice treated with the co-stimulatory blockade (CSB) agents anti-CD40L mAb and CTLA4g-4 demonstrated virtually no graft rejection during the 28 day experiment, and had significant increases in myelination, connexin-32 expression, and Akt phosphorylation compared with any other group. These results indicate that even with SC rejection, nerve regeneration can occur to some degree, particularly with FK506 treatment. However, we found that co-stimulatory blockade facilitate optimal myelin formation and maturation of SCs as indicated by protein expression of myelin basic protein (MBP), connexin-32 and phospho-Akt.

Keywords

allograft; Schwann cell; migration; nestin; myelin; phenotype; transgenic; regeneration

Corresponding author: Terence M. Myckatyn, MD, Division of Plastic and Reconstructive Surgery, Washington University School of Medicine, 660 South Euclid Ave, Campus Box 8238, Saint Louis, MO, 63110, myckatynt@wudosis.wustl.edu, tel: 314-362-4263, fax: 314-362-4536.

^{*} Authors share same address and affiliation as corresponding author

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Introduction

Allografts are a viable option for reconstructing extensive peripheral nerve injuries based on numerous animal studies (Brenner, et al., 2004, Zalewski and Gulati, 1980), and a growing clinical experience (Mackinnon, et al., 2001, Mackinnon and Hudson, 1992). Cold preservation (CP) of nerve allografts confers a graded reduction in graft antigenicity. The type 1 T-helper cell response (Th1), primarily mediated by interferon- γ (IFN- γ), is nearly completely obliterated by 4 weeks of CP, while a 7 week course is required to suppress the T-helper Type-2 response (Th2) mediated by interleukin-4 (IL-4) and results in an acellular nerve allograft (Fox, et al., 2005). It is unclear if Th2 cell activity is dispensable for allograft survival in the context of organ transplantation (Kist-van Holthe, et al., 2002, Larsen, et al., 1996). In the context of nerve allografts, however, a 7 week period of CP not only suppresses the Th2 response, but also eliminates intercellular adhesion molecule-1 and major histocompatibility complex II (MHC-II) expression (Atchabahian, et al., 1999), while supporting nerve regeneration through a 1.5 cm allograft (Fox, et al., 2005).

A prolonged course of CP alone may be sufficient to facilitate regeneration through relatively short nerve allografts due to the presence of migratory Schwann cells (SCs) (Kimura, et al., 2005). Unsuccessful nerve regeneration through acellularized nerve grafts greater than 3 cm, however, suggests that the neurotrophic support afforded by SCs is required to support regeneration across longer gaps (Brenner, et al., 2005). FK506 may be uniquely suited to treat peripheral nerve allografts, functioning both as an immunosuppressant (Mackinnon, et al., 2001) and as an agent that can accelerate nerve regeneration (Gold, et al., 1994, Jost, et al., 2000, Pan, et al., 2003, Steiner, et al., 1997). Blockade of the costimulatory signals regulating the activation and expansion of alloreactive T cells represents an alternative method of suppressing the host alloreactive response (Ansari and Abdi, 2003). Ligation of the CD40 pathway alone induces a permissive state (Brenner, et al., 2004), but requires synergism with simultaneous blockade of the B7/CD28 pathway to achieve indefinite immune hyporesponsiveness (Kirk, et al., 1997, Larsen, et al., 1996).

In nerve autografts, non-myelinating SCs are preserved, and assume a myelinating phenotype (Aguayo, et al., 1976). In nerve allografts, systemic immunosuppression preserves donor-derived SCs unless rejection ensues, or the immunosuppressant is withdrawn in which case acute rejection occurs (Midha, et al., 1994). Since these studies were performed, numerous phenotypic SC markers have been characterized in developing (Jessen and Mirsky, 2005), and injured peripheral nerves (Atanasoski, et al., 2006, Atanasoski, et al., 2001, Chen, et al., 2007) but not in nerve allografts. In the present study, we characterize the time course over which SCs assume proliferative, migratory, or myelinating phenotypes in nerve allografts that are acutely rejected, CP, or treated with either FK506 or synergistic co-stimulatory blockade (CSB). We hypothesize that CSB will be most effective for preventing SC rejection and promoting differentiation to a mature SC phenotype post-injury. We will elucidate the relative contributions of these treatment modalities to SC migration and maturation and accelerated nerve regeneration to refine future therapeutic strategies and ensure the rapid regeneration of myelinated axons.

Methods

Mice

Transgenic mice expressing green fluorescent protein (GFP) in their SCs, and cyan fluorescent protein (CFP) in their axons were inbred on a C57BL/6 background. Six to 10 week old mice weighing 22–30 grams both received and donated allografts from a fluorescent-naïve dysgenic Balb/C counterpart (Fig. 1A). Homozygous S100-GFP^{+/+} mice (n=96) were used to evaluate differentiated SCs (Jessen and Mirsky, 2005, Zuo, et al., 2004), while Nestin-GFP^{+/+} mice

(n=15) were used to identify SC precursors characterized by upregulated GFP expression in denervated and graft tissue (Mignone, et al., 2004). To study the course of SC dedifferentiation in isografts, fluorescent-naïve Nestin-GFP^{-/-} mice (n=4) donated or received isografts from Nestin-GFP^{+/+} littermates (n=4). GFP-S100^{+/+} mice bred to Thy1-CFP(23) mice (Thy1-CFP^{+/+}/S100-GFP^{+/+}) were used for simultaneous *in vivo* imaging of peripheral axons and SCs (n=20) (Feng, et al., 2000), as we have previously described (Hayashi, et al., 2007). Nestin-GFP mice were gifted by Dr. G. Enikolopov (Cold Spring Harbor, NY), while all other strains were purchased from The Jackson Laboratory (Bar Harbor, ME). Genotyping was performed using quantitative PCR performed on DNA extracts from mouse tails using primers provided by Jackson Laboratories as well as GFP-expression directly observed in retinal glial cells with fluorescent goggles. All experiments were performed in accordance with protocols approved by the Division of Comparative Medicine at the Washington University School of Medicine.

Surgical Procedures

All animals were anesthetized with subcutaneous injections of ketamine (Fort Dodge, IA) and medetomidine (Pfizer, NY). For nerve grafting, the sciatic nerves were exposed in a pair of MHC- and fluorescence-expressing mismatched mice using sterile microsurgical technique. Nerve grafts were fashioned by transecting the sciatic nerve 5 and 15 mm distal to the sciatic notch. The resulting 10 mm grafts were swapped and interposed in a reversed orientation with 11-0 microsutures under 40x magnification. Baseline *in vivo* imaging was performed, muscle and skin closed with 8-0 and 6-0 nylon sutures, and the mice recovered with antisedan (Pfizer, NY) on a warming pad.

To confirm MHC mismatch, two distinct 1 cm² skin grafts were harvested from anesthetized pairs of C57BL/6 background and Balb/C mice not used for nerve grafting. One skin graft was replaced as an autograft and the other swapped with its mismatched counterpart as an allograft. Skin grafts were sutured with 6-0 nylon sutures, mice recovered, and monitored daily for rejection.

Immunosuppressive Regimens

In one group (Fig 1B), nerve allografts were CP at 4°C for 7 weeks in 15 cc aliquots of University of Wisconsin solution (NPBI International BV, Emmer Compascuum, The Netherlands) supplemented with penicillin G (200,000 U/L), regular insulin (40 U/L), and dexamethasone (16 mg/L) (Fox, et al., 2005). In another group, systemic immunosuppression was achieved with FK506 (Fujisawa, Tokyo, Japan) administered subcutaneously 6 days per week at 2 mg/kg/day (Grand, et al., 2002). To achieve long-term nerve allograft immune hyporesponsiveness, the CD40 and CD28/B7 pathways were ligated with anti-CD40 (BioExpress, Lebanon, NH) and CTLA4-Ig (BioExpress, Lebanon, NH). Three doses of anti-CD40 ligand (0.5mg/day) monoclonal antibody and CTLA4-Ig (500mg/day) fusion protein were administered intraperitoneally on postoperative days 0, 2, and 4 (Ansari and Abdi, 2003, Brenner, et al., 2004, Magott-Procelewska, 2004).

In-Vivo Serial Imaging

In vivo imaging of nerve grafts repopulated by GFP-labeled SCs and CFP-labeled axons was performed at the time of surgery and 5, 10, 15, and 28 days later with an SMZ-1500 fluorescence dissecting microscope under 450 and 488 nm fluorescent light (Nikon Instruments Inc, Melville, NY). The sciatic nerve graft was briefly exposed under anesthesia and imaged with a CoolSNAP-ES CCD (Photometrics, Tucson, AZ) controlled by MetaMorph version 7.1 software (Universal Imaging Corporation, Downingtown, PA). Acquisition was standardized to generate a 12-bit (0-4095 pixel intensity) image of the nerve graft bordered proximally and distally by host sciatic nerve. A 20 pixel wide line scan longitudinally bisected the engrafted sciatic nerve and a graph plotting graft position on the x-axis versus pixel intensity on y-axis

generated (Hayashi, et al., 2007). Group comparisons were made by subdividing individual grafts into 5 segments, calculating mean fluorescent intensity within each segment, subtracting mean background intensity, and then calculating the mean intensity for that particular segment for the entire treatment group (n=6 per group).

Immunohistochemistry

Mice were deeply anesthetized and transcardially perfused with heparinized 0.01 M phosphate buffered saline (PBS) followed by 4% paraformaldehyde in 0.1 M PBS. The engrafted sciatic nerves and extensor digitorum longus (EDL) muscles were harvested and postfixed for 30 minutes. Engrafted sciatic nerves designated for sectioning were frozen in OCT compound at -80°C or embedded in paraffin, while the muscle specimens were evaluated as whole mounts. Frozen sections were blocked with 5% normal goat serum and diluted with 0.3% Triton-X in PBS for one hour. To confirm the phenotype of GFP-expressing cells, SCs were co-labeled with rabbit polyclonal anti-S100 antibody (DAKO, Carpinteria, CA). Myelin basic protein (MBP) was labeled with rabbit polyclonal anti-MBP antibody (CHEMICON, Temecula, CA) and macrophages were labeled with rat monoclonal anti-F4/80 antibody (Serotec, Oxford, UK) all diluted 1:100 and incubated overnight at 4°C . Secondary antibodies were diluted 1:500 and included Cy3-conjugated goat anti-rabbit antibody for S100 and Cy3-conjugated donkey anti-rat antibody for F4/80 labeled-macrophages (Jackson ImmunoResearch, West Grove, PA). To label muscle fiber motor endplates, whole mounts of EDL muscles were soaked for 30 minutes in a 1:100 solution of Alexa 594-conjugated alpha-bungarotoxin (BTX; Molecular Probes, Eugene, OR).

For phospho-mitogen-activated protein kinase (phospho-MAPK) and phospho-Akt detection, allografts were embedded in paraffin and the avidin-biotin-peroxidase complex (ABC) method was used for staining. The sections were deparaffinized in xylene and rehydrated in graded ethanol concentrations. Antigen unmasking was performed by sub-boiling slides for 15 minutes. Sections were immersed in 0.3% aqueous peroxide solution for 10 minutes, and blocked for 1 hour in 5% goat serum. The slides were then incubated at 4°C overnight with rabbit anti-phospho-MAPK or phospho-Akt antibody. After serial washes in PBS, the sections were incubated with biotinylated anti-rabbit IgG (Vector Laboratories, Burlingame, CA) for 30 minutes, and subsequently incubated with avidin-biotin-peroxidase complex (ABC-kit, Vector Laboratory, Burlingame, CA) for 30 minutes. Antibodies were visualized using 0.02% 3,3-diaminobenzidine (DAB) (Sigma, St. Louis, MO), after 0.005% peroxide was added for 1 minute. Sections were washed in distilled water, followed by dehydration in a series of graded ethanol solutions and xylene. As a control, primary antibody was replaced with preimmune serum, which resulted in negative staining (data not shown). Sections were evaluated with confocal microscopy using an Olympus FV1000 spectral scanning microscope with 20x, 40x, 40x oil, and 60x oil objectives with numerical apertures of 0.75, 0.9, 1.3, and 1.42 respectively. The lasers used included a multi-line 458, 488, and 515 nm laser as well as 405 nm, 561 nm, and 633 nm lasers.

Histomorphometry and Non-Biased Stereology

Allografts (n=24) evenly distributed amongst the four treatment groups were harvested 10 and 28 days following engraftment and assessed with light microscopy (LM) and electron microscopy (EM). The 28 day time point was chosen since in a short, murine nerve allograft, earlier time points correlate more closely than later time points (≥ 6 weeks) with outcomes noted in clinically-relevant long nerve allografts in higher animal models (Brenner, et al., 2004, Grand, et al., 2002, Myckatyn, et al., 2002, Myckatyn, et al., 2003, Myckatyn and MacKinnon, 2004). Specimens were preserved in glutaraldehyde, postfixed in osmium tetroxide, and embedded in Araldite 502 resin. For LM, 1 μm sections were harvested ~3 mm from the proximal suture line in 10 day old grafts and ~5 mm from the suture line in 28 day old grafts.

Quantitative analysis was performed on toluidine blue stained sections with the aid of a semi-automated digital image-analysis system linked to LECO metallurgical software adapted for nerve morphometry as we have previously described in detail (Hunter, et al., 2007). Briefly, sectioned nerves were divided into $5.5 \times 10^4 \mu\text{m}^2$ frames and evaluated using eight-bitplane digital pseudocoloring and thresholding-based algorithms to select for myelinated axons, calculate their density, area, myelin thickness, and widths (Hunter, et al., 2007). Equivalent diameters (ED) were used to calculate width ($\text{ED} = \sqrt{4(\text{axon area}/\pi)}$) since myelinated axons are not perfect circles and therefore have variable widths. G-ratios were calculated from this data ($\text{g-ratio} = \text{axonal ED}/\text{fiber ED}$). The entire section was counted and manually checked to exclude any selection bias or sampling error, and total myelinated axon numbers were plotted against width as a frequency distribution with the aid of Prism software (Version 4.0, GraphPad Software Inc.).

EM was performed on 90 nm sections stained with uranyl acetate and lead citrate. For each harvested specimen, 15 ultramicrographs were taken with a Zeiss 902 electron microscope (Zeiss Instruments, Chicago, IL) at 4360x magnification, scanned at 400 dots per inch resolution and evaluated with MicroBright Field Stereo Investigator software (MBF Bioscience StereoInvestigator version 7.0, Williston, Vermont). Using the fractionator technique, the sampling frame grid was set to $80 \mu\text{m}^2$ and axon area (Gundersen, et al., 1988), and diameter thus calculated. Since the entire nerve was not counted with EM, unmyelinated axonal density/ mm^2 was calculated and also plotted against axonal width as a frequency distribution. All unmyelinated axons associated with a single SC were also manually counted in these ultramicrographs specimens and a distribution of the number of axons per Remak bundle calculated for every treatment group. Stereologic and morphometric data was then imported to Prism to calculate the frequency distribution of axons based on width.

Motor Endplate Evaluation

We calculated the total number of reinnervated endplates as a percentage of the total number of endplates 6 weeks following engraftment in Thy1-CFP^{+/+}/S100-GFP^{+/+} mice. Described previously (Magill, et al., 2007), the deep surface of BTX-labeled EDL muscle whole mounts were surveyed using fluorescent microscopy at 10x and 20x to identify labeled terminal axons and endplates and counted using a 40x oil objective (1.3 N.A.) and 458, 488, and 561 nm lasers. To differentiate GFP from CFP, Olympus FV1000 software was used to capture emissions from a 20 nm bandwidth at the peak of the two emission profiles. These GFP and CFP emission profiles were independently plotted and differentiated with a spectral unmixing algorithm (Hayashi, et al., 2007). Z-series stacks from 20–25 areas ($100 \mu\text{m}^2$) known to contain motor endplates (≥ 300 endplates per group) were evaluated and the number of innervated and denervated motor endplates counted.

Western Blot

Nerve allografts (n=24) were harvested from each treatment group 5 and 28 days following engraftment and subdivided into proximal, middle, and distal segments for separate assessment. Graft segments were homogenized in lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% NP40, 10% Glycerol, 1 mM EGTA, protease inhibitor cocktail, and 1 mM sodium orthovanadate). Lysates were centrifuged at 15000g for 15 minutes and supernatants retained for analysis. The samples were mixed with SDS-PAGE buffer (Bio-Rad, Hercules, CA) and boiled for 3 minutes. The same amount of protein (10 μg) was loaded and resolved in 4% to 12% SDS-PAGE gels (Invitrogen, Carlsbad, CA). Resolved proteins were transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA), and incubated with a blocking solution containing 5% Bovine Serum Albumin (Sigma, St. Louis, MO) in TBS-Tween buffer for 30 minutes, and further incubated with primary antibodies in the same buffer overnight. Immunoblots were incubated with secondary antibody conjugated with HRP [Goat anti-rabbit

(1:5,000 dilution; Santa Cruz, Santa Cruz, CA)] and immune complexes detected by enhanced chemiluminescence (LumiGLO, Cell signaling, Danvers, MA). Protein loading was normalized to GAPDH signal. For quantification, immunoreactive bands obtained in autoradiographic films were scanned at 400 dpi (Epson America, Long Beach, CA), and analyzed for signal intensity by Gel-pro analyzer version 4.5 (Media Cybernetics, Bethesda, MD). The following primary antibodies were used: anti-phospho-p44/42 MAPK, anti-p44/42 MAPK, anti-phospho-Akt, anti-Akt, anti-Caspase-3 (rabbit polyclonal; 1:500 dilution; all from Cell signaling, Danvers, MA), anti-Connexin-32 (rabbit polyclonal; 1:1000 dilution; Invitrogen Carlsbad, CA), and anti-GAPDH (rabbit polyclonal; Santa Cruz, Santa Cruz, CA).

Enzyme-Linked Immunospot (ELISPOT) Assay

The IFN- γ -mediated Th1 response to nerve allografts was assessed by ELISPOT. Donor splenocytes were harvested from S100-GFP^{+/+} mice 10 days after they were engrafted with Balb/C nerves (n=3 per group). Splenocytes were added to multiscreen 96-well filtration plates (Millipore, Bedford, ME), coated overnight at 4°C with the capture antibody anti-mouse interferon gamma (IFN- γ ; Endogen, Woburn, MA). The plates were then washed with PBS and blocked with 1% bovine serum albumin (BSA) for 2 hours at room temperature. Equal numbers of stimulator allogenic BALB-c splenocytes were co-incubated in quadruplicate at 37°C for 24 hours with responder (transplant recipient) splenocytes. Biotinylated IFN- γ was added to co-incubated cells, and 24 hours later labeled with horseradish-peroxidase-labeled streptavidin (Endogen, Woburn, MA). Controls, also plated in quadruplicate, included cell-free culture media and irradiated (2000 rad) stimulator allogenic BALB/c splenocytes (200,000 cells/well) (negative controls), and concavalin A (positive control). The plates were then developed with BD Elispot AEC Substrate Set (BD Biosciences, San Diego, CA). Plates were dried and the number of spots in each well - representing the number of cytokine-producing cells - counted using ImmunoSpot Analyzer 3 (Cellular Technology, Cleveland, OH).

Functional Recovery

Walking-track analysis was performed on five animals from each of the four groups using methods similar to those previously described (Brown, et al., 1989, Hare, et al., 1992, Inserra, et al., 1998). Animals undergoing walking tracks were walked every week up to a 6 week endpoint. The hind paws were dipped in D-76 film developer (Eastman-Kodak, Rochester, NY) and the mice walked down a 4×43 cm x-ray paper-lined corridor. Print length was measured from the heel to toe tip on the normal side (NPL) and the experimental side (EPL) using a calibrated SummaSketch II Plus digitizing tablet (GTCO Cal Comp Peripherals, Columbia, MD). These values were used to calculate the print length factor (PLF) where $PLF = (EPL - NPL) / NPL$.

Statistical Analysis

Statistical analysis was performed in Statistica (Version 7.1, StatSoft, Tulsa, OK), where a $p < 0.05$ was considered significant. ELISPOT, densitometric, morphometric and motor endplate reinnervation data was evaluated with a one-way analysis of variance (ANOVA) followed by a *post-hoc* Scheffe test. The PLF was evaluated with a one-way repeated measures ANOVA and pair-wise multiple comparisons were made *post-hoc* using the Student-Newman-Keuls method. Error bars, where present, represent standard deviation.

Results

Preventing Allograft Rejection

A major-MHC mismatch between donor and untreated recipient mice was confirmed when swapped skin allografts began to reject by 9 days, but autografts survived indefinitely (>42

days) (Fig. 2A). ELISPOT assays, performed 10 days following nerve engraftment showed that the Th1-mediated IFN- γ allograft response was significantly higher ($p<0.001$) in untreated mice than those receiving CP allografts, or systemic treatment with FK506 or costimulatory blockade (CSB) with anti-CD40L mAb and CTL4-Ig (Fig. 2B). Unlike Wallerian degeneration, which is caspase-3 independent (Finn, et al., 2000), cleavage of activated caspase-3 into 17- and 19-kDa subunits is indispensable for rejection-mediated apoptosis (Gottlieb and Kitsis, 2001). By Western blot, activated caspase-3 was not detected in treated or untreated allografts 5 days post-engraftment (Fig. 2C), but by 28 days, untreated allografts demonstrated significantly elevated levels of activated caspase-3 compared to all other groups ($p<0.05$). A mild elevation in activated caspase-3 noted in mice treated with FK506 was thought to be due to early subacute rejection, but did not reach statistical significance. Neural debris, defined as all toluidine-blue stained non-neural and non-vascular material within the nerve allograft, was significantly higher ($p<0.05$) in untreated allografts compared to all other groups at 28 days (Supplemental Data 1). Seven week CP allografts, which were devoid of the by-products of Wallerian degeneration, had significantly less neural debris than all other groups ($p<0.05$). Taken together, these data suggest that untreated nerve allografts were undergoing acute immune-mediated rejection while the treated allografts were not.

In Vivo Imaging of SC Migration

To study the origin and phenotype of SCs contained within nerve allografts over time, S100-GFP^{+/+} and Nestin-GFP^{+/+} mice were paired with fluorescence naïve Balb-C mice (Fig. 1A). Based on 12-bit image analysis (0-4095), background intensity from randomly sampled regions of interest in non-neural tissue ranged from 140.2 ± 20.3 (n=12) on day 0 to 183.5 ± 34.2 (n=12) on day 15 and was not significantly different from a fluorescent naïve Balb/c mouse on day 0 (153.4 ± 21.1 ; n=24). SCs expressing S100-GFP traversed the allograft in untreated mice, significantly ($p<0.05$) increasing fluorescent intensity throughout the graft on day 15 (Fig. 3A). CP allografts were repopulated with S100-GFP expressing SCs between 5 and 10 days ($p<0.05$), and like untreated allografts demonstrated a progressive increase in graft intensity with each successive imaging session (Fig. 3A). Allografts in mice treated with FK506 were largely devoid of S100-GFP until 28 days at which point a significant ($p<0.05$) increase in migration was noted in the proximal (0–2 mm) and distal (8–10 mm) graft cuffs. In the CSB group, a qualitative increase in proximal (0–2 mm) graft intensity was noted but did not reach statistical significance until the 28 day time point ($p<0.05$), similar to FK506-treated mice. These data suggest that SCs migrate into allografts that possess rejecting or no SCs, but do not migrate if graft-based SCs are present and tolerated by the immune system.

To specifically evaluate dedifferentiated SCs, Nestin-GFP^{+/+} mice were similarly evaluated. Prior to injury, Nestin-GFP^{+/+} mice demonstrated no significant increase in fluorescent intensity over background, confirming that the nestin promoter was not detectably active in uninjured adult mouse peripheral nerve (Fig. 4A). Fluorescent-naïve Balb-C allografts demonstrated a significant increase in migrated Nestin-GFP expression by 28 days (579 ± 68) over baseline levels in untreated mouse allografts ($p<0.05$). This was however, significantly less ($p<0.05$) relative to the CP allograft group (1087 ± 103). Nestin-GFP^{+/+} mice treated with FK506 or CSB failed to demonstrate significant host Nestin-GFP^{+/+} SC infiltration into fluorescence-naïve allografts. This suggested that the preservation of allograft-based SCs applied to both differentiated (S100-GFP) and dedifferentiated (Nestin-GFP) phenotypes for at least 28 days.

Except for CP allografts, which were devoid of GFP-expressing SCs, Nestin-GFP^{+/+} and S100-GFP^{+/+} allografts were also placed in fluorescent naïve Balb/c hosts (Fig. 3B&4B). In this scenario, GFP-expression would be graft-derived, and a reduction in graft fluorescence would be attributable to either host SC migration or reduced graft-derived S100- or nestin-promoter

activity. SCs populating untreated allografts demonstrated a dramatic reduction in S100-GFP expression at 10 days likely due to denervation (Fig. 3B). By 20 days, renewed S100-GFP expression was noted, but was lost by 28 days. The transient increase in GFP-intensity noted at 20 days in the figure shown may have been derived from macrophages resident to the nerve allograft. It is known that a small percentage of macrophages express GFP in the S100-GFP line (Hayashi, et al., 2007, Zuo, et al., 2004), and that resident macrophages contribute to nerve graft degeneration (Shen, et al., 2000). While the response of resident macrophages to infiltrating host immune cells has not, to our knowledge, been specifically studied in nerve allografts, we suspect that the proliferation of resident macrophages of which some were GFP-labeled, contributed to this increase in fluorescent intensity by 20 days before they were eradicated by the immune response at 28 days. This finding merits further future investigation. At 5 days, Nestin-GFP^{+/+} allografts were the brightest in untreated mice (Fig. 4B), with minimal GFP expression by 15 days. Taken together, these data suggest that allograft-based SCs dedifferentiated in the first 5 days with increased nestin promoter activity and a reciprocal reduction in S100 promoter activity. Residual donor SCs that were reinnervated differentiated back to an S100-expressing phenotype between 15 and 20 days, but were rejected within 28 days. Conversely, mice treated with FK506 retained graft-derived S100-GFP expression during the 28 day study (Fig. 3B) while Nestin-GFP expression was noted within 5 days of engraftment, likely due to dedifferentiation of denervated SCs and persisted for at least 28 days (Fig. 4B). CSB-treated mice demonstrated a similar trend (Fig. 3B&4B). These data suggest that donor allograft SCs dedifferentiated in response to nerve injury, but were largely preserved with immunosuppression and capable of restoring a relatively differentiated, S100-GFP expressing phenotype.

We took advantage of the heterozygosity of the Nestin-GFP line to determine whether dedifferentiation of host and graft SCs was a function of rejection or denervation by repeating these experiments in a nerve isograft. Nerve isografts from Nestin-GFP^{-/-} and Nestin-GFP^{+/+} littermates were swapped and serial *in vivo* imaging repeated. Shown in Figs. 4A&B, GFP-expression in isografts was similar to that seen in CSB treated mice that had received allografts. The similarity in SC phenotype between isografts and adequately treated allografts suggests that the dedifferentiation of SCs observed in FK and CSB treated nerve allografts was related to denervation rather than rejection.

In Vivo Imaging of Schwann Cell-Axonal Relationships

To quantify the rate of regeneration through nerve grafts in the various treatment groups and relate this to SC migration, engrafted host Thy1-CFP^{+/+}/S100-GFP^{+/+} mice were serially imaged (Supplemental Data 2). The most rapid regeneration occurred in FK506-treated mice where in some cases axons crossed the entire 1 cm graft within 10 days (1 mm/day). Axons regenerated up to 7 mm in CSB treated mice at the 10 day time point (0.7 mm/day) and were observed distal to the graft by 28 days. Regeneration was relatively poor in both untreated and CP allografts. At 10 days, axons had regenerated 4 mm, and only approximated the distal graft surgical coaptation at 28 days. Treatments with FK506 or CSB, that protected graft-based SCs and did not rely on migration, were maximally supportive of early nerve regeneration.

Quantitative Assessment of Regeneration and Motor Endplate Reinnervation

Nerve cross sections were evaluated 10 and 28 days after engraftment for regenerated myelinated and unmyelinated axons, their size distribution, Remak bundles, and g-ratios. The FK506 and CSB treatment groups demonstrated significantly more unmyelinated (Fig. 5A) and myelinated (Fig. 5B) axons overall than untreated and CP allografts in cross sections taken 3 mm into the allograft at 10 days at all fiber widths ($p < 0.05$). No myelinated axons were noted in untreated and CP allografts at this early timepoint, and very few myelinated axons in FK506 and CSB treated mice (1% of that seen at 28 days). By 28 days (Fig. 5C), there were

significantly more unmyelinated axons in the center of CP than untreated allografts ($p<0.05$) but fewer myelinated axons than FK506 or CSB-treated mice, particularly at widths of 1–3 μm (Fig. 5D). While the number of myelinated axons at 28 days was significantly higher in CSB than CP allografts overall, regeneration of myelinated axons in the CP group was significantly higher than the CSB group at fiber widths of $\leq 1 \mu\text{m}$ and 3–6 μm ($p<0.05$).

Evaluation of Remak bundles and g-ratios lent further support to our suspicion that the delayed reinnervation and myelination noted in CP allografts related to their reliance on SC migration. Not only was there a disproportionately high number of unmyelinated versus myelinated axons at 28 days in CP allografts, but there was a significantly higher percentage of 1:1 relationships with SCs ($p<0.05$) compared to all other groups, suggesting that at least some of these axons would eventually become myelinated (Table 1). Moreover, mean g-ratio – inversely proportional to myelin thickness – was highest in CP allografts, also suggesting a deficiency in myelinated axons. G-ratios in CSB treated mice were significantly lower than all other groups ($p<0.05$) suggesting increased myelination (Table 2). The overall number of axons was reduced in untreated allografts, but those that were present still demonstrated some myelination presumably from surviving graft-based or migrated host SCs.

To confirm that axons regenerating across allografts were reinnervating their targets, we quantified the percentage of reinnervated motor endplates at 6 weeks (Fig. 6A–E). Endplate reinnervation of the EDL was significantly ($p<0.01$) better in FK506-treated mice where $64 \pm 12\%$ of labeled endplates were reinnervated compared with $4 \pm 2\%$ in untreated, $23 \pm 7\%$ in CP allografts, and $27 \pm 9\%$ in CSB-treated mice (Fig. 6E). Functional recovery, based on recovery of the print length factor (Fig. 6F), was also significantly faster in FK506 treated mice by 3 weeks post-engraftment ($p<0.05$). Functional recovery in untreated mice showed little improvement and was significantly worse than all other groups by 4 weeks ($p<0.05$), corroborating our finding of minimal endplate reinnervation by regenerating sciatic nerve branches.

Confirmation of Graft Versus Host Derived SCs

To confirm our *in vivo* findings, the origin of SCs within nerve allografts was evaluated by immunolabeling with anti-S100 antibody. Host-derived SCs were double-labeled with constitutively expressed GFP and anti-S100 antibody while donor-derived SCs were singly-labeled with anti-S100 antibody (Fig. 7A–D). Both untreated (Fig. 7A) and CP allografts (Fig. 7B) demonstrated labeling with both constitutive GFP and anti-S100 antibody. Untreated allografts, however, had visibly less antibody labeling, with increased fragmentation of the labeled SCs. In the setting of rejection, intrinsically expressed GFP may have persisted for a week or greater in the absence of S100 promoter activity or protein expression thus explaining the imbalance between constitutive and immune labeling (Feng, et al., 2000, Pan, et al., 2003). Allografts in FK506 and CSB treated mice were predominantly populated by donor-derived SCs with minimal host-derived SC migration (Fig. 7C&D). FK506-treated mice, like untreated allografts demonstrated increased fragmentation of S100-labeling, suggesting the presence of mild subacute rejection. Glial fibrillary acidic protein (GFAP) expression, used to identify non-myelinating and dedifferentiated SCs was significantly elevated in CP allografts at 5 days, and in the distal segment of allografts maintained with CSB. By 28 days, however, only CSB-treated mice demonstrated significantly ($p<0.05$) elevated GFAP relative to controls suggesting the maintenance of dedifferentiated and non-myelinating SCs in this treatment group (Supplemental Data 3).

Myelination is Affected by Allograft Treatment Modality

To study myelination, we evaluated MBP and connexin-32 expression in nerve allografts. MBP immunolabeling was robust and contiguously expressed along the course of regenerated axons

in the allografts of CSB-treated mice compared to all other groups (Fig. 8A–D). At 5 days, the mean expression from all treatment groups of connexin-32, a gap junction protein that is downregulated with nerve injury and upregulated with reinnervation (Chandross, et al., 1996, Mambetisaeva, et al., 1999, Scherer, et al., 1995), was $39.8 \pm 6.7\%$ of GAPDH signal with no significant differences between any group (data not shown). However, at 28 days, its expression was significantly increased in the proximal ($p < 0.01$) and middle ($p < 0.05$) third of CSB-treated allografts ($p < 0.05$) with incrementally less expression from proximal to distal (Fig. 8E). These data suggest that upregulation of connexin-32 expression mirrors reinnervation of myelinating allograft-based SCs in CSB-treated mice, and that a delay in migration (CP treated) or mild (FK506 treated) or severe rejection (untreated) attenuates its expression.

Distribution of MAPK and Akt Signaling in Treated and Untreated Allografts

The MAPK and Akt intracellular signaling pathways are differentially modulated by numerous stimuli in multiple cell types (Besset, et al., 2000, Bonni, et al., 1999, Harrisingh, et al., 2004, Mograbi, et al., 2001, Veit, et al., 2004, Xia, et al., 1995, Zawadzka and Kaminska, 2003). Of particular relevance to the present study, a reciprocal relationship characterized by the downregulation of MAPK signaling in the face of Akt phosphorylation occurs in the context of SC maturation and myelination (Ogata, et al., 2004). We confirmed increased phosphorylated MAPK signaling throughout untreated allografts at 28 days (Fig. 9A), relative to other groups. At 5 days, Western blot analysis demonstrated increased MAPK activity in untreated and FK506 treated mice (data not shown) and by 28 days confirmed a significant increase in phosphorylated MAPK in all segments of untreated allografts. FK506 treated mice also demonstrated a mild but not statistically significant increase in phosphorylated MAPK signaling by 28 days. Phosphorylated-Akt signaling was noted in all allografts at 28 days (Fig. 9B). The ratio of phosphorylated Akt to Akt was significantly elevated in the proximal and middle thirds of CSB treated mouse allografts relative to FK506 treatment ($p < 0.05$). Nerve allografts involve numerous cell types including neuronal projections, SCs, vascular endothelium, and inflammatory cells whose heterogeneous intracellular signaling programs respond to a diverse series of stimuli. Thus, there is no doubt that the absolute degree to which phosphatidylinositol-3-kinase (PI3K) -Akt and extracellular signal-regulated kinase (Erk) pathways are activated in the context of a surgically reconstructed nerve will be confounded by this heterogeneous *in vivo* milieu. However, our data still suggests a trend towards increased MAPK signaling in the context of acute rejection with untreated, and possibly subacute rejection with FK506-treated nerve allografts. It also suggests an increase in Akt-signaling commensurate with improved myelination in CSB-treated mice.

Discussion

SC phenotype has been extensively studied during development and following traumatic denervation, but not in the unique context of peripheral nerve allografts. Allograft-derived SCs are affected not only by a requisite period of denervation, but also the modality used to prevent graft rejection. Acellular CP nerve allografts offer the unique opportunity to study host SC migration in adults, while donor SCs residing in fresh allografts that are maintained by immunosuppressive or tolerance-inducing therapies possess a disparate SC phenotype profile. We confirm that acellular CP nerve allografts are not rejected, but support delayed nerve regeneration characterized by a deficiency or delay in reinnervation by myelinated axons. Not unexpectedly, FK506-treated mice demonstrated rapid regeneration and reinnervation of motor endplate targets. Allografts in FK506-treated mice, however, also showed signs of mild subacute graft rejection and poor myelination by 28 days consistent with previous work demonstrating mild chronic allograft rejection with systemic Cyclosporin A immunosuppression. Conversely, preservation of allograft-based SCs in mice treated with CSB

translated into a significant elevation in MBP, connexin-32, and phospho-Akt expression as well as a significant reduction in g-ratio.

Numerous SC-specific markers have been described to characterize their multiple phenotypes (Jessen and Mirsky, 2005). We chose to study the S100-GFP and nestin-GFP lines based on strong constitutive GFP-expression, distinct phenotype-based differences in promoter activity, and availability of a transgenic mouse model. S100 expression is downregulated following nerve injury (Hayashi, et al., 2007, Perez and Moore, 1968), suggesting that it cannot be exclusively used as a marker to track migrating and potentially dedifferentiated SCs. Nestin, a 200 kDa intermediate filament protein, has been identified in proliferating neural crest, peripheral (Hockfield and McKay, 1985), and terminal SCs (Kang, et al., 2007). Using Nestin-GFP^{+/+} mice to identify dedifferentiated SCs, we confirmed that a reduction in S100 expression occurs coincident with an increase in nestin promoter activity. In 1976, Aguayo reported engrafting sural nerves with cervical sympathetic trunk autografts to demonstrate that non-myelinating graft SCs remained segregated from host SCs, but were sufficiently plastic to eventually assume a myelinating phenotype (Aguayo, et al., 1976). By swapping nerve isografts between Nestin-GFP^{+/+} and Nestin-GFP^{-/-} littermates, we showed that denervated SCs upregulate nestin promoter activity in the absence of graft rejection. Further, we confirmed Aguayo's work that viable, isologous SCs remained segregated within their respective host and graft tissue without migrating (Aguayo, et al., 1976, Aguayo, et al., 1976). Based on GFP expression, Nestin promoter activity in allografts treated with CSB resembled that observed in isografts suggesting a similarity in SC phenotype between adequately treated allografts and isografts.

SC migration has been extensively studied during development, where it is highly dependent on interactions between Neuregulin-1 and ErbB2 (Lyons, et al., 2005). While regulation of SC phenotype following nerve injury may, in part, recapitulate developmental processes (Hayworth, et al., 2006), other evidence suggests that developing and denervated SCs differ (Chen, et al., 2007). For example, while ErbB2 signaling is found to be indispensable for SC maturation and myelination during development (Jessen and Mirsky, 2005), and its ablation leads to attenuated expression of its downstream signaling molecules phosphorylated MAPK and cyclin D1, it is unnecessary for preserving SC proliferation or survival after nerve injury (Atanasoski, et al., 2006). Moreover, in adults, neuregulins are implicated in the downregulation of MAG by strongly activating Erk pathways. Conversely, stimulation of PI3K-Akt pathways in mature, well differentiated SCs leads to the upregulation of MAG expression (Ogata, et al., 2004, Taveggia, et al., 2005).

Mature SCs, capable of producing neurotrophic factors and myelinating axons, are critical to nerve regeneration and functional recovery after traumatic nerve injury. We showed that CSB-treated mice demonstrated an overall increase in myelin expression coincident with increased Akt phosphorylation. The expression of connexin-32, a gap junction protein that is upregulated in mature Nodes of Ranvier and Schmidt-Lantermann incisures and downregulated following injury (Chandross, et al., 1996), was also increased with CSB therapy. While markers for the restoration of mature SC-axonal relationships were most prominent in CSB-treated mice, it was the FK506-treated mice that demonstrated significantly more large diameter ($\geq 3 \mu\text{m}$) myelinated axons by 28 days, and faster reinnervation of motor endplates and recovery by 6 weeks despite some evidence for subacute rejection. These findings would suggest that exogenously administered agents like FK506 can stimulate axons to regenerate, at least in part, independent of the local SC environment, thus activating relevant signaling mechanisms confined exclusively to the neuron and its axonal projections. While its neuroregenerative, SC-independent mechanism of action is still being investigated, FK506 does appear to directly influence injured axons by binding the neuroimmunophilin ligands FKBP-12 or FKBP-52 (Gold, 1999, Steiner, et al., 1997). Recently, it has also been suggested that a dipeptide analogue

of FK506 binds heat shock cognate 70 (Hsc70) whose downstream signaling results in an increase in cAMP-response element binding protein (CREB) phosphorylation, which in turn may play a role in upregulating GDNF expression (Cen, et al., 2006). Intuitively, the co-administration of FK506 and CSB therapy could provide an environment favorable for both remyelination and reinnervation. Unfortunately, FK506 can abrogate CSB-induced immune hyporesponsiveness and lead to the rejection of nerve (Brenner, et al., 2005), and other organ transplants (Kirk, et al., 1999, Larsen, et al., 1996).

The nerve allograft offers a unique opportunity to evaluate mature, denervated, rejecting, and migrating SC phenotype in an adult model. These nerve allografts, however, must be sufficiently large to enable surgical manipulation and are therefore characterized by a relatively heterogeneous ensemble of migrating SCs, regenerating axons, and the markers that define these phenotypes. We were able to show that markers of SC differentiation, like connexin-32 and Akt expression were more pronounced in the proximal and middle graft segments than the distal segments and reflected the course of axonal regeneration. In the absence of immune-privileged SCs, reinnervation was preceded by a migratory SC phase that delayed the appearance of the myelinating SC phenotype. Still, even untreated nerve allografts supported a small degree of regeneration as well as phospho-Akt, and connexin-32 expression by 28 days. These findings are explained by the fact that potentially thousands of axons and SCs within a nerve graft reconstruction are independently influenced by differences in their immediate microenvironment. This inherent weakness in detecting subtle differences between experimental groups in a large graft model compared with *in vitro* or nerve crush studies using smaller nerves is justified, however, by the fact that the relevance of any signaling mechanism or therapeutic intervention will ultimately be assessed based on its effects on larger human peripheral nerves.

Data from this study suggests that modulation of the immune response significantly affects SC phenotype and nerve regeneration at both the morphologic and molecular levels. Some investigators have altered MAG expression in untreated rat sciatic nerve allografts by modulating MAPK and PI3K-Akt signaling with adenoviral vectors (Ogata, et al., 2004). By contrast, we found the regeneration of myelinated axons to be poor and MAPK signaling to be elevated when allografts were not treated. Differences between studies may be explained by the fact that allografts in outbred Wistar rats used in their study likely possessed varying degrees of MHC-disparity resulting in only modest rejection. Like Ogata et al. (2004) who evaluated their allografts at 5 weeks, we show that untreated allografts eventually support some axonal regeneration, albeit less than that detected in treated allografts which had far more robust regeneration.

By modulating their treatment environment, peripheral nerve allografts provide a useful model for studying the plasticity of SC phenotype in adults. Apoptosis, SC rejection, elevated MAPK levels, scant nerve regeneration and endplate reinnervation with minimal recovery characterize untreated allografts. We confirm that cold preserved allografts are characterized by a SC migratory phase with upregulation of nestin promoter activity, delayed myelination, and modest motor endplate reinnervation. Interestingly, mice treated with FK506 demonstrated the most robust regeneration and functional recovery, but at the expense of mild subacute rejection as evidenced by a mild elevation in cleaved caspase-3 and MAPK expression, mild host SC migration at 28 days, and fewer myelinated axons compared to CSB-treated mice. The ability of FK506 to enhance regeneration in the context of a suboptimal SC environment merits further investigation at later time points, and in higher animal models with more rigorous behavioral analysis. Our current findings would suggest that preventing even mild SC rejection is necessary to facilitate complete SC differentiation and myelin maturation, but is not necessary to promote nerve regeneration through short nerve allografts. Clinical translation of our data suggests that despite its drawbacks, FK506 will remain the best currently available option for

preventing nerve allograft rejection until co-stimulatory blockade regimens become readily available in humans. Future studies will evaluate whether nerve allografts in mice treated with either continuous FK506 therapy or CSB will eventually become repopulated by host SCs, and what, if any impact this will have on SC phenotype, and myelin formation. The impact of FK506 withdrawal on SC phenotype after graft reinnervation will also be studied utilizing the novel transgenic and imaging tools now available for the study of peripheral nerve regeneration. These studies will help to identify a treatment modality that minimizes host morbidity by preserving autologous tissue, minimizing immunosuppression, and enhancing nerve regeneration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to acknowledge Dr. Jeffrey Milbrandt (Washington University) for his thoughtful review of this manuscript and Dr. G. Enikolopov (Cold Springs Harbor, NY) for providing our lab with breeding pairs of Nestin-GFP mice and genotyping information. Also, we would like to thank Ms. J. Luciano for assistance with animal breeding, genotyping, and walking track analysis, and Mr. J. Koob and Dr. D. Liu for their preliminary work on serial imaging of nerves. This work was funded by a grant from the National Institutes of Health (5RO1NS033406-14) awarded to Dr. Mackinnon, the American Association of Plastic Surgeons John E. Hoopes Academic Scholar awarded to Dr. Myckatyn, and the American Academy of Otolaryngology –Head and Neck Surgery awarded to Dr. Moradzadeh and Dr. Hayashi.

References

1. Aguayo AJ, Charron L, Bray GM. Potential of Schwann cells from unmyelinated nerves to produce myelin: a quantitative ultrastructural and radiographic study. *J Neurocytol* 1976;5:565–573. [PubMed: 978232]
2. Aguayo AJ, Epps J, Charron L, Bray GM. Multipotentiality of Schwann cells in cross-anastomosed and grafted myelinated and unmyelinated nerves: quantitative microscopy and radioautography. *Brain Res* 1976;104:1–20. [PubMed: 1247896]
3. Ansari MJ, Abdi R. Emerging immunomodulatory therapies targeting the co-stimulatory pathways for the prevention of transplant rejection. *IDrugs* 2003;6:964–969. [PubMed: 14534853]
4. Atanasoski S, Scherer SS, Sirkowski E, Leone D, Garratt AN, Birchmeier C, Suter U. ErbB2 signaling in Schwann cells is mostly dispensable for maintenance of myelinated peripheral nerves and proliferation of adult Schwann cells after injury. *J Neurosci* 2006;26:2124–2131. [PubMed: 16481445]
5. Atanasoski S, Shumas S, Dickson C, Scherer SS, Suter U. Differential cyclin D1 requirements of proliferating Schwann cells during development and after injury. *Mol Cell Neurosci* 2001;18:581–592. [PubMed: 11749035]
6. Atchabahian A, Mackinnon SE, Hunter DA. Cold preservation of nerve grafts decreases expression of ICAM-1 and class II MHC antigens. *Journal of Reconstructive Microsurgery* 1999;15:307–311. [PubMed: 10363556]
7. Besset V, Scott RP, Ibanez CF. Signaling complexes and protein-protein interactions involved in the activation of the Ras and PI3K pathways by the c-Ret receptor tyrosine kinase. *J Biol Chem*. 2000
8. Bonni A, Brunet A, West AE, Datta SR, Takasu MA, Greenberg ME. Cell survival promoted by the Ras-MAPK signaling pathway by transcription-dependent and -independent mechanisms [see comments]. *Science* 1999;286:1358–1362. [PubMed: 10558990]
9. Brenner MJ, Lowe JB 3rd, Fox IK, Mackinnon SE, Hunter DA, Darcy MD, Duncan JR, Wood P, Mohanakumar T. Effects of Schwann cells and donor antigen on long-nerve allograft regeneration. *Microsurgery* 2005;25:61–70. [PubMed: 15481042]
10. Brenner MJ, Mackinnon SE, Rickman SR, Jaramillo A, Tung TH, Hunter DA, Mohanakumar T. FK506 and anti-CD40 ligand in peripheral nerve allotransplantation. *Restor Neurol Neurosci* 2005;23:237–249. [PubMed: 16082080]

11. Brenner MJ, Tung TH, Mackinnon SE, Myckatyn TM, Hunter DA, Mohanakumar T. Anti-CD40 ligand monoclonal antibody induces a permissive state, but not tolerance, for murine peripheral nerve allografts. *Exp Neurol* 2004;186:59–69. [PubMed: 14980810]
12. Brown CJ, Mackinnon SE, Evans PJ, Bain JR, Makino AP, Hunter DA, Hare GM. Self-evaluation of walking-track measurement using a Sciatic Function Index. *Microsurgery* 1989;10:226–235. [PubMed: 2796719]
13. Cen X, Nitta A, Ohya S, Zhao Y, Ozawa N, Mouri A, Ibi D, Wang L, Suzuki M, Saito K, Ito Y, Kawagoe T, Noda Y, Ito Y, Furukawa S, Nabeshima T. An analog of a dipeptide-like structure of FK506 increases glial cell line-derived neurotrophic factor expression through cAMP response element-binding protein activated by heat shock protein 90/Akt signaling pathway. *J Neurosci* 2006;26:3335–3344. [PubMed: 16554484]
14. Chandross KJ, Kessler JA, Cohen RI, Simburger E, Spray DC, Bieri P, Dermietzel R. Altered connexin expression after peripheral nerve injury. *Mol Cell Neurosci* 1996;7:501–518. [PubMed: 8875432]
15. Chen ZL, Yu WM, Strickland S. Peripheral Regeneration. *Annu Rev Neurosci*. 2007
16. Feng G, Mellor RH, Bernstein M, Keller-Peck C, Nguyen QT, Wallace M, Nerbonne JM, Lichtman JW, Sanes JR. Imaging neuronal subsets in transgenic mice expressing multiple spectral variants of GFP. *Neuron* 2000;28:41–51. [PubMed: 11086982]
17. Finn JT, Weil M, Archer F, Siman R, Srinivasan A, Raff MC. Evidence that Wallerian degeneration and localized axon degeneration induced by local neurotrophin deprivation do not involve caspases. *J Neurosci* 2000;20:1333–1341. [PubMed: 10662823]
18. Fox IK, Jaramillo A, Hunter DA, Rickman SR, Mohanakumar T, Mackinnon SE. Prolonged cold-preservation of nerve allografts. *Muscle Nerve* 2005;31:59–69. [PubMed: 15508128]
19. Gold BG. FK506 and the role of the immunophilin FKBP-52 in nerve regeneration. *Drug Metab Rev* 1999;31:649–663. [PubMed: 10461545]
20. Gold BG, Storm-Dickerson T, Austin DR. The immunosuppressant FK506 increases functional recovery and nerve regeneration following peripheral nerve injury. *Restor Neurol Neurosci* 1994;6:287–296.
21. Gottlieb RA, Kitsis RN. Seeing death in the living. *Nat Med* 2001;7:1277–1278. [PubMed: 11726959]
22. Grand AG, Myckatyn TM, Mackinnon SE, Hunter DA. Axonal regeneration after cold preservation of nerve allografts and immunosuppression with tacrolimus in mice. *J Neurosurg* 2002;96:924–932. [PubMed: 12005401]
23. Gundersen HJ, Bagger P, Bendtsen TF, Evans SM, Korbo L, Marcussen N, Moller A, Nielsen K, Nyengaard JR, Pakkenberg B, et al. The new stereological tools: disector, fractionator, nucleator and point sampled intercepts and their use in pathological research and diagnosis. *Apmis* 1988;96:857–881. [PubMed: 3056461]
24. Hare GM, Evans PJ, Mackinnon SE, Best TJ, Bain JR, Szalai JP, Hunter DA. Walking track analysis: a long-term assessment of peripheral nerve recovery. *Plast Reconstr Surg* 1992;89:251–258. [PubMed: 1732892]
25. Harrisingh MC, Perez-Nadales E, Parkinson DB, Malcolm DS, Mudge AW, Lloyd AC. The Ras/Raf/ERK signalling pathway drives Schwann cell dedifferentiation. *Embo J* 2004;23:3061–3071. [PubMed: 15241478]
26. Hayashi A, Koob JW, Liu DZ, Tong AY, Hunter DA, Parsadanian A, Mackinnon SE, Myckatyn TM. A double-transgenic mouse used to track migrating Schwann cells and regenerating axons following engraftment of injured nerves. *Exp Neurol*. 2007
27. Hayworth CR, Moody SE, Chodosh LA, Krieg P, Rimer M, Thompson WJ. Induction of neuregulin signaling in mouse schwann cells in vivo mimics responses to denervation. *J Neurosci* 2006;26:6873–6884. [PubMed: 16793894]
28. Hockfield S, McKay RD. Identification of major cell classes in the developing mammalian nervous system. *J Neurosci* 1985;5:3310–3328. [PubMed: 4078630]
29. Hunter DA, Moradzadeh A, Whitlock EL, Brenner MJ, Myckatyn TM, Wei CH, Tung TH, Mackinnon SE. Binary imaging analysis for comprehensive quantitative histomorphometry of peripheral nerve. *J Neurosci Methods*. 2007

30. Inserra MM, Bloch DA, Terris DJ. Functional indices for sciatic, peroneal, and posterior tibial nerve lesions in the mouse. *Microsurgery* 1998;18:119–124. [PubMed: 9674927]
31. Jessen KR, Mirsky R. The origin and development of glial cells in peripheral nerves. *Nat Rev Neurosci* 2005;6:671–682. [PubMed: 16136171]
32. Jost SC, Doolabh VB, Mackinnon SE, Lee M, Hunter D. Acceleration of peripheral nerve regeneration following FK506 administration. *Restor Neurol Neurosci* 2000;17:39–44. [PubMed: 11490076]
33. Kang H, Tian L, Son YJ, Zuo Y, Procaccino D, Love F, Hayworth C, Trachtenberg J, Mikesch M, Sutton L, Ponomareva O, Mignone J, Enikolopov G, Rimer M, Thompson W. Regulation of the intermediate filament protein nestin at rodent neuromuscular junctions by innervation and activity. *J Neurosci* 2007;27:5948–5957. [PubMed: 17537965]
34. Kimura A, Ajiki T, Takeuchi K, Hakamata Y, Murakami T, Hoshino Y, Kobayashi E. Transmigration of donor cells involved in the sciatic nerve graft. *Transplant Proc* 2005;37:205–207. [PubMed: 15808594]
35. Kirk AD, Burkly LC, Batty DS, Baumgartner RE, Berning JD, Buchanan K, Fechner JH Jr, Germond RL, Kampen RL, Patterson NB, Swanson SJ, Tadaki DK, TenHoor CN, White L, Knechtle SJ, Harlan DM. Treatment with humanized monoclonal antibody against CD154 prevents acute renal allograft rejection in nonhuman primates. *Nature Medicine* 1999;5:686–692.
36. Kirk AD, Harlan DM, Armstrong NN, Davis TA, Dong Y, Gray GS, Hong X, Thomas D, Fechner JH Jr, Knechtle SJ. CTLA4-Ig and anti-CD40 ligand prevent renal allograft rejection in primates. *Proc Natl Acad Sci U S A* 1997;94:8789–8794. [PubMed: 9238056]
37. Kist-van Holthe JE, Gasser M, Womer K, Najafian N, Dong V, Samsonov DV, Geehan CS, Chandraker A, Sayegh MH, Waaga AM. Regulatory functions of alloreactive Th2 clones in human renal transplant recipients. *Kidney Int* 2002;62:627–631. [PubMed: 12110027]
38. Larsen CP, Elwood ET, Alexander DZ, Ritchie SC, Hendrix R, Tucker-Burden C, Cho HR, Aruffo A, Hollenbaugh D, Linsley PS, Winn KJ, Pearson TC. Long-term acceptance of skin and cardiac allografts after blocking CD40 and CD28 pathways. *Nature* 1996;381:434–438. [PubMed: 8632801]
39. Lyons DA, Pogoda HM, Voas MG, Woods IG, Diamond B, Nix R, Arana N, Jacobs J, Talbot WS. *erbb3* and *erbb2* are essential for schwann cell migration and myelination in zebrafish. *Curr Biol* 2005;15:513–524. [PubMed: 15797019]
40. Mackinnon SE, Doolabh VB, Novak CB, Trulock EP. Clinical outcome following nerve allograft transplantation. *Plast Reconstr Surg* 2001;107:1419–1429. [PubMed: 11335811]
41. Mackinnon SE, Hudson AR. Clinical application of peripheral nerve transplantation. *Plast Reconstr Surg* 1992;90:695–699. [PubMed: 1410009]
42. Magill CK, Tong A, Kawamura D, Hayashi A, Hunter DA, Parsadanian A, Mackinnon SE, Myckatyn TM. Reinnervation of the tibialis anterior following sciatic nerve crush injury: A confocal microscopic study in transgenic mice. *Exp Neurol*. 2007
43. Magott-Procelewska M. Costimulatory pathways as a basic mechanisms of activating a tolerance signal in T cells. *Ann Transplant* 2004;9:13–18. [PubMed: 15759541]
44. Mambetisaeva ET, Gire V, Evans WH. Multiple connexin expression in peripheral nerve, Schwann cells, and Schwannoma cells. *J Neurosci Res* 1999;57:166–175. [PubMed: 10398294]
45. Midha R, Mackinnon SE, Becker LE. The fate of Schwann cells in peripheral nerve allografts. *J Neuropathol Exp Neurol* 1994;53:316–322. [PubMed: 8176414]
46. Mignone JL, Kukekov V, Chiang AS, Steindler D, Enikolopov G. Neural stem and progenitor cells in nestin-GFP transgenic mice. *J Comp Neurol* 2004;469:311–324. [PubMed: 14730584]
47. Mograbi B, Bocciardi R, Bourget I, Busca R, Rochet N, Farahi-Far D, Juhel T, Rossi B. Glial cell line-derived neurotrophic factor-stimulated phosphatidylinositol 3-kinase and Akt activities exert opposing effects on the ERK pathway: importance for the rescue of neuroectodermic cells. *J Biol Chem* 2001;276:45307–45319. [PubMed: 11535584]
48. Myckatyn TM, Ellis RA, Grand AG, Sen SK, Lowe JB 3rd, Hunter DA, Mackinnon SE. The effects of rapamycin in murine peripheral nerve isografts and allografts. *Plast Reconstr Surg* 2002;109:2405–2417. [PubMed: 12045568]
49. Myckatyn TM, Hunter DA, Mackinnon SE. The effects of cold preservation and subimmunosuppressive doses of FK506 on axonal regeneration in murine peripheral nerve isografts. *Can J Plast Surg* 2003;11:15–22.

50. Myckatyn TM, MacKinnon SE. A review of research endeavors to optimize peripheral nerve reconstruction. *Neurol Res* 2004;26:124–138. [PubMed: 15072631]
51. Ogata T, Iijima S, Hoshikawa S, Miura T, Yamamoto S, Oda H, Nakamura K, Tanaka S. Opposing extracellular signal-regulated kinase and Akt pathways control Schwann cell myelination. *J Neurosci* 2004;24:6724–6732. [PubMed: 15282275]
52. Pan YA, Misgeld T, Lichtman JW, Sanes JR. Effects of neurotoxic and neuroprotective agents on peripheral nerve regeneration assayed by time-lapse imaging in vivo. *J Neurosci* 2003;23:11479–11488. [PubMed: 14673013]
53. Perez VJ, Moore BW. Wallerian degeneration in rabbit tibial nerve: changes in amounts of the S-100 protein. *J Neurochem* 1968;15:971–977. [PubMed: 5682514]
54. Scherer SS, Deschenes SM, Xu YT, Grinspan JB, Fischbeck KH, Paul DL. Connexin-32 is a myelin-related protein in the PNS and CNS. *J Neurosci* 1995;15:8281–8294. [PubMed: 8613761]
55. Shen ZL, Lassner F, Bader A, Becker M, Walter GF, Berger A. Cellular activity of resident macrophages during Wallerian degeneration. *Microsurgery* 2000;20:255–261. [PubMed: 11015722]
56. Steiner JP, Connolly MA, Valentine HL, Hamilton GS, Dawson TM, Hester L, Snyder SH. Neurotrophic actions of nonimmunosuppressive analogues of immunosuppressive drugs FK506, rapamycin, and cyclosporin A. *Nature Medicine* 1997;3:421–428.
57. Taveggia C, Zanazzi G, Petrylak A, Yano H, Rosenbluth J, Einheber S, Xu X, Esper RM, Loeb JA, Shrager P, Chao MV, Falls DL, Role L, Salzer JL. Neuregulin-1 type III determines the ensheathment fate of axons. *Neuron* 2005;47:681–694. [PubMed: 16129398]
58. Veit C, Genze F, Menke A, Hoeffert S, Gress TM, Gierschik P, Giehl K. Activation of phosphatidylinositol 3-kinase and extracellular signal-regulated kinase is required for glial cell line-derived neurotrophic factor-induced migration and invasion of pancreatic carcinoma cells. *Cancer Res* 2004;64:5291–5300. [PubMed: 15289335]
59. Xia Z, Dickens M, Raingeaud J, Davis RJ, Greenberg ME. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science* 1995;270:1326–1331. [PubMed: 7481820]
60. Zalewski AA, Gulati AK. Survival of nerve and Schwann cells in allografts after cyclosporin A treatment. *Exp Neurol* 1980;70:219–225. [PubMed: 6968689]
61. Zawadzka M, Kaminska B. Immunosuppressant FK506 affects multiple signaling pathways and modulates gene expression in astrocytes. *Mol Cell Neurosci* 2003;22:202–209. [PubMed: 12676530]
62. Zuo Y, Lubischer JL, Kang H, Tian L, Mikesch M, Marks A, Scofield VL, Maika S, Newman C, Krieg P, Thompson WJ. Fluorescent proteins expressed in mouse transgenic lines mark subsets of glia, neurons, macrophages, and dendritic cells for vital examination. *J Neurosci* 2004;24:10999–11009. [PubMed: 15590915]

Figure 1.

A. Nerve allografts were swapped between fluorescence naïve Balb/C mice (black; – GFP) and those with GFP expression driven by either the S100 or nestin promoter. While GFP-S100 is constitutively active (white; + GFP) in these uninjured adults, Nestin-GFP promoter expression depended upon denervation, becoming activated within 5 days of denervation and engraftment. **B.** Summary of treatment modalities, abbreviations, and outcome measures.

Figure 2.

Nerve pretreatment with 7 weeks of CP, FK506, and CSB all effectively prevented nerve allograft rejection. **A.** Recipient S100-GFP^{+/+} mice inbred on a C57BL/6 background received skin autografts and allografts from a Balb/C partner to confirm major-MHC mismatch shown at t=14d. Autografts survived indefinitely (>42d). Skin allografts rejected within 9 days and began to slough and bleed by 14 days. **B.** ELISPOT assays confirmed a strong IFN- γ response to nerve allografts at 10 days, but silencing of the Th1 response in all treatment groups ($p < 0.001$). **C.** Apoptosis of rejected cells within the nerve allografts was confirmed by the presence of cleaved caspase-3. Representative results from single allograft segments are shown. Untreated allografts demonstrated significant elevation in cleaved caspase-3 normalized to the GAPDH signal (* $p < 0.05$). P for proximal, M for middle, and D for distal third graft segments.

Figure 3.

A. S100-GFP^{+/+} mice bred on a C57BL/6 background received fluorescence naïve allografts that were left untreated (No Rx), cold preserved for 7 weeks (CP), or subjected to FK506 (FK) or CSB therapy with anti-CD40L mAb and CTLA4-4. Fluorescent allograft intensity was measured with line scanning of pixel intensity on days 0, 5 (not shown), 10, 15, and 28. Mean pixel intensity from mice in each group (n=6) was calculated per graft segment and plotted. Cold preserved allografts were repopulated within 5 days. Untreated allografts within 15 days, and the proximal and distal cuffs of allografts were repopulated in immunosuppressed mice by 28 days. **B.** S100-GFP^{+/+} allografts, except for CP allografts which were devoid of SCs, were also transplanted into their fluorescence-naïve Balb-C counterparts. GFP intensity in

untreated allografts was reduced at 10 days, increased at 20 days before disappearing at 28 days. Conversely, FK506- and CSB-treated mice retained GFP signal intensity throughout the 28 day experiment, except for the proximal and distal cuffs of the allograft. * $p<0.05$.

Figure 4.

A. Nestin-GFP^{+/+} mice bred on a C57BL/6 background received fluorescence naïve allografts that were left untreated, CP, or treated with FK506 or CSB. A fifth group received isografts (ISO) from Nestin-GFP^{-/-} littermates. Fluorescent intensity was measured 0, 5, 10, 15, and 28 days later (days 0 and 28 shown). CP allografts demonstrated the most robust migration of Nestin-GFP expressing cells while minimal GFP expression was noted in immunosuppressed allografts and isografts during the 28 day experiment. **B.** Nestin-GFP^{+/+} grafts were also transplanted into fluorescent naïve hosts as allografts or isografts. Untreated allografts expressed GFP within 5 days but then lost this expression by 15 days. Conversely, allografts in immunosuppressed mice and isografts demonstrated Nestin-GFP expression by 15 days that persisted for at least 28 days.

Figure 5.

A. Non-biased stereology was used to estimate the density of unmyelinated axons 3 mm distal to the proximal suture line 10 days after engraftment. FK506- and CSB-treated allografts demonstrated significantly higher fiber counts than untreated or CP allografts ($p < 0.005$). **B.** A few myelinated axons were noted in FK506- and CSB-treated allografts at 10 days. **C.** By 28 days, the mid-point (5 mm) of CP allografts were noted to support regeneration of unmyelinated axons. **D.** Based on total fiber counts, the mid-point of FK506- and CSB-treated allografts demonstrated superior regeneration of myelinated axons compared to all other groups at 28 days although CP allografts had significantly more regeneration at some fiber diameters. (* denotes significantly more axons, at least $p < 0.05$, compared to CP allografts, Δ denotes significantly fewer axons, $p < 0.05$ compared to CP allografts). **E.** EM at 28 days of untreated allograft with few myelinated and unmyelinated axons. **F.** EM of CP allograft less densely populated with myelinated axons than **G.** FK506- or **H.** CSB-treated allografts at 28 days. Scale bar 1 μm.

Figure 6.

Motor endplate reinnervation was evaluated following rhodamine-BTX labeling in the EDL muscle 6 weeks following surgery in Thy1-CFP^{+/+}/S100-GFP^{+/+} hosts. **A.** Untreated allografts displayed minimal reinnervation. **B.** The CP group was marked by patchy reinnervation of endplates. **C.** FK506 treated mice demonstrated robust reinnervation by the majority of its terminal arbors. **D.** CSB group mice also demonstrated patchy reinnervation. Scale bars 100 μ m. **E.** Quantitative analysis of ≥ 300 endplates revealed that reinnervation was significantly better in FK506-treated mice relative to all other groups (* $p < 0.01$ compared to CP and CSB). CP and CSB groups were significantly better than untreated allografts (**; $p < 0.01$). **F.** Restoration of plantar flexion was significantly faster in FK506 treated mice compared to all other groups starting 3 weeks post-engraftment based on the print-length factor (*; $p < 0.05$). Untreated mice had significantly worse recovery starting 4 weeks post-engraftment (Δ ; $p < 0.05$).

Figure 7.

To differentiate donor from graft-derived SCs at 28 days, nerve allografts from S100-GFP^{+/+} hosts were labeled with anti-S100 antibody. Host-derived SCs were co-labeled with constitutively expressed GFP and anti-S100 antibody (green and magenta), while donor-derived graft SCs were singly labeled with anti-S100 antibody (magenta only). **A.** Untreated allografts demonstrated significant GFP and anti-S100 co-labeling (inset demonstrates co-labeled SCs), **B.** as did CP allografts. **C.** Allografts in FK506 and **D.** CSB treated mice were predominantly populated by donor-derived SCs (magenta) with minimal host-derived SC migration. Increased fragmentation of S100-labeling was noted in untreated and FK506 treated mice. Scale bars 100μm.

Figure 8.

Thy1-CFP^{+/+}/S100-GFP^{+/+} hosts were labeled with MBP to colocalize axons (cyan), and host SCs (green), with MBP (orange). **A.** Untreated allografts demonstrated minimal MBP expression. Exclusively noted in untreated allografts, GFP-labeled cells with a sponge-like morphology were noted to be macrophages (Hayashi, et al., 2007). **B.** CP allografts demonstrated modest MBP expression and colocalization with migrated host SCs (yellow). **C.** Clumped and relatively scant MBP expression was noted in FK506 treated allografts. **D.** CSB treated mice had robust, contiguous MBP expression in allografts that colocalized with regenerated axons. Scale bars 100 μ m. **E.** Connexin-32 expression was significantly increased in the proximal (* $p < 0.01$) and middle (** $p < 0.05$) thirds of CSB-treated mouse allografts. Its

expression was reduced in all other groups at the 28 day time point. Prox. for proximal, Mid. for middle, and Dist. for distal third graft segments.

Figure 9.

A. Phosphorylated-MAPK signal, a measure of de-differentiated SCs, was significantly elevated by Western blot in all segments of untreated allografts at 28 days (* $p < 0.05$). **B.** The ratio of pAkt to Akt was significantly elevated in the proximal and middle thirds of CSB treated mouse allografts compared to FK506 treated mice (* $p < 0.05$). Prox. for proximal, Mid. for middle, and Dist. for distal third graft segments.

Table 1

Percentage distribution of #unmyelinated axons/SC (Remak bundles) at 28 days

# Unmyelinated Axons/SC	1/1	2/1	3/1	4/1	5/1	>6/1
No Rx	43.5±18.1%	21.1±7.3%	17.2±4.7%	11.7±3.4	6.5±2.1	0
CP	65.7±13.1%	17.7±5.1%	13.08±4.1%	1.86±1.1%	0	0
FK	47.5±12.6%	22.2±7.1%	16.4±3.2%	7±2.3%	2.9±1.1%	3.8±1.3%
CSB	58.8±13.4%	24.4±6.4%	10.5±2.9%	3.3±1.2%	1.1±0.4%	0

Table 2

Mean g-ratios for myelinated axons at 28 days.

No Rx	60.0 ± 0.4
CP	63.1 ± 2.8*
FK	55.9 ± 3.6
CSB	51.0 ± 3.8**

* CP allografts had significantly higher g-ratios (relatively thinner myelin) than FK506-and CSB-treated allografts ($p < 0.05$).

** CSB-treated mice had significantly lower g-ratios (thicker myelin) than all other groups ($p < 0.05$).