A novel model for evaluating nerve regeneration in the composite tissue transplant: the murine heterotopic limb transplant

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Abstract

Purpose For individuals who have experienced debilitating upper extremity injury or amputation, hand transplantation holds the potential for drastic quality of life improvement. This potential depends on adequate nerve regeneration into the transplant and reanimation of graft musculature. In this study, we demonstrate the use of a murine heterotopic limb transplant model for evaluation of nerve regeneration in a composite tissue allograft (CTA). We also compare the effects of various immunosuppressive regimens on nerve regeneration in this model.

Methods The study consisted of five groups of mice, all of which underwent heterotopic limb transplant with coaptation of the recipient and donor sciatic nerves. The groups received the following immunosuppressive regimens: group A (positive control)—syngeneic transplant, no immunosuppression; group B (negative control)—allogeneic transplant, no immunosuppression; group C—allogeneic transplant, FK-506+MR1; group D—allogeneic transplant, MR1+CTLA4-Ig; group E—syngeneic transplant, FK-506 treatment with preloading.

Results Group B animals showed signs of transplant rejection as early as 5 days postoperatively. Except for one mouse from group C and one mouse from group D, all

other animals had viable transplants and nerve regeneration present in the donor sciatic nerve at the 3-week endpoint of the study.

Conclusions To our knowledge, this represents the first report of the use of a mouse CTA model for evaluation of nerve regeneration. The mouse heterotopic limb transplant model will be a valuable tool for CTA research since it can be performed with more ease, and with less host morbidity and mortality than the mouse orthotopic model.

Keywords CTA · Composite tissue allograft · Limb transplantation · Mouse · Nerve regeneration · FK-506 · Costimulatory blockade

Introduction

For those individuals who have experienced debilitating upper extremity injuries or amputations, hand transplantation holds the potential for drastic quality of life improvement. At least 50 hand transplantation procedures have been performed worldwide since the first successful case was reported in September 1998 [12]. Initially, successful composite tissue allograft (CTA) transplantation was limited by the availability of an immunosuppressant regimen able to prevent rejection of the CTA, which is highly immunogenic due to its heterogeneous tissue composition that includes skin, subcutaneous tissues, muscle, vascularized bone, and blood vessels [19]. Now, the success of CTA as a permanent tool in the reconstructive armamentarium will depend on further breakthroughs in immunomodulation, such as development of new protocols that minimize maintenance immunosuppression or induce a state of graft tolerance. Just as important to its ultimate success, however,

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will be enhancing long-term functional results through improving nerve regeneration in the CTA.

Analysis of functional recovery has been reported for hand transplants performed after 2002, which have been logged in the International Registry on Hand and Composite Tissue Transplantation [17, 23]. All patients demonstrated protective sensibility and most showed discriminative sensibility. Extrinsic muscle recovery occurred prior to intrinsic muscle recovery and most patients were able to use their transplanted hand for most activities of daily living. Even with these promising results, however, the functionality of hand transplants, especially for fine tactile movements, has yet to approach that of the uninjured hand. There is a need, therefore, for gaining a better understanding of nerve regeneration in CTAs and for developing strategies to enhance it. This will especially be true as the level of transplantation moves more and more proximally—from the hand to the forearm to the upper arm—and poses a greater regenerative challenge.

Little experimental data has been published to date on nerve regeneration in CTAs. On the other hand, research on immunosuppressive regimens in CTAs has been ongoing for years, and rodents have proven to be a good model for these investigations. Rodents have also served as a reliable model for the study of nerve regeneration through nerve allografts, isografts, and conduits. Rodents, it follows, could serve as a valuable model for evaluating nerve regeneration in CTA. While useful in theory, a rodent orthotopic hind limb CTA model requires a long learning curve and is time consuming and very technically demanding. We have previously described the use of a heterotopic murine hindlimb CTA model for evaluation of immunosuppressive regimens [26, 27]. The primary aim of this study was to determine whether we could use this same model, with the added modification of coaptation of the donor and recipient sciatic nerves, for evaluation of nerve regeneration in a CTA. To our knowledge, this represents the first report of the use of a mouse limb transplantation model for evaluation of nerve regeneration in a CTA.

To test the utility of this model, we investigated the effects of various immunosuppressive regimens, specifically costimulatory blockade (CSB) and tacrolimus (FK-506), on nerve regeneration. Costimulatory blockade allows host T cells to recognize donor antigens but prevents T cell activation. Tacrolimus, a calcineurin-inhibitor [4, 20], inhibits the activation of T-cell proliferation [33], and is widely used in clinical solid organ transplantation. Tacrolimus is also known to enhance peripheral nerve regeneration [9] although the exact mechanism of its neuroregenerative properties remains unclear. It has been shown to enhance peripheral nerve regeneration in multiple models including transection [14], crush [18, 32], chronic axotomy [25], isograft [6], and

allograft [7, 21]. We hypothesized that the different immunosuppressive regimens would lead to differing degrees of nerve regeneration in the CTA, with acceleration of sciatic nerve regeneration in groups treated with tacrolimus and CSB [8].

Material and Methods

Animal Care

All housing, care, and surgical procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the experimental protocols were approved by the university's Animal Studies Committee. Animals were housed in a central animal care facility with access to standard rodent chow and water ad libitum. All animals were evaluated daily for signs of rejection or distress. The criteria used to diagnose rejection have been previously detailed for the rat model [5, 16, 22, 26, 27]. These include erythema, progressive edema, and exudation, followed by eschar formation, necrosis, mummification, and slough. For the purposes of this study, rejection was defined as skin changes beyond edema and erythema.

Animal Groups

Full MHC disparity was present between recipient and donor animals. The transplants were performed in 2–3-month old C57Bl/6 mice (H-2K^b) weighing 20–25 g. Balb/c mice (H-2K^d) (Jackson Laboratory, Bar Harbour, ME, USA) of equivalent age and weight served as donors for allogeneic hind limb transplants.

There were five study groups (Table 1). Group A animals underwent syngeneic heterotopic limb transplantation and served as a positive control (transplanted nerve=isograft). Group B animals underwent allogeneic heterotopic limb transplantation without immunosuppression and served as a negative control (transplanted nerve=untreated allograft). Group C animals underwent allogeneic heterotopic limb transplantation and received CSB plus FK-506. Specifically, 0.5 mg of MR1 (hamster anti-mouse anti-CD40L mAb; Bio Express, Inc, West Lebanon, NH, USA) was administered by intraperitoneal (IP) injection on days 0, 3, 6, 9, 12, and 15 and 2 mg/kg of FK-506 (Astellas USA, Inc., Deerfield, IL, USA) was administered subcutaneously daily from the time of surgery until the study end point. Group D animals underwent allogeneic heterotopic limb transplantation and received IP administration of 0.5 mg MR1 and 0.5 mg CTLA4-Ig (Bio Express, Inc) on days 0, 3, 6, 9, 12, and 15. Group E animals received syngeneic heterotopic limb transplantation and received 2 mg/kg [2, 3, 6, 7, 10, 18, 36]

Table 1 Animal groups, treatments, and rejection

	Transplantation	Group ID	Animal number (<i>n</i>)	Donor	Recipient	Treatment	Rejection	Days ^d
Group A	Syngeneic	Syn	5	C57Bl/6	C57Bl/6	No	0	
Group B	Allogeneic	Allo	1	Balb/c	C57Bl/6	No	1	5
Group C	Allogeneic	Allo/M+F	6	Balb/c	C57Bl/6	MR1+FK506 ^a	1/6	13
Group D	Allogeneic	Allo/M+C	7	Balb/c	C57Bl/6	MR1+CTLA4Ig ^b	1/7	8
Group E	Syngeneic	Syn/FK Pre	8	C57Bl/6	C57Bl/6	FK506 (2 mg/kg) ^c	0	

^a MR1 (0.5 mg, i.p.) treated at 0, 3, 6, 9, 12, and 15 days after surgery, FK506 (2 mg/kg, s.c. daily) treatment started on the surgery day till the end time point

^b MR1 and CTLA4Ig (both 0.5 mg, i.p.) treated at 0, 3, 6, 9, 12, and 15 days after surgery

^c FK506 (2 mg/kg, s.c. daily) started 3 days before surgery till end time point

^d The days when rejection sign showed after limb transplantation

of FK-506 daily, beginning 3 days before surgery (preload) and continuing until the study end point (Table 1). Three days of FK-506 preloading was chosen based on previous histologic and histomorphometric data showing superior regeneration with preloading over postsurgical administration alone [24]. The end point of 3 weeks was chosen according to our previous published data [1, 35] and with the aim to avoid the “blow-through” effect present in the rodent model (when differences between treatment groups are lost due to the rodent’s innate superlative regeneration capacity) [3]. All mice were killed at the end point of 3 weeks post-transplantation, except for group B, which were killed 8 days postoperatively because of acute rejection.

Surgical Technique

Anesthesia was administered via subcutaneous injection of ketamine (75 mg/kg) and medetomidine (1.0 mg/kg). All heterotopic limb transplantations were performed according to our previously established protocol [28–31] with the modification of sciatic nerve coaptation between the recipient animal and the donor hind limb. Briefly, for the donor surgery, a longitudinal incision was made along the medial aspect of the thigh to expose and mobilize the femoral vessels up to the inguinal ligament. A second longitudinal incision was made along the lateral aspect of the thigh to expose the sciatic nerve, which was neurolyzed and transected 10 mm proximal to its trifurcation. Two additional circumferential skin incisions were made, the first at 2–3 mm proximal to knee joint and the second at the level of ankle joint. The femur was transected with a scalpel 2–3 mm proximal to knee joint, taking care to preserve the caudal tibial vessels in order to maintain satisfactory blood supply to the grafted lower limb. The ankle joint was disarticulated in order to completely mobilize the lower limb. To facilitate inseting the CTA with the skin externalized, a longitudinal skin incision was made on the

lateral aspect of the donor lower leg and skin flaps dissected both anteriorly and posteriorly, while maintaining the blood supply from the medial side [26–28, 31]. The donor limb was then flushed with cold heparinized saline (50 U/mL) via the abdominal aorta, which was exposed through a midline abdominal incision. The femoral vessels were then divided in the groin just distal to the inguinal ligament. The lower limb was wrapped in cold saline-soaked gauze and kept on ice while the recipient animal was prepared.

In the recipient animals, the femoral vessels were exposed and mobilized in the same manner as in the donor animals. Branches of the femoral artery in the proximal thigh were carefully preserved to maintain collateral supply to the native limb after division of the femoral artery. To prepare the recipient sciatic nerve, a longitudinal incision was made along the lateral aspect of the thigh. The sciatic nerve was exposed and mobilized, then transected 5 mm proximal to its trifurcation. Microvascular clamps were used to obtain proximal and distal control of the femoral vessels, and the femoral artery was divided just distal to the lateral caudal femoral branches. The transplant limb was brought into the field. The venous anastomosis was performed first in an end-to-side fashion using 11-0 nylon suture on a 50 µm needle (SharpPoint, Reading, PA, USA). This usually required eight to ten interrupted sutures. The arterial anastomosis was performed second in an end-to-end manner, using four to six interrupted 11-0 nylon sutures. The anastomoses were performed “underwater,” with the vessels submerged in heparinized saline (50 U/mL), as previously described [27], for better filling and visualization of the lumen. For inseting, skin from the ipsilateral flank of the recipient was excised, and a subcutaneous tunnel was made from the groin incision to the flank. The transplanted limb was then passed through this tunnel and delivered through the flank incision, without any tension on the vascular pedicle. The transplant limb was positioned longitudinally with its distal end pointing in the cephalad direction, and the donor skin paddle was inset to

the flank skin using 6-0 nylon suture. Then, a second tunnel was made through the interval between the pectineal and adductor muscles towards the lateral thigh muscles and the sciatic nerve. A single 9-0 nylon suture at the proximal aspect of the donor nerve stump was used to guide the donor sciatic nerve through the tunnel for coaptation with the recipient sciatic nerve. The nerve coaptation was performed between the proximal recipient stump and the donor sciatic nerve using four to five interrupted 11-0 nylon sutures. All surgery was performed under magnification from X6 to X25, as necessary.

Recipient animals received a total of 0.5 ml of normal saline by subcutaneous injection before release of the vascular clamps. After completion of the transplant, animals were allowed to recover on a warming pad for 24 h prior to return to the animal facility. No dressings, immobilization, or systemic anticoagulation was used. The grafted skin paddle was examined daily for evidence of ischemia, necrosis, or signs of rejection.

Histomorphometric Analysis

The entire transplanted limb and recipient/donor sciatic nerve complex were harvested and fixed in cold, buffered 3% (weight/volume) cold glutaraldehyde (Polysciences Inc., Warrington, PA, USA) solution for 24 h, then postfixed in 1% osmium tetroxide, dehydrated in graded ethanol solutions [15] and embedded in Araldite 502 (Polysciences, Inc., Warrington, PA, USA). One-micron-thick nerve cross-sections were obtained 3 mm distal to the nerve repair site using an LKB III Ultramicrotome (LKB-Produkter AB, Bromma, Sweden), stained with toluidine blue, and evaluated under light microscopy for neural architectural characteristics. The 3 mm evaluation distance distally was chosen based on the short-end point of 3 weeks. An observer blinded to the experimental groups obtained all measurements. Further analyses of microscopic images were performed with an automated digital image analysis system linked to histomorphometry software [13] to quantify the total nerve fiber count (total nerve number), fiber density, and percentage neural tissue. Eight to ten randomly selected fields under $\times 1,000$ magnification were analyzed per nerve.

Statistical Analysis

The mean $\pm$ SD is represented in all data in this study. A two-tailed analysis of variance was performed to determine the differences within groups. Histomorphometric calculations were performed using Statistica statistical software (StatSoft, Inc.). A Student–Newman–Keuls test was then performed to compare groups. Statistical significance was established at $p < 0.05$.

Results

Surgical Case Length and Postoperative Hindlimb Function

Donor limb harvest required approximately 1.5 h. Preparation of the recipient site and transplantation of the donor limb, including microvascular surgery and inseting, required approximately 2–2.5 h. Vascular clamp time was approximately 60 min. Flaccid paralysis below the level of the knee was present in the ipsilateral hindlimb due to sacrifice of the sciatic nerve. However, all animals maintained function of the proximal ipsilateral hindlimb and were therefore able to ambulate after surgery. No signs of ischemia were present in the ipsilateral hindlimb. Autotomy was not observed.

Animal survival, as well as transplant survival, improved after an initial learning curve. Animal surgeries were performed sequentially, beginning with group A and ending with group E. Of the ten mice in group A who underwent heterotopic hind limb transplantation, one died on postoperative day 2 and another died on postoperative day 19. Three additional mice were killed due to failed vascular anastomoses distinguished by postoperative day 3. The overall success rate in group A was therefore 50% (5/10). Without immunosuppression, the allogeneic transplant in group B was expected to undergo early rejection; transplant survival analysis therefore did not apply. Of the ten mice in group C, three died between postoperative days 3–5 and one was killed at 4 days postoperatively because of surgical site infection; the overall success rate was 60% (6/10). Of the eight mice in group D, one mouse was killed on postoperative day 3 due to a failed vascular anastomosis; the overall success rate was 87.5% (7/8). Of the ten mice in group E, two mice died within 2 days postoperatively. No failed anastomoses occurred in this group. The overall success rate was 80% (8/10). For the entire study, the success rate was 69.3%.

Limb Transplant Survival and Rejection

All transplanted limbs experienced some edema, which generally resolved within the first postoperative week. Groups A and E, which had received syngeneic transplants (nerve isograft equivalent), showed no signs of rejection (Fig. 1a). Group B, which had received allogeneic transplants (nerve allograft equivalent) without immunosuppressant treatment experienced rejection (erythema to eschar formation) as early as post-operative day 5 (Fig. 1b). In group C, which had received allogeneic transplants with MR1+FK-506 treatment, one of six mice showed donor skin eschar formation and necrosis at the grafted skin edge by postoperative day 13. In group D, which had received allogeneic transplants and MR1 plus CTLA4Ig treatment, one of seven mice developed donor skin eschar formation 8 days postopera-

Fig. 1 Survival and rejection of transplanted limbs. Photos were taken at the time of sacrifice.

Right side panels show magnified images of the grafted areas. **a** Group A (syngeneic transplant, no immunosuppression) mouse at the 3-week endpoint. **b** Group B (allogeneic transplant, no immunosuppression) mouse at 8 days. An early end point was chosen due to frank rejection of the transplant. **c** Representative image of a group C and/or D (allogeneic transplant+immunosuppression) mouse at the 3-week endpoint. At 3 weeks post-transplantation, new hairs are beginning to grow up in grafted limb (**a** and **c**)

A, Syngeneic Group

B, Allogeneic Group without treatment

C, Allogeneic Group with treatment

tively following an acute dusky discoloration of the grafted skin. Correspondingly, the grafted sciatic nerve in these two mice showed little (group D mouse) to no (group C mouse) nerve regeneration (Table 1). Except for these two mice, all other mice receiving syngeneic transplants or allogeneic transplants with immunosuppression showed new hair growth in the grafted limb by 3 weeks postoperatively (Fig. 1a and c). In these mice, histologic specimens

demonstrated normal viable tissue in the grafted limb (Fig. 2).

Histology

Nerve sections from distal to the sciatic coaptation site were analyzed in all groups (Fig. 3). Group A (syngeneic transplant) mice showed normal myelinated nerve architecture with robust nerve regeneration (Fig. 3a). Group B mice (allogeneic transplant, no immunosuppression) demonstrated an absence of regeneration of myelinated fibers. Only Wallerian degeneration with nonviable fibers was seen at an early time frame and myelin debris at a later time frame. Lymphocytic cellular infiltrate was seen within the endoneurium (Fig. 3b). Except for the two mice that developed rejection, group C and D mice (allogeneic transplant, C—MR1+FK-506, D—MR1+CTLA4Ig), showed increased nerve regeneration compared to group B. Regenerating small myelinated fibers were seen simultaneous with ongoing Wallerian degeneration of pre-existing myelinated fibers, without any cellular infiltrate (Fig. 3c, d). Group E (syngeneic transplant, FK-506 with preloading) showed robust nerve regeneration similar to that of group A (Fig. 3e).

Fig. 2 The viable grafted tissue. Histologic specimens demonstrated viable femoral bone and surrounding tissues

Fig. 3 Histograms for nerve fibers. **a** Group A (positive control—syngeneic transplant, no immunosuppression) with normal myelinated nerve architecture at 3 weeks post-operation. Robust regeneration of myelinated fibers is shown. **b** Group B (negative control—allogeneic transplant, no immunosuppression) showing an absence of regeneration of myelinated fibers. The time frame of Wallerian degeneration is shown with nonviable fibers at early time frame and myelin debris at later time frame. Cellular infiltrate is seen in endoneurium. **c** Group C

(allogeneic transplant with MR-1+FK506 treatment) demonstrating increase nerve regeneration compared to group B. Regenerating small myelinated fibers are present along with ongoing Wallerian degeneration of previously existing myelinated fibers. Cellular infiltrate is absent. **d** Group D (allogeneic transplant with MR-1+CTLA4Ig treatment) with nerve regeneration similar to group C. **e** Group E (syngeneic transplant with FK506+preload) showing similar robust nerve regeneration as group A

Histomorphometry

Group B (allogeneic transplant, no immunosuppression) showed no viable axons in the transplanted sciatic nerves (Figs. 3b and 4). All other groups showed moderate to good nerve regeneration based on total nerve number, fiber density, and percent nerve tissue. Group E (syngeneic transplant, FK-506 with preloading) showed excellent regeneration and a trend towards surpassing group A (syngeneic transplant, no immunosuppression) in total nerve number ($2,046 \pm 841.55$ vs. $1,619.77 \pm 325.96$), fiber density (fibers/mm², $14,188.84 \pm 6,480.50$ vs. $11,913.76 \pm 2,975.05$), and percent nerve tissue (% nerve, 11.90 ± 5.83 vs. 8.97 ± 2.27), although it did not achieve statistical significance (Fig. 4). These results were consistent with our previously published data which showed FK-506 treatment and preload to be effective for promoting nerve regeneration [6, 7, 9, 14, 18, 24, 32]. Group D (allogeneic transplant, MR1+CTLA4Ig) showed very good nerve regeneration that was not significantly different from group A ($1,952.88 \pm 1,318.15$ vs. $1,619.77 \pm 325.96$ for total nerve number; $9,669.77 \pm 5,804.41$ vs. $11,913.76 \pm 2,975.05$ for fiber density; and 9.69 ± 6.83 vs. 8.97 ± 2.27 for percent nerve tissue). Group C (allogeneic transplant, MR1+FK-506), showed lower values across all three parameters compared with group A ($1,254.86 \pm 1,077.58$ vs. $1,619.77 \pm 325.96$ for total nerve number; $10,807.03 \pm 7,153.10$ vs.

$11,913.76 \pm 2,975.05$ for fiber density; and 7.39 ± 6.50 vs. 8.97 ± 2.27 for percent nerve tissue), but this difference was not statistically significant. Additionally, nerve fiber widths were analyzed, and except for group B animals, there was no difference among the groups (data not shown). Size histograms for nerve fibers from all groups are shown in Fig. 5; there were no significant differences between groups.

Discussion

The mouse remains a challenging model for CTA transplantation, demanding a high level of microsurgical skill for dissection and anastomosis of its small caliber vessels. The mouse is also a physiologically fragile animal, with little room for blood loss given its approximate circulatory volume of 1.4–2 ml. As a result, large procedures such as limb transplantation are often accompanied by problems with high mortality and morbidity. The availability of and relative ease in creating transgenic and knockout murine strains makes the mouse a desirable model, however, compared to the rat. For nerve regeneration research in particular, existing transgenic murine strains include an *S100-green fluorescent protein (GFP)* strain whose Schwann cells (SCs) express GFP under control of the SC-specific S100 promoter. These have been used to dynamically quantify host SC migration into nerve allografts [34] and could similarly be used to study host SC

Fig. 4 Histomorphometric analysis in the study. **a** Total nerve number, **b** fiber density (fibers/mm²), and **c** percent nerve (%) are shown. Error bars represent standard deviation

migration into the nerves of a CTA. A *thy1-cyan fluorescent protein (CFP)* murine strain exists whose axons express CFP. Our lab has previously combined these two strains to create a

Fig. 5 Size histograms for nerve fibers. No significant differences were detected in comparisons of fiber distributions between groups. All nerve fragment groups demonstrated a similar fiber distribution profile

double-transgenic *thy1-CFP/S100-GFP* mouse which allowed for serial assessment of the SC-axonal relationship during regeneration via serial live imaging [11]. The ability to utilize such transgenic lines as the above to study nerve regeneration in CTA would be welcome. In this study, we present the first murine model of nerve regeneration in a hind limb CTA, and we hope that this model will facilitate the application of the greater genetic resources available in the mouse to the field of CTA.

We first described the use of this heterotopic hind limb transplantation model in 2001, performed at that time without the sciatic nerve coaptation, for analysis of CTA rejection under various immunosuppressive regimens [26, 27]. Orthotopic limb transplantation in mice has been previously described as well, first by Zhang et al. [37]. Orthotopic limb transplantation is more commonly performed in a rat model, however, due to issues with size and physiologic fragility in the mouse. In the mouse, we feel that a heterotopic model has multiple advantages over an orthotopic model. First, heterotopic transplantation is associated with significantly less morbidity, allowing the mouse to keep its hind limbs. Not only does this allow for ambulation immediately after surgery, but it also reduces surgical blood loss and decreases the physiologic stress of the surgery. Secondly, the end-to-side venous anastomosis of the extremely thin and delicate mouse femoral vein is technically easier and faster to perform than an end-to-end anastomosis. This easier venous anastomosis, as well as the less complicated inset of a heterotopic limb compared to an orthotopic limb, leads to shorter surgical times. The end-to-side technique can also shorten the ischemia time of the donor tissue, which may significantly affect subsequent functional recovery. Finally, the subcutaneous position of the graft, which is not possible with orthotopic placement, prevents autotomy during the period of reinnervation.

We used this new mouse heterotopic CTA model to test the effects on CTA nerve regeneration of various immunosuppressive regimens, which had been previously studied in rodent nerve allograft models. Prior experimental research, as well as clinical experience, has demonstrated the ability of FK-506 to improve nerve regeneration across many different models of nerve injury and repair [6, 7, 14, 18, 21, 25, 32].

Preloading with FK-506, beginning 3 days preoperatively, further improves its ability to enhance nerve regeneration and functional recovery [24]. In this study, we similarly found that FK-506 treatment with preloading in group E trended toward better nerve regeneration compared to no treatment in group A (Fig. 4), even though the difference did not quite reach statistical significance.

In previous studies, we had demonstrated that CSB prolonged limb allograft survival, even though tolerance was not achieved [28, 29, 31]. In this study, CSB with the combination of MR1 and CTLA4Ig treatment in group D provided effective immunosuppression and permitted axonal regeneration that was equivalent to the group A positive control (Fig. 4). Treatment with both MR1 and FK-506 in group C, however, trended toward impaired axonal regeneration (Fig. 4), although this did not reach statistical significance. Poorer regeneration in this group might suggest abrogation of both the ability of CSB to permit regeneration and FK-506 to enhance regeneration. This phenomenon, in which dual immunosuppressive therapy impairs nerve regeneration, has been previously described [2]. The results of this study were ultimately consistent with our previous non-CTA data [2, 6, 7, 9, 14, 18, 24, 32] that showed immunosuppressant treatment, FK-506 or CSB, was able to promote nerve regeneration after nerve injury.

We acknowledge limitations to our study. The poor nerve regeneration seen in the two mice from groups C and D increased the standard deviations for their respective groups, making it more difficult to distinguish differences between groups. We speculate that rejection of donor Schwann cells from the CTA nerve, despite immunosuppression, may have contributed to the poor nerve regeneration. Schwann cells play crucial role in nerve regeneration after injury but also are the strongest antigenic component in nerve tissue. In the two mice with poor nerve regeneration from the C and D groups, rejection signs were observed in the grafted tissue postoperatively, likely depleting the number of Schwann cells available to support regeneration. Although we chose the number of animals for each group based on an a priori power calculation, a greater number of animals per group may have proven or disproven what appear to be trends. Finally, we acknowledge that this was a short-term study of only 3 weeks duration post-transplantation. Treatment with CSB has been previously shown to prevent acute rejection and prolong limb allograft survival, but not to prevent chronic rejection [28, 29, 31]. A longer follow-up period would therefore be necessary to evaluate the long-term ability of CSB to support nerve regeneration and donor SC survival in CTA. In the future, we plan to perform long-term studies using this model. Specifically, we plan to use the *S100-GFP/thy1-YFP* double-transgenic mice [11] to study the relationship

between Schwann cell migration and axonal regeneration in the CTA. Additionally, reinnervation of motor targets will be evaluated. The axons of Thy1-YFP mice, which are commercially available, can be reliably visualized at the neuromuscular junction with fluorescent microscopy. Counterstaining of the muscular component of the neuromuscular junction can be performed with α -bungarotoxin. By visualizing the overlap of the axonal fluorescence and the α -bungarotoxin stain, the extent of neuromuscular junction reinnervation can be quantitatively assessed. This allows for assessment of muscle end-organ reinnervation outside of the usual functional analyses such as walking track or muscle force.

Conclusion

The mouse heterotopic limb transplant model is a useful tool for CTA research since it can be performed with more ease, in less time, and with less host morbidity and mortality than the orthotopic model. The ability to reliably perform CTA surgery in the mouse will allow investigators to take advantage of its more extensive genetic database, including all of the knockout and transgenic strains. Preliminary study of nerve regeneration in this CTA model has been consistent with previous data from nerve allograft studies, demonstrating the ability of FK-506 and CSB immunosuppressant treatments to support nerve regeneration.

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