Published in final edited form as:

J Neurosurg. 2010 February ; 112(2): 386–393. doi:10.3171/2009.7.JNS09187.

The role of T helper cell differentiation in promoting nerve allograft survival with costimulation blockade

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Abstract

Object—Peripheral nerve allografts provide a temporary scaffold for host nerve regeneration and allow for the repair of significant segmental nerve injuries. Despite this potential, nerve allograft transplantation requires temporary systemic immunosuppression. Characterization of the immunological mechanisms involved in the induction of immune hyporesponsiveness to prevent nerve allograft rejection will help provide a basis for optimizing immunomodulation regimens or manipulating donor nerve allografts to minimize or eliminate the need for global immunosuppression.

Methods—The authors used C57Bl/6 mice and *STAT4* and *STAT6* gene BALB/c knockout mice. A nonvascularized nerve allograft was used to reconstruct a 1-cm sciatic nerve gap in the murine model. A triple costimulatory blockade of the CD40, CD28/B7, and inducible costimulatory (ICOS) pathways was used. Quantitative assessment was performed at 3 weeks with nerve histomorphometry, walking track analysis, and the enzyme-linked immunospot assay.

Results—The *STAT6* ^{−/−} mice received 3 doses of costimulation-blocking antibodies and had axonal regeneration equivalent to nerve isografts, while treated *STAT4* ^{−/−} mice demonstrated moderate axonal regeneration but inferior to the T helper cell Type 2-deficient animals. Enzyme-linked immunospot assay analysis demonstrated a minimal immune response in both *STAT4* ^{−/−} and *STAT6* ^{−/−} mice treated with a costimulatory blockade.

Conclusions—The authors' findings suggest that Type 1 T helper cells may play a more significant role in costimulatory blockade-induced immune hyporesponsiveness in the nerve allograft model, and that Type 2 T helper differentiation may represent a potential target for directed immunosuppression.

Keywords

allograft; peripheral nerve; immunosuppression; costimulatory blockade

The repair of significant segmental nerve injuries is often limited by the amount of expendable autogenous donor supply. The limited availability of donor autografts can be circumvented by the use of cadaveric peripheral nerve sources. Peripheral nerve allografts provide a temporary

scaffold for host SC migration and nerve regeneration, but require temporary global attenuation of the immune system. Host immunosuppression is required until host SCs have replaced donor SCs and regeneration through the nerve allograft is complete.^{5,7,19,25,35,38,39,43} Donor SCs behave as facultative APCs and represent the primary target of host allograft rejection.^{15,16,35,38,49,57,63} Allograft pretreatment with cold preservation has been shown to diminish SC expression of major histocompatibility complex Class II alloantigens and decrease the required dose of systemic immunosuppression.^{9,10,13,52}

Costimulatory blockade has shown promise for the induction of donor-specific immune hyporesponsiveness with significantly less morbidity than conventional nonspecific immunosuppression. Costimulatory blockade induces a transient period of donor immune unresponsiveness by blocking the activation and expansion of T cells. Although the importance of both the direct and indirect pathway in allograft rejection is well understood, the relative contribution of each pathway and the role of the T cell subsets in mediating nerve allograft rejection remain unclear. Costimulatory signals regulate the expansion of alloreactive T cells and are necessary to initiate a full immune response.^{2,56} The effect of costimulatory blockade on T helper cells in peripheral nerve allotransplantation is poorly understood, and may be related to changes in antigen presentation and T-cell differentiation and activation.

There are at least 2 functionally distinct subsets of CD4⁺ T helper cells (Th1 and Th2); the proliferation and differentiation of these subsets is mediated through cytokines and *STAT* genes. The *STAT4* and *STAT6* genes are activated primarily by IL-12 and IL-4, causing a deviation toward a Th1 or Th2 response, respectively (Fig. 1A). Significant work in knockout mice has suggested that *STAT6*^{-/-} mice preferentially develop CD4⁺ Th1 cells (Fig. 1B) because of deficient production and responsiveness to cytokine IL-4.^{25,28,36,48,55,59} This preferential development of Th1 cells leads to increased resistance to certain tumor types,^{26,45,46,54} as well as a more robust rejection in graft versus host disease models.⁴¹ Because of a disrupted response to IL-12, *STAT4*^{-/-} mice preferentially develop CD4⁺ Th2 cells (Fig. 1C).^{27,29,55} An impaired IL-12 response in *STAT4*^{-/-} mice leads to disrupted IFN- γ production, altered IL-12-induced lymphocyte proliferation, and disrupted IL-12-enhanced natural killer cell activity.²⁷

In the present study, we used *STAT4* and *STAT6* knockout mice to characterize the role of T-cell phenotype in nerve allograft survival with costimulatory blockade. We hypothesized that costimulatory blockade may be dependent on immune deviation of the T helper cytokine profile for the induction of immune hyporesponsiveness and nerve allograft survival.

Methods

Animal Preparation and Care

Male *STAT4*^{-/-} and *STAT6*^{-/-} knockout mice with a BALB/c background and C57Bl/6 (B6) mice were obtained from Jackson Laboratory. The animals were housed in a central animal care facility with access to water and standard rodent feed ad libitum. All housing, care, and surgical procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the specific protocol met with the approval of the Washington University Animal Studies Committee.

Blocking Agents

All costimulatory blocking agents were obtained from BioXcell. Hamster MR-1, which blocks CD40–CD40 L interaction, human CTLA-4-Ig, which blocks the B7-CD28 T-cell costimulation pathway, and anti-ICOSL, which blocks ICOS–ICOSL interaction (Fig. 2).

Animals treated with costimulatory blockade received therapy on postoperative Days 0, 2, and 4.

Surgical Procedure

As detailed in Table 1, *STAT4*^{-/-} and *STAT6*^{-/-} mice were used as both donors and recipients with a fully allogeneic WT C57Bl/6 (B6) recipient or donor. A non-vascularized, 1-cm sciatic nerve allograft was harvested from the donor and transplanted in reverse orientation into the recipient. Wild-type controls with C57Bl/10 (B10) and C57Bl/6 (B6) mice were used as donors and recipients, respectively, in isograft and allograft control groups (Groups I and II). In Group III, C57Bl/10 (B10) mice were recipients of WT C57Bl/6 donor nerve allografts. This group received costimulatory blockades on Days 0, 2, and 4. In Group IV, *STAT6*^{-/-} mice were recipients of WT C57Bl/6 donor nerve allografts, in Group V, *STAT4*^{-/-} mice were recipients of WT C57Bl/6 donor nerve allografts, and in Group VI *STAT6*^{-/-} mice received WT C57Bl/6 nerve allografts while receiving co-stimulatory blockades (MR1, CTLA4-Ig, and anti-ICOS) on Days 0, 2 and 4. Similarly, in Group VII, *STAT4*^{-/-} mice were the recipients of WT C57Bl/6 nerve allografts, and received costimulatory blockades (MR1, CTLA4-Ig, and anti-ICOS) on Days 0, 2 and 4.

Histomorphometrical Analysis

Sciatic nerve segments were harvested en bloc. Labeled nerve specimens from distinct regions along the graft reconstruction and proximal and distal to the graft were preserved in glutaraldehyde, postfixed in osmium tetroxide, and embedded in Araldite 502, and 1- μ m cross sections were taken with an LKB III Ultramicrotome. Nerves were qualitatively assessed for the preservation of nerve architecture, quality and quantity of regenerated nerve fibers, extent of myelination, and the presence of ongoing Wallerian degeneration. The system used a series of algorithms to distinguish axons, myelin, nerve sheath, and debris from each other, and was double-checked by a histomorphometrist.²² Eight-bitplane digital pseudocoloring and custom algorithms were used to distinguish axons, myelin, and debris from one another. Processed cross sections were digitized and assessed for total fascicular area and total number of fibers.

In this study, all data are presented as means $\pm$ SDs. A 2-tailed ANOVA was used to determine the differences between individual groups. Histomorphometric calculations were performed using commercially available software (Statistica; StatSoft, Inc.). If significant, a Student-Newman-Keuls test was performed to compare groups. Statistical significance was established at $p < 0.05$.

Walking Track Analysis

All animals were walked at weekly intervals, (1, 2, and 3 weeks) to generate an SFI. Paired measures of the print length and toe spread (first to fifth toe) were recorded for the normal (control) foot and the corresponding experimental foot. The elongation and subsequent normalization of EPL have been shown to correlate with tibial nerve injury and recovery in both mice and rats.^{11,17,18,24,62} The NPL is compared with the injured side (the EPL) and a print length factor is derived according to the following formula: $(EPL - NPL)/NPL$. The SFI utilizes measurements of toe spread and print length, and relates the overall toe spread of the experimental (ETS and EPL) versus the normal (NTS and NPL) sides, and is calculated as $SFI = 118.9 [(ETS - NTS)/NTS] - 51.2 [(EPL - NPL)/NPL] - 7.5$.^{11,17,18,24,62}

Enzyme-Linked Immunospot Assay

Donor and recipient spleens were harvested for splenocytes prior to planned death. Splenocytes were added to multiscreen 96-well filtration plates and coated with capture antibodies to IFN- γ . Cells and antibodies were incubated in quadruplicate for 3 days. The cells were then

aspirated, the detection antibody added, and incubation proceeded for an additional 24 hours. Substrate avidin-horseradish peroxidase was added and the plates were developed for spots. The plates air-dried overnight before image analysis was performed with a Series 3 ImmunoSpot Analyzer (Cellular Technology) specifically designed for automated evaluation of ELISPOT results.³

Results

Axonal Regeneration and Polarity in T Helper Cell Differentiation

It is generally accepted that rejection in allotransplantation is dependent on both Th1 and Th2 responses. The classic paradigm suggests that the Th1 phenotype plays a predominant role in mediating acute rejection, whereas a Th2 profile is associated with allogenic tolerance. This is supported by several studies that have linked a Th1 cytokine profile with rejection, while demonstrating that the presence of Th2 cytokines was associated with tolerance.^{39,57} As expected, we found that Group V mice, which received fully allogenic donor nerve grafts, demonstrated poor histomorphometric evidence of distal graft axonal regeneration (Figs. 3 and 4A), similar to that observed in Group II, the control allograft group (90 ± 120 and 183 ± 133 total distal fibers, respectively; Fig. 5) consistent with acute rejection. Similarly, Group IV mice, which received fully allogenic nerve allografts, demonstrated marginally increased (but not significantly greater [$p < 0.09$]) axonal regeneration (248 ± 234 distal fibers) than that seen in the untreated *STAT6*^{-/-} group. The findings suggest that redundancy in the immune system permits acute nerve allograft rejection despite the lack of either Th1 or Th2 differentiation.

Role of STAT4 in Costimulatory Blockade–Induced Immune Hyporesponsiveness

As demonstrated in Fig. 3, *STAT4*^{-/-} and *STAT6*^{-/-} mice treated with triple costimulatory blockade (Groups VI and VII) demonstrated markedly improved histomorphometric axonal regeneration and improved functional recovery as measured by walking track analysis (with the SFI) (Fig. 6). The *STAT6*^{-/-} mice treated with co-stimulatory blockade demonstrated neural regeneration equivalent to that of Group I, the isograft control (1412 ± 482 and 1570 ± 439 fibers, respectively; Fig. 5), yet significantly better than in *STAT4*^{-/-} mice treated with costimulatory blockade. These findings suggest that the presence of an inducible Th1 phenotype (STAT6 pathway) may be required for triple costimulatory blockade–induced hyporesponsiveness in this model.

Moderate Immune Response, IFN- γ Suppression, and Axonal Regeneration

The ELISPOT results are summarized in Fig. 7. Allograft controls demonstrated a robust immune response; IFN- γ production was 134 ± 30 spots/million cells, in contrast to isograft controls, which generated 15 ± 7 spots/million cells. In general, Th1 cells produce IL-2 and IFN- γ , while Th2 cells secrete IL-4, IL-5, IL-10, and IL-13.²⁷ The *STAT4*^{-/-} mice are known to generate IFN- γ in an IL-12–independent manner,³⁰ yet interestingly, the untreated *STAT4*^{-/-} group receiving fully allogenic nerve allografts generated a stronger immune response (104 ± 33 spots/million cells) than the untreated *STAT6*^{-/-} group, which generated only 68 ± 23 spots/million cells. Notably, both the *STAT4*^{-/-} and *STAT6*^{-/-} groups treated with costimulatory blockade demonstrated a blunted immune response (Fig. 7), and both of these responses were significantly lower than the response seen in the allograft and untreated control mice ($p < 0.05$). However, only *STAT6*^{-/-} mice demonstrated neural regeneration comparable to isograft controls (1412 ± 482 vs 1570 ± 439 fibers). Our findings suggest that even in the setting of moderate rejection, neural regeneration can be dramatically disrupted.

Discussion

The allotransplantation of nerve tissue represents a unique challenge, and in contrast to solid organ or reconstructive transplants, requires only transient immune suppression. The mechanism of costimulatory blockade–induced immune hyporesponsiveness in nerve transplantation has not been fully elucidated. Our results support the work done in cytokine knockout mice and call into question the Th1/Th2 classic paradigm of rejection.⁶⁴ Our findings suggest that a Th1 phenotype plays a predominant role in costimulatory blockade–induced immune hyporesponsiveness, while Th2 polarity may be more important in the alloimmune response to the nerve allograft.

The activation of the immune response requires 2 distinct signals: the interaction of the T-cell receptor (first signal) and costimulatory molecules. Costimulatory molecules of APCs are surface molecules that bind to specific receptors on T cells. These costimulatory signals provide the mitogenic signals necessary for subsequent T-cell activation, clonal expansion, and maintenance of mature T cells. It is known that the use of the CD40 L blockade induces an immune hyporesponsiveness^{4,44} from enhanced apoptosis of antigen-reactive T cells.^{12,36,60} Although several animal studies have demonstrated allograft acceptance with the CD40 L blockade,^{32,59,65} the induced hyporesponsiveness caused by this blockade appears to be transient, and in several studies has failed to prevent chronic rejection.^{8,14} The mechanism of action for CTLA4-Ig is the binding of CD80 and CD86, reducing IL-2 production, and inhibiting T-cell activation.¹ The CTLA4-Ig blockade of CD28-B7 has been shown to block both the Th1^{31,33,50} and Th2 responses.^{20,33,48} Kishimoto et al.³³ treated *STAT4*–/– and *STAT6* –/– mice receiving fully allogeneic cardiac allografts with a single injection of CTLA4-Ig on posttransplant Day 2. Treatment of both groups resulted in long-term graft survival and the induction of donor-specific tolerance. However, the authors of multiple studies have demonstrated that T-cell activation and allograft rejection can occur in the absence of both CD40 and CD28 signals.^{37,40,61} The inducible costimulatory (ICOS) molecule is expressed on activated T cells,²³ is constitutively expressed on APCs, and regulates both Th1 and Th2 cell differentiation.^{6,42,47} We recently demonstrated the synergistic potential of adding anti-ICOS to the CD40 and CD28/B7 blockade (triple costimulatory blockade). We found that the mice that received the triple costimulatory blockade had significantly more nerve regeneration when compared with blockade of CD40 and CD28/B7, or of either agent alone.⁵³

In the present study we found that the induction of a triple costimulatory blockade–induced immune hyporesponsiveness may require an intact *STAT4* pathway for the proper differentiation of a Th1 phenotype and production of a Th1 cytokine profile. The authors of recent studies have suggested a critical importance of IFN- γ expression for induction of tolerance. In their study, Konieczny and colleagues³⁴ compared the survival of murine skin and cardiac allografts in WT and *IFN- γ* –/– mice treated with a costimulatory blockade (B7-CD28 and CD40–CD40 L). They found that induction of long-term allograft survival by costimulatory blockade required some degree of IFN- γ expression. Similar findings obtained by Hassan et al.²¹ using a CD28/B7 blockade alone in a murine cardiac allograft model also suggest that IFN- γ may play a critical role in the induction of tolerance. These studies suggest that costimulatory blockade–induced tolerance requires at least a partial Th1 response (IFN- γ production) or an intact *STAT4* pathway. Our data obtained when the *STAT4* and *STAT6* mice received triple costimulatory blockade corroborates these findings and suggests the critical importance of the *STAT4* pathway for tolerance induction. In addition, because *STAT6*-deficient mice are unable to upregulate MHC Class II molecules,^{28,51} the manner of antigen presentation may provide an additional explanation for our findings of improved allograft tolerance and significantly better nerve regeneration in treated *STAT6*–/– animals (Group VII).

Data from ELISPOT analysis revealed that both untreated *STAT4*^{-/-} and *STAT6*^{-/-} groups generated moderate immune responses (104 ±33 and 68 ±23 spots/million cells, respectively) compared with allograft controls (134 ±30 spots/million cells). Our previous experience with FK-506, costimulatory blockade, and the current results suggest that both moderate and high cytokine responses can generate a histological phenotype of nerve rejection, underscoring an interesting discrepancy between the ELISPOT and histomorphometric data with regard to immunosuppressive effect. Based on IFN- γ production, a significant reduction in the host immune response was readily seen in both the treated *STAT4*^{-/-} and *STAT6*^{-/-} groups, appearing to provide equivalent immunosuppression with minimal response seen in in vitro cultures. However, the histomorphometric data of axonal regeneration through the nerve allograft demonstrates a much greater difference between the regimens, with the *STAT4*^{-/-} group achieving only half as many regenerating axons as the *STAT6*^{-/-}. There are 2 potential explanations for these findings. The first is that histomorphometric analysis of axonal regeneration is simply a more sensitive indicator of the magnitude of the immune response than in vitro cytokine production in response to donor antigen. We have previously demonstrated that the cytokine profile of the immune response to nerve tissue is similar to that of skin with predominantly Th1 activation, in contrast to that of muscle and bone, which show a Th2 immune deviation.⁵⁸ Although the quantitative ELISPOT assay accurately reflects the status of the immune response, nerve tissue appears to be much more antigenic than previously believed, and may require more profound immunosuppression (similar to skin) for satisfactory regeneration and function. Both tissue types share an abundance of an immunologically active cell population. In particular, the Langerhans cells of the skin and the SCs in the nerves both act as APCs which facilitate the immune response. The second explanation is that the *STAT4* pathway may have an unidentified role in nerve regeneration that is independent of its role in immune regulation. As such, although costimulatory blockade may be equally immunosuppressive to the acute response in both *STAT4*^{-/-} and *STAT6*^{-/-} hosts, the *STAT6*^{-/-} animals may have an advantage in terms of neuroregenerative ability.

Conclusions

Our findings demonstrate the importance of an intact Th1 pathway for triple costimulatory blockade-induced immune hyporesponsiveness. Moreover, it appears that both a moderate and high cytokine response can produce a histological phenotype of nerve rejection. In addition, peripheral nerve allograft survival may also require either an intact *STAT4* pathway or some level of IFN- γ expression.

Abbreviations used in this paper

APC	antigen-presenting cell
ELISPOT	enzyme-linked immunospot assay
EPL	experimental print length
ETS	experimental toe spread
ICOS	inducible costimulatory
NPL	normal print length
NTS	normal toe spread
SC	Schwann cell
SFI	sciatic functional index
Th1	T helper cell Type 1

Th2 T helper cell Type 2
WT wild-type

Acknowledgments

Disclosure

This work was supported by NIH Grant 2R01NS033406-13A1 to Dr. Mackinnon.

References

1. Alegre ML, Frauwirth KA, Thompson CB. T-cell regulation by CD28 and CTLA-4. *Nat Rev Immunol* 2001;1:220–228. [PubMed: 11905831]
2. Bluestone JA. New perspectives of CD28-B7-mediated T cell costimulation. *Immunity* 1995;2:555–559. [PubMed: 7540940]
3. 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]
4. Brown DL, Bishop DK, Wood SY, Cederna PS. Short-term anti-CD40 ligand costimulatory blockade induces tolerance to peripheral nerve allografts, resulting in improved skeletal muscle function. *Plast Reconstr Surg* 2006;117:2250–2258. [PubMed: 16772925]
5. Buttemeyer R, Rao U, Jones NF. Peripheral nerve allograft transplantation with FK506: functional, histological, and immunological results before and after discontinuation of immunosuppression. *Ann Plast Surg* 1995;35:396–401. [PubMed: 8585683]
6. Dong C, Juedes AE, Temann UA, Shresta S, Allison JP, Ruddle NH, et al. ICOS co-stimulatory receptor is essential for T-cell activation and function. *Nature* 2001;409:97–101. [PubMed: 11343121]
7. Doolabh VB, Mackinnon SE. FK506 accelerates functional recovery following nerve grafting in a rat model. *Plast Reconstr Surg* 1999;103:1928–1936. [PubMed: 10359255]
8. Ensminger SM, Witzke O, Spriewald BM, Morrison K, Morris PJ, Rose ML, et al. CD8+ T cells contribute to the development of transplant arteriosclerosis despite CD154 blockade. *Transplantation* 2000;69:2609–2612. [PubMed: 10910284]
9. Evans PJ, Mackinnon SE, Levi AD, Wade JA, Hunter DA, Nakao Y, et al. Cold preserved nerve allografts: changes in basement membrane, viability, immunogenicity, and regeneration. *Muscle Nerve* 1998;21:1507–1522. [PubMed: 9771677]
10. Evans PJ, MacKinnon SE, Midha R, Wade JA, Hunter DA, Nakao Y, et al. Regeneration across cold preserved peripheral nerve allografts. *Microsurgery* 1999;19:115–127. [PubMed: 10231120]
11. George LT, Myckatyn TM, Jensen JN, Hunter DA, Mackinnon SE. Functional recovery and histomorphometric assessment following tibial nerve injury in the mouse. *J Reconstr Microsurg* 2003;19:41–48. [PubMed: 12582966]
12. Graca L, Honey K, Adams E, Cobbold SP, Waldmann H. Cutting edge: anti-CD154 therapeutic antibodies induce infectious transplantation tolerance. *J Immunol* 2000;165:4783–4786. [PubMed: 11045999]
13. 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]
14. Guillot C, Guillonnet C, Mathieu P, Gerdes CA, Menoret S, Braudeau C, et al. Prolonged blockade of CD40-CD40 ligand interactions by gene transfer of CD40Ig results in long-term heart allograft survival and donor-specific hyporesponsiveness, but does not prevent chronic rejection. *J Immunol* 2002;168:1600–1609. [PubMed: 11823487]
15. Gulati AK. Immune response and neurotrophic factor interactions in peripheral nerve transplants. *Acta Haematol* 1998;99:171–174. [PubMed: 9587399]

16. Gulati AK, Cole GP. Nerve graft immunogenicity as a factor determining axonal regeneration in the rat. *J Neurosurg* 1990;72:114–122. [PubMed: 2294170]
17. Hare GM, Evans PJ, Mackinnon SE, Best TJ, Bain JR, Szalai JP, et al. Walking track analysis: a long-term assessment of peripheral nerve recovery. *Plast Reconstr Surg* 1992;89:251–258. [PubMed: 1732892]
18. Hare GM, Evans PJ, Mackinnon SE, Best TJ, Midha R, Szalai JP, et al. Walking track analysis: utilization of individual footprint parameters. *Ann Plast Surg* 1993;30:147–153. [PubMed: 8489179]
19. Hare GM, Mackinnon SE, Midha R, Wong PY, Au B, Munro C, et al. Cyclosporine A inhibits lymphocyte migration into ovine peripheral nerve allografts. *Microsurgery* 1996;17:697–705. [PubMed: 9588715]
20. Harris NL, Peach RJ, Ronchese F. CTLA4-Ig inhibits optimal T helper 2 cell development but not protective immunity or memory response to *Nippostrongylus brasiliensis*. *Eur J Immunol* 1999;29:311–316. [PubMed: 9933113]
21. Hassan AT, Dai Z, Konieczny BT, Ring GH, Baddoura FK, Abou-Dahab LH, et al. Regulation of alloantigen-mediated T-cell proliferation by endogenous interferon-gamma: implications for long-term allograft acceptance. *Transplantation* 1999;68:124–129. [PubMed: 10428279]
22. Hunter DA, Moradzadeh A, Whitlock EL, Brenner MJ, Myckatyn TM, Wei CH, et al. Binary imaging analysis for comprehensive quantitative histomorphometry of peripheral nerve. *J Neurosci Methods* 2007;166:116–124. [PubMed: 17675163]
23. Hutloff A, Dittrich AM, Beier KC, Eljaschewitsch B, Kraft R, Anagnostopoulos I, et al. ICOS is an inducible T-cell co-stimulator structurally and functionally related to CD28. *Nature* 1999;397:263–266. [PubMed: 9930702]
24. 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]
25. Jensen JN, Mackinnon SE. Composite tissue allotransplantation: a comprehensive review of the literature—part 1. *J Reconstr Microsurg* 2000;16:57–68. [PubMed: 10668755]
26. Kacha AK, Fallarino F, Markiewicz MA, Gajewski TF. Cutting edge: spontaneous rejection of poorly immunogenic P1.HTR tumors by Stat6-deficient mice. *J Immunol* 2000;165:6024–6028. [PubMed: 11086033]
27. Kaplan MH, Grusby MJ. Regulation of T helper cell differentiation by STAT molecules. *J Leukoc Biol* 1998;64:2–5. [PubMed: 9665267]
28. Kaplan MH, Schindler U, Smiley ST, Grusby MJ. Stat6 is required for mediating responses to IL-4 and for development of Th2 cells. *Immunity* 1996;4:313–319. [PubMed: 8624821]
29. Kaplan MH, Sun YL, Hoey T, Grusby MJ. Impaired IL-12 responses and enhanced development of Th2 cells in Stat4-deficient mice. *Nature* 1996;382:174–177. [PubMed: 8700209]
30. Kaplan MH, Wurster AL, Grusby MJ. A signal transducer and activator of transcription (Stat)4-independent pathway for the development of T helper type 1 cells. *J Exp Med* 1998;188:1191–1196. [PubMed: 9743537]
31. Khoury SJ, Akalin E, Chandraker A, Turka LA, Linsley PS, Sayegh MH, et al. CD28-B7 costimulatory blockade by CTLA4Ig prevents actively induced experimental autoimmune encephalomyelitis and inhibits Th1 but spares Th2 cytokines in the central nervous system. *J Immunol* 1995;155:4521–4524. [PubMed: 7594447]
32. Kirk AD, Burkly LC, Batty DS, Baumgartner RE, Berning JD, Buchanan K, et al. Treatment with humanized monoclonal antibody against CD154 prevents acute renal allograft rejection in nonhuman primates. *Nat Med* 1999;5:686–693. [PubMed: 10371508]
33. Kishimoto K, Dong VM, Issazadeh S, Fedoseyeva EV, Waaga AM, Yamada A, et al. The role of CD154-CD40 versus CD28-B7 costimulatory pathways in regulating allogeneic Th1 and Th2 responses in vivo. *J Clin Invest* 2000;106:63–72. [PubMed: 10880049]
34. Konieczny BT, Dai Z, Elwood ET, Saleem S, Linsley PS, Baddoura FK, et al. IFN-gamma is critical for long-term allograft survival induced by blocking the CD28 and CD40 ligand T cell costimulation pathways. *J Immunol* 1998;160:2059–2064. [PubMed: 9498741]
35. Lassner F, Schaller E, Steinhoff G, Wonigeit K, Walter GF, Berger A. Cellular mechanisms of rejection and regeneration in peripheral nerve allografts. *Transplantation* 1989;48:386–392. [PubMed: 2781604]

36. Li Y, Li XC, Zheng XX, Wells AD, Turka LA, Strom TB. Blocking both signal 1 and signal 2 of T-cell activation prevents apoptosis of alloreactive T cells and induction of peripheral allograft tolerance. *Nat Med* 1999;5:1298–1302. [PubMed: 10545997]
37. Lin H, Rathmell JC, Gray GS, Thompson CB, Leiden JM, Alegre ML. Cytotoxic T lymphocyte antigen 4 (CTLA4) blockade accelerates the acute rejection of cardiac allografts in CD28-deficient mice: CTLA4 can function independently of CD28. *J Exp Med* 1998;188:199–204. [PubMed: 9653096]
38. Mackinnon S, Hudson A, Falk R, Bilbao J, Kline D, Hunter D. Nerve allograft response: a quantitative immunological study. *Neurosurgery* 1982;10:61–69. [PubMed: 7035993]
39. Mackinnon SE, Midha R, Bain J, Hunter D, Wade J. An assessment of regeneration across peripheral nerve allografts in rats receiving short courses of cyclosporin A immunosuppression. *Neuroscience* 1992;46:585–593. [PubMed: 1545911]
40. Mandelbrot DA, Oosterwegel MA, Shimizu K, Yamada A, Freeman GJ, Mitchell RN, et al. B7-dependent T-cell co-stimulation in mice lacking CD28 and CTLA4. *J Clin Invest* 2001;107:881–887. [PubMed: 11285307]
41. Mariotti J, Foley J, Ryan K, Buxhoeveden N, Kapoor V, Amarnath S, et al. Graft rejection as a Th1-type process amenable to regulation by donor Th2-type cells through an IL-4/STAT6 pathway. *Blood* 2008;112:4756–4775.
42. McAdam AJ, Chang TT, Lumelsky AE, Greenfield EA, Boussiotis VA, Duke-Cohan JS, et al. Mouse inducible costimulatory molecule (ICOS) expression is enhanced by CD28 co-stimulation and regulates differentiation of CD4+ T cells. *J Immunol* 2000;165:5035–5040. [PubMed: 11046032]
43. 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]
44. Mungara AK, Brown DL, Bishop DK, Wood SY, Cederna PS. Anti-CD40L monoclonal antibody treatment induces long-term, tissue-specific, immunologic hyporesponsiveness to peripheral nerve allografts. *J Reconstr Microsurg* 2008;24:189–195. [PubMed: 18459087]
45. Ostrand-Rosenberg S, Clements VK, Terabe M, Park JM, Berzofsky JA, Dissanayake SK. Resistance to metastatic disease in STAT6-deficient mice requires hemopoietic and nonhemopoietic cells and is IFN-gamma dependent. *J Immunol* 2002;169:5796–5804. [PubMed: 12421960]
46. Ostrand-Rosenberg S, Grusby MJ, Clements VK. Cutting edge: STAT6-deficient mice have enhanced tumor immunity to primary and metastatic mammary carcinoma. *J Immunol* 2000;165:6015–6019. [PubMed: 11086031]
47. Ozkaynak E, Gao W, Shemmeri N, Wang C, Gutierrez-Ramos JC, Amaral J, et al. Importance of ICOS-B7RP-1 costimulation in acute and chronic allograft rejection. *Nat Immunol* 2001;2:591–596. [PubMed: 11429542]
48. Padrid PA, Mathur M, Li X, Herrmann K, Qin Y, Cattamanchi A, et al. CTLA4Ig inhibits airway eosinophilia and hyperresponsiveness by regulating the development of Th1/Th2 subsets in a murine model of asthma. *Am J Respir Cell Mol Biol* 1998;18:453–462. [PubMed: 9533932]
49. Pollard JD, Gye RS, McLeod JG. An assessment of immunosuppressive agents in experimental peripheral nerve transplantation. *Surg Gynecol Obstet* 1971;132:839–845. [PubMed: 4995335]
50. Sayegh MH, Akalin E, Hancock WW, Russell ME, Carpenter CB, Linsley PS, et al. CD28-B7 blockade after alloantigenic challenge in vivo inhibits Th1 cytokines but spares Th2. *J Exp Med* 1995;181:1869–1874. [PubMed: 7536798]
51. Shimoda K, van Deursen J, Sangster MY, Sarawar SR, Carson RT, Tripp RA, et al. Lack of IL-4-induced Th2 response and IgE class switching in mice with disrupted Stat6 gene. *Nature* 1996;380:630–633. [PubMed: 8602264]
52. Siemionow M, Bozkurt M, Kulahci Y. Current status of composite tissue allotransplantation. *Handchir Mikrochir Plast Chir* 2007;39:145–155. [PubMed: 17602375]
53. Tai CY, Weber RV, Mackinnon SE, Tung TH. Multiple co-stimulatory blockade in the peripheral nerve allograft. *Neurol Res.* 2009 [epub ahead of print].
54. Terabe M, Matsui S, Noben-Trauth N, Chen H, Watson C, Donaldson DD, et al. NKT cell-mediated repression of tumor immunosurveillance by IL-13 and the IL-4R-STAT6 pathway. *Nat Immunol* 2000;1:515–520. [PubMed: 11101874]

55. Thierfelder WE, van Deursen JM, Yamamoto K, Tripp RA, Sarawar SR, Carson RT, et al. Requirement for Stat4 in interleukin-12-mediated responses of natural killer and T cells. *Nature* 1996;382:171–174. [PubMed: 8700208]
56. Thompson CB. Distinct roles for the costimulatory ligands B7-1 and B7-2 in T helper cell differentiation? *Cell* 1995;81:979–982. [PubMed: 7541314]
57. Trumble TE, Shon FG. The physiology of nerve transplantation. *Hand Clin* 2000;16:105–122. [PubMed: 10696580]
58. Tung TH, Mohanakumar T, Mackinnon SE. TH1/TH2 cytokine profile of the immune response in limb component transplantation. *Plast Reconstr Surg* 2005;116:557–566. [PubMed: 16079692]
59. van Maurik A, Herber M, Wood KJ, Jones ND. Cutting edge: CD4+CD25+ alloantigen-specific immunoregulatory cells that can prevent CD8+ T cell-mediated graft rejection: implications for anti-CD154 immunotherapy. *J Immunol* 2002;169:5401–5404. [PubMed: 12421913]
60. Wells AD, Li XC, Li Y, Walsh MC, Zheng XX, Wu Z, et al. Requirement for T-cell apoptosis in the induction of peripheral transplantation tolerance. *Nat Med* 1999;5:1303–1307. [PubMed: 10545998]
61. Yamada A, Kishimoto K, Dong VM, Sho M, Salama AD, Anosova NG, et al. CD28-independent costimulation of T cells in alloimmune responses. *J Immunol* 2001;167:140–146. [PubMed: 11418642]
62. Yao M, Inserra MM, Duh MJ, Terris DJ. A longitudinal, functional study of peripheral nerve recovery in the mouse. *Laryngoscope* 1998;108:1141–1145. [PubMed: 9707232]
63. Yu LT, Rostami A, Silvers WK, Larossa D, Hickey WF. Expression of major histocompatibility complex antigens on inflammatory peripheral nerve lesions. *J Neuroimmunol* 1990;30:121–128. [PubMed: 2172305]
64. Zhai Y, Ghobrial RM, Busuttil RW, Kupiec-Weglinski JW. Th1 and Th2 cytokines in organ transplantation: paradigm lost? *Crit Rev Immunol* 1999;19:155–172. [PubMed: 10352902]
65. Zhai Y, Meng L, Gao F, Wang Y, Busuttil RW, Kupiec-Weglinski JW. CD4+ T regulatory cell induction and function in transplant recipients after CD154 blockade is TLR4 independent. *J Immunol* 2006;176:5988–5994. [PubMed: 16670307]

FIG. 1.

Flow charts illustrating T-cell differentiation. **A:** CD4⁺ T helper cells consist of at least 2 functionally distinct subsets (Th1 and Th2). The subsequent proliferation and differentiation of these is mediated through the cytokine environment and *STAT* genes. **B and C:** *STAT4*^{-/-} mice preferentially develop Th2 cells (B), and *STAT6*^{-/-} mice preferentially develop Th1 cells (C). TCR = T-cell receptor.

FIG. 2.

Diagram showing the costimulation blockade. MR-1 blocks the CD40–CD40 L interaction, CTLA-4-Ig blocks the B7-CD28 T-cell costimulation pathway, and anti-ICOSL blocks the ICOS–ICOSL interaction.

FIG. 3.

Photomicrographs. **A:** Sample obtained in allograft control mouse demonstrates disorganized architecture with no clear axonal regeneration. **B:** Sample obtained in isograft control mouse demonstrates near normal nerve architecture with numerous myelinated fibers. **C:** Sample obtained in *STAT6*^{-/-} mouse without costimulatory blockade demonstrates disorganized architecture and high collagen concentration. **D:** Sample obtained in *STAT6*^{-/-} mouse with costimulatory blockade demonstrates simultaneous Wallerian degeneration and areas of myelinated fibers. **E:** Sample obtained in *STAT4*^{-/-} mouse without costimulatory blockade is similar in appearance to the allograft control, with dense collagen concentration and few myelinated fibers. **F:** Sample obtained in *STAT4*^{-/-} mouse with costimulatory blockade does not appear to be qualitatively significantly different from the *STAT6*^{-/-} with costimulatory blockade. Toluidine blue stain, original magnification $\times 400$ for all panels.

FIG. 4.

Electron microscopy. Distal nerve graft obtained in an *STAT6*^{-/-} mouse without costimulation blockade, demonstrating diffuse cellular infiltrate and large mast cells (*left*), and distal nerve graft obtained in a *STAT6*^{-/-} mouse with costimulatory blockade, demonstrating multiple myelinated fibers (*right*, *arrows*).

FIG. 5.

Bar graph showing histomorphometrical analysis. Total number of distal nerve fibers 3 weeks after nerve grafting. *Asterisk* indicates statistical significance ($p < 0.05$).

FIG. 6.
Graph showing walking track analysis. Assessment of neural regeneration with the SFI.
Measurements were made at 1-, 2-, and 3-week time points.

FIG. 7. Bar graph showing ELISPOT data. Interferon- γ production is quantified 3 weeks after nerve grafting. Values are expressed in spots per million cells. *Asterisk* indicates statistical significance ($p < 0.05$).

TABLE 1

Summary of treatment groups*

Group No.	Treatment	No. of Mice
I	isograft control	8
II	allograft control	8
III	allograft w/ CB	8
IV	<i>STAT6</i> ^{-/-} mice w/ allograft	6
V	<i>STAT4</i> ^{-/-} mice w/ allograft	6
VI	<i>STAT6</i> ^{-/-} mice w/ allograft & CB	6
VII	<i>STAT4</i> ^{-/-} mice w/ allograft & CB	6

* CB = costimulatory blockade.