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Costimulation Blockade Inhibits the Indirect Pathway of Allorecognition in Nerve Allograft Rejection

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Abstract

Background—Nerve allografts provide a temporary scaffold for host nerve regeneration. The need for systemic immunosuppression limits clinical application. Characterization of the immunological mechanisms that induce immune hyporesponsiveness may provide a basis for optimizing immunomodulating regimens.

Methods—We utilized wild type and MHC class II – deficient mice, as both recipients and donors. Host treatment consisted of triple costimulatory blockade. Quantitative assessment was made at three weeks using nerve histomorphometry, and muscle testing was performed on a subset of animals at seven weeks.

Results—Nerve allograft rejection occurred as long as either the direct or indirect pathway were functional. Indirect antigen presentation appeared to be more important.

Conclusion—Nerve allograft rejection occurs in the absence of a normal direct or indirect immune response but may be more dependent on indirect allorecognition. The indirect pathway is required to induce costimulatory blockade immune hyporesponsiveness.

Keywords

Allograft; peripheral nerve; immunosuppression; cold preservation; costimulatory blockade

Introduction

Manipulation of the immune system to prevent allograft rejection while minimizing the morbidity of immunosuppression is essential for the advancement of reconstructive transplantation. The blockade of costimulatory signals to permit donor-specific allograft survival showed promise in experimental models of organ and musculoskeletal tissue transplantation, but it has not met with the same success in humans. It has been studied more extensively in the organ transplantation model than in composite tissue allograft in which

different and heterogeneous tissue composition elicits a somewhat different immune response. To further define the potential role of costimulation blockade in clinical transplantation, a better understanding of its effect on rejection of the reconstructive allograft is necessary to identify complementary therapies that will improve outcomes.

There are two known pathways by which donor antigen peptides are recognized by host T cells. In the direct pathway of allorecognition, *donor* antigen presenting cells (APCs) present donor antigen to host T cells in the context of class II major histocompatibility complex (MHC) molecules. Schwann cells (SCs) are known to act as facultative APCs and are a primary target of the immune response to the nerve allograft[1-7]. In indirect allorecognition *host* APC's present processed donor antigen to host T cells with class II MHC molecules (Figure 1). Both pathways are known to play a role in allograft rejection, but their relative roles in differing settings are incompletely understood. The significance of the direct pathway has long been understood, and there is now better appreciation for an important role for indirect recognition[8-10]. This study investigates the relative contribution of each pathway in nerve allograft rejection and how they are affected by blocking costimulatory signals. The combination of MHC^{-/-} allografts placed in wild type recipients was used to isolate the indirect immune pathway, and wild type allografts placed into MHC^{-/-} mice were used to isolate the direct immune pathway[11]. Delineation of the immunological mechanisms involved in rejection of the nerve allograft will allow the design of strategies to manipulate the host immune system in order to broaden the indications for nerve allotransplantation.

Materials and Methods

Mice

This study utilized male *MHC*^{-/-} knockout mice with a *C57Bl/6* background and wild type *C57Bl/6(B6)* and *Balb/c* mice (Jackson Laboratory, Bar Harbor, ME). 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.

Reagents

All costimulatory blocking agents were obtained from BioXcell, West Lebanon, NH, and included: Hamster MR-1 to block the CD40-CD40L interaction, Human CTLA-4-Ig to block the B7-CD28 T cell costimulation pathway, and Anti-ICOSL to block the ICOS-ICOSL interaction. Animals treated with costimulatory blockade received therapy on postoperative days 0, 2, and 4, administered at a dose of 0.5 mg by intraperitoneal (IP) injection.

Surgical Procedure

All surgeries were performed on anesthetized eight week old mice, with the right hindquarter shaved and depilated (Nair Lotion). A skin incision was made parallel to the femur, and the biceps femoris muscle was split. Under 16-40x magnification, the sciatic nerve was exposed with microinstruments to include the sciatic notch proximally and its trifurcation to tibial, peroneal, and sural nerves distally. The sciatic nerve was transected 5 mm proximal to its trifurcation, and a peripheral nerve allograft was reversed in orientation and interposed between the transected ends and secured with 11-0 microsutures under 40x magnification. The muscle was closed using 8-0 vicryl suture and skin with 6-0 nylon suture (Ethicon, NJ) and mice recovered with injections of Antisedan (Novartis, Canada) on a warming pad. Nerve grafts were obtained from isogeneic (isografts) or dysgeneic

(allografts) strains which were harvested using the same anesthetic and surgical approach. Nerves were harvested bilaterally to minimize animal use. They were oriented with an 11-0 microsuture proximally, after which donor animals were immediately sacrificed. The harvested, non-vascularized 1 cm sciatic nerve allograft was transplanted in reverse orientation into the recipient.

As detailed in Table 1, *MHC*^{-/-} deficient mice were used as both donor and recipient with a fully allogenic wild-type *Balb/c* recipient or donor. Wild type controls with *C57Bl/6* (*B6*) and *Balb/c* mice were also each used as donors and recipients in isograft and allograft control groups (groups 1-4). Groups 5-7 received fresh allografts and IP costimulatory blockade (MR1, CTLA4-Ig, and anti-ICOS) on days 0, 2 and 4, and group 5 served as the wild-type control. Two additional controls were performed with (Groups 8 and 9) *MHC*^{-/-} recipients. Group 8 received an *MHC*^{-/-} donor nerve (additional isograft control), and Group 9 received the adoptive transfer of CD8-depleted splenocytes and an allograft with the previously described triple costimulatory blockade protocol. Prior to nerve transplantation, the *MHC*^{-/-} recipients were treated with three doses (100 µg intraperitoneal) of Anti-CD4 (GK 1.5; rat anti-mouse CD4) and anti-CD8 (2.43; rat anti-mouse CD8) on -6, -3, -1 days before transplantation, in order to deplete recipient native CD4⁺/CD8⁺ T-cells. CD8-depleted spleen cells (100×10^6) from wild-type B6 mice were transferred intravenously into *MHC*^{-/-} recipients on the day of surgery[12,13].

Histomorphometry

Sciatic nerve segments were harvested *en bloc*. Labeled nerve specimens from distinct regions along the graft itself and proximal and distal to the graft were preserved in glutaraldehyde, postfixed in osmium tetroxide and embedded in Araldite 502, and 1 µm 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 presence of ongoing Wallerian degeneration. The system utilizes a series of custom algorithms and eight-bitplane digital pseudocoloring to distinguish axons, myelin, nerve sheath, and debris from each other, and results are double-checked by our histomorphometrist[14]. Processed cross sections were digitized and assessed for total fascicular area and total fiber number. The mean and standard deviation was used to present all data in this study. A two-tailed analysis of variance (ANOVA) was made to determine the differences between individual groups. Histomorphometric calculations were performed using Statistica (StatSoft, Inc., Tulsa, OK). If significant, a Student-Newman-Keuls test was performed to compare groups. Statistical significance was established at $p < 0.05$.

Tetanic muscle force measurement

A subset of animals (n=3-4) (Table 2) from each group, (except groups 5, 8 and 9) underwent maximum tetanic force/normalized maximum power testing, and wet weights of the extensor digitorum longus (EDL) were measured. These animals were maintained for seven weeks prior to muscle testing and sacrifice. The EDL and nerve graft were exposed, retaining the distal tendon. The exposed hindlimb was mounted horizontally and attached by the distal tendinous insertions to the lever measurement post. Muscle length was adjusted to the length (L_0) at which maximal twitch force was reached. Stimulation was delivered via electrodes positioned along the length of the repaired nerve graft. Maximal tetanic force and power were determined using an escalating level of pulse stimulation (Hz). After the maximum force of stimulation was measured, the muscle was removed and weighed.

Results

Nerve rejection will occur in the absence of a normal direct or indirect pathway

The redundancy in the immune system facilitates host rejection in other allotransplantation models even in the absence of a normal direct or indirect pathway[11]. As expected, we found that untreated wild-type *BalbC* mice (Group 3) that received fully allogenic *MHC*^{-/-} donor nerve grafts demonstrated poor histomorphometric evidence of distal graft axonal regeneration similar to that observed in the allograft controls (Group 2) (total number of distal total fibers: 37 ± 87 and 135 ± 300). Similarly, untreated *MHC*^{-/-} mice (Group 4) that received fully allogenic *BalbC* nerve allografts demonstrated marginally increased axonal regeneration (total number of distal fibers: 699 ± 440 , Figure 2), but significantly less ($p < 0.009$) than isograft controls (1570 ± 440) and not statistically different than allograft controls ($p < 0.052$). Although functional assessment with muscle testing was not powered to observe any statistical difference among the groups, functional assessment correlated well with histomorphometric results in the isograft and allograft control groups (Maximum tetanic force 435 vs. 162 Fo, mN –Table 2).

When treated with costimulatory blockade, elimination of the indirect pathway resulted in diminished nerve regeneration

As demonstrated in Figure 2, the presence of the indirect pathway is necessary for costimulatory blockade-induced immune hyporesponsiveness. *MHC*^{-/-} mice that received *BalbC* nerve allografts treated with triple costimulatory blockade and with or without adoptive transfer of CD8-depleted splenocytes (Groups 7 and 9) demonstrated relatively poor histomorphometric axonal regeneration (Total distal fiber counts 512 ± 160 and 65 ± 57). Allograft controls (Group 5) treated with costimulatory blockade had significantly lower fiber counts (983 ± 336 , $p < 0.021$) than *Wild-type BalbC* mice (Group 6) that received *MHC*^{-/-} nerve grafts treated with costimulatory blockade (total distal fiber counts: 1722 ± 610). Histomorphometric results correlated well with muscle force and power measurements (Table 2).

Adoptive transfer of Wild-type C57Bl/6 (B6) CD4+ T-cells did not improve costimulatory mediated neural regeneration

An additional experiment was performed to assess whether the above findings were due to a low level of functional CD4+ T cells in *MHC*^{-/-} recipient mice. Group 8 served as an additional isograft control using *MHC*^{-/-} mice as both donor and recipient. Group 9 consisted of *MHC*^{-/-} recipients depleted of native CD4+ and CD8+ T cells (materials and methods) prior to receiving allotransplantation. In addition, each animal received triple costimulatory blockade and underwent adoptive transfer of CD-8 depleted T cells from wild type *C57Bl/6* mice. As demonstrated in Figure 2, the decreased response to costimulatory blockade in *MHC*^{-/-} recipients was not due to an impaired immune response secondary to low levels of functional CD4+ T cells, but rather it was due to an immune response based on direct allorecognition which is less dependent on costimulation.

Discussion

Inhibition of T-cell activation by costimulatory blockade has demonstrated significant potential in both solid organ transplantation[11,15-17] and nerve allotransplantation[18-20] models. While solid organ transplants requires a lifetime of aggressive immunosuppression, nerve allotransplantation only requires temporary systemic immunosuppression. Under sufficient immunosuppression, donor SCs assist migrating host SCs as axonal regeneration occurs across the nerve allograft[21-23]. Once adequate host SC migration has occurred and the target end-organ has been reinnervated, immunosuppression can be withdrawn[21-24].

Blockade of these costimulatory signals has proven effective at decreasing the required dose of other more traditional immunosuppressive agents, achieving temporary allotransplantation tolerance and preventing T-cell clonal expansion and maturation[25-30].

Anti-CD40L mAb (MR-1) has been studied extensively, and prolonged graft survival and tolerance induction in the murine cardiac model have been demonstrated [31,32]. CTLA4-Ig causes arrest of T cells in the G1 phase of the cell cycle by inhibiting T cell activation through a reduction in IL-2 production and IL-2 receptor expression[33,34], and it has been shown to produce long term cardiac allograft survival in mouse and rat models[35,36]. ICOS is a structurally analogous costimulatory molecule that is not constitutively expressed on T cells but is induced following T cell activation[37-39]. ICOS blockade has been shown to prolong liver allograft survival in a murine model[40]. Recent work in our lab has demonstrated that combination therapy with multiple costimulatory blocking agents (MR-1, CTLA-4-Ig, and Anti-ICOSL), produces neural regeneration not significantly different from isograft controls[41].

In this study we investigated the role of the direct and indirect pathways in the immune hyporesponsiveness produced by costimulation-blocking antibodies. We found that the redundancy of the immune system facilitates nerve rejection in the absence of a normal direct or indirect pathway. The significance of the direct pathway has long been understood, and there is now better appreciation for an important role for indirect recognition[12,28]. Both pathways are known to play a role in allograft rejection[42], but their relative roles in differing settings are incompletely understood. Immunological factors such as antigen presentation may vary with the cellular composition of the allograft and may contribute to differential tissue resistance to immunomodulation. The parenchymal component constitutes a much smaller proportion of the entire peripheral nerve allograft than an organ or composite tissue allograft. The immune response is directed at the cellular components, which in the nerve allograft include SCs, endothelial cells, and perivascular macrophages. In the untreated control groups, the strength of the rejection response based only on the indirect pathway was just as strong as when both pathways were intact. However when the indirect pathway was eliminated leaving only the direct pathway intact, total axonal regeneration was improved and was very close to reaching statistical significance. Indirect antigen presentation therefore seems to play a greater role in the nerve allograft rejection than expected. The non-vascularized nature and relative lack of professional APC's on the nerve allograft may explain a weaker direct component. Professional APC's include dendritic cells, macrophages and other mononuclear phagocytes and B lymphocytes, while non-professional APC's include epithelial and mesenchymal cells. The role of non-professional APC's in antigen presentation remains unclear, and it is unlikely that they play a major part in most T cell responses[24]. The nerve allograft is more homogenous in tissue composition and is quantitatively smaller than an organ allograft which also contains sizeable blood vessels for surgical revascularization. Microvascular endothelial cells may also present antigen and may be significant during allograft rejection. Non-vascularized nerve grafts contain microvessels in the epi- and perineurium, but they are miniscule and not directly exposed to host blood initially. As such, the nerve allograft is more analogous to a non-vascularized skin allograft except that donor skin also contains Langerhan cells which, as dendritic cells, are professional APC's. Because SC's are only facultative APC's, the relative lack of donor professional APC's may account for a strong indirect component of the host response to the nerve allograft.

The use of transgenic mice that lack class II MHC molecules allowed us to eliminate either the direct or indirect pathways of antigen recognition to evaluate their relative roles with and without treatment with costimulation blockade[11]. In this model, triple costimulatory blockade provides a permissive environment for neural regeneration in the nerve allograft.

This effect was not achieved when only direct allorecognition was functional and was further enhanced when only the indirect pathway was available. As demonstrated in the solid organ literature, the presence of the indirect pathway appears necessary for costimulatory blockade induced immune hyporesponsiveness[11,43,44]. Similarly, we found that the capacity to mount an indirect response is necessary to achieve costimulatory blockade-mediated neural regeneration. The direct pathway of allorecognition appears to be more resistant to costimulation blockade because it is less dependent on CD40 and CD28 costimulation.

One potential limitation of this study involves the relative number of circulating CD4⁺ T cells in MHC deficient mice. *MHC*^{-/-} mice are known to have lower numbers of CD4⁺ cells with an increased number of CD8⁺ T cells[45], which may impair the immune response or impart a selective resistance to costimulatory blockade. The threshold level of required CD4⁺ T cells to generate an adequate allograft rejection response is not precisely known. In order to account for these findings we performed an additional control experiment utilizing the adoptive transfer of wild-type CD4⁺ T cells into *MHC*^{-/-} recipients receiving costimulatory blockade (Group 9). Our results corroborate similar experiments done with cardiac transplantation[11], and we found that costimulatory blockade-induced immune hyporesponsiveness in the absence of an indirect pathway is not due to an abnormal level of CD4⁺ T cells. MHC deficient mice that received wild-type CD4⁺ T cells with costimulatory blockade still failed to have any meaningful neural regeneration. Our results suggest, therefore, that the indirect pathway is necessary for costimulatory blockade induced immune hyporesponsiveness.

Conclusion

We have demonstrated in this experiment that the indirect pathway is required for the induction of transient hyporesponsiveness afforded by costimulatory blockade. A combination of costimulatory blockade with other immunomodulating modalities that are known to effect both the indirect and direct pathways such as cold preservation may provide a suitable alternative to systemic immunosuppression.

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Abbreviations

(APCs)	Antigen presenting cells
(MHC)	Major histocompatibility complex
(SCs)	Schwann cells
(EDL)	Extensor digitorum longus

References

1. Gulati AK. Immune response and neurotrophic factor interactions in peripheral nerve transplants. *Acta Haematol* 1998;99(3):171–4. [PubMed: 9587399]
2. Gulati AK, Cole GP. Nerve graft immunogenicity as a factor determining axonal regeneration in the rat. *J Neurosurg* 1990;72(1):114–22. [PubMed: 2294170]
3. Lassner F, et al. Cellular mechanisms of rejection and regeneration in peripheral nerve allografts. *Transplantation* 1989;48(3):386–92. [PubMed: 2781604]

4. Mackinnon S, et al. Nerve allograft response: a quantitative immunological study. *Neurosurgery* 1982;10(1):61–9. [PubMed: 7035993]
5. Pollard JD, Gye RS, McLeod JG. An assessment of immunosuppressive agents in experimental peripheral nerve transplantation. *Surg Gynecol Obstet* 1971;132(5):839–45. [PubMed: 4995335]
6. Trumble TE, Shon FG. The physiology of nerve transplantation. *Hand Clin* 2000;16(1):105–22. [PubMed: 10696580]
7. Yu LT, et al. Expression of major histocompatibility complex antigens on inflammatory peripheral nerve lesions. *J Neuroimmunol* 1990;30(2-3):121–8. [PubMed: 2172305]
8. Auchincloss H Jr, Sultan H. Antigen processing and presentation in transplantation. *Curr Opin Immunol* 1996;8(5):681–7. [PubMed: 8902394]
9. Gould DS, Auchincloss H Jr. Direct and indirect recognition: the role of MHC antigens in graft rejection. *Immunol Today* 1999;20(2):77–82. [PubMed: 10098326]
10. Shoskes DA, Wood KJ. Indirect presentation of MHC antigens in transplantation. *Immunol Today* 1994;15(1):32–8. [PubMed: 8136009]
11. Yamada A, et al. Recipient MHC class II expression is required to achieve long-term survival of murine cardiac allografts after costimulatory blockade. *J Immunol* 2001;167(10):5522–6. [PubMed: 11698419]
12. Auchincloss H Jr, et al. Prevention of alloantibody formation after skin grafting without prolongation of graft survival by anti-L3T4 in vivo. *Transplantation* 1988;45(6):1118–23. [PubMed: 3289152]
13. Ghobrial RR, et al. In vivo use of monoclonal antibodies against murine T cell antigens. *Clin Immunol Immunopathol* 1989;52(3):486–506. [PubMed: 2788053]
14. Hunter DA, et al. Binary imaging analysis for comprehensive quantitative histomorphometry of peripheral nerve. *J Neurosci Methods* 2007;166(1):116–24. [PubMed: 17675163]
15. Guillonau C, et al. Inhibition of chronic rejection and development of tolerogenic T cells after ICOS-ICOSL and CD40-CD40L co-stimulation blockade. *Transplantation* 2005;80(4):546–54. [PubMed: 16220623]
16. Guillonau C, et al. Anti-CD28 antibodies modify regulatory mechanisms and reinforce tolerance in CD40Ig-treated heart allograft recipients. *J Immunol* 2007;179(12):8164–71. [PubMed: 18056359]
17. Metzler B, et al. Combinations of anti-LFA-1, everolimus, anti-CD40 ligand, and allogeneic bone marrow induce central transplantation tolerance through hemopoietic chimerism, including protection from chronic heart allograft rejection. *J Immunol* 2004;173(11):7025–36. [PubMed: 15557200]
18. Kvist M, et al. Costimulation blockade in transplantation of nerve allografts: long-term effects. *J Peripher Nerv Syst* 2008;13(3):200–7. [PubMed: 18844786]
19. Kvist M, et al. Immunomodulation by costimulation blockade inhibits rejection of nerve allografts. *J Peripher Nerv Syst* 2007;12(2):83–90. [PubMed: 17565532]
20. Ray WZ, et al. The role of T helper cell differentiation in promoting nerve allograft survival with costimulation blockade. *J Neurosurg* 2010;112(2):386–93. [PubMed: 19663546]
21. Mackinnon SE, et al. An assessment of regeneration across peripheral nerve allografts in rats receiving short courses of cyclosporin A immunosuppression. *Neuroscience* 1992;46(3):585–93. [PubMed: 1545911]
22. Midha R, et al. Temporary immunosuppression for peripheral nerve allografts. *Transplant Proc* 1993;25(1 Pt 1):532–6. [PubMed: 8438403]
23. Midha R, Mackinnon SE, Becker LE. The fate of Schwann cells in peripheral nerve allografts. *J Neuropathol Exp Neurol* 1994;53(3):316–22. [PubMed: 8176414]
24. Atchabadian A, et al. Regeneration through long nerve grafts in the swine model. *Microsurgery* 1998;18(6):379–82. [PubMed: 9847001]
25. Foster RD, et al. Long-term acceptance of composite tissue allografts through mixed chimerism and CD28 blockade. *Transplantation* 2003;76(6):988–94. [PubMed: 14508367]
26. Gao W, et al. Stimulating PD-1-negative signals concurrent with blocking CD154 co-stimulation induces long-term islet allograft survival. *Transplantation* 2003;76(6):994–9. [PubMed: 14508368]

27. Kosuge H, et al. Induction of immunologic tolerance to cardiac allograft by simultaneous blockade of inducible co-stimulator and cytotoxic T-lymphocyte antigen 4 pathway. *Transplantation* 2003;75(8):1374–9. [PubMed: 12717233]
28. Lakkis FG, et al. Blocking the CD28-B7 T cell costimulation pathway induces long term cardiac allograft acceptance in the absence of IL-4. *J Immunol* 1997;158(5):2443–8. [PubMed: 9036995]
29. Larsen CP, et al. A new look at blockade of T-cell costimulation: a therapeutic strategy for long-term maintenance immunosuppression. *Am J Transplant* 2006;6(5 Pt 1):876–83. [PubMed: 16611323]
30. Vincenti F, et al. Costimulation blockade with belatacept in renal transplantation. *N Engl J Med* 2005;353(8):770–81. [PubMed: 16120857]
31. Larsen CP, et al. Long-term acceptance of skin and cardiac allografts after blocking CD40 and CD28 pathways. *Nature* 1996;381(6581):434–8. [PubMed: 8632801]
32. van Maurik A, et al. 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(10):5401–4. [PubMed: 12421913]
33. Krummel MF, Allison JP. CD28 and CTLA-4 have opposing effects on the response of T cells to stimulation. *J Exp Med* 1995;182(2):459–65. [PubMed: 7543139]
34. Walunas TL, et al. CTLA-4 can function as a negative regulator of T cell activation. *Immunity* 1994;1(5):405–13. [PubMed: 7882171]
35. Kita Y, et al. Prolonged cardiac allograft survival in rats systemically injected adenoviral vectors containing CTLA4Ig-gene. *Transplantation* 1999;68(6):758–66. [PubMed: 10515375]
36. Turka LA, et al. T-cell activation by the CD28 ligand B7 is required for cardiac allograft rejection in vivo. *Proc Natl Acad Sci U S A* 1992;89(22):11102–5. [PubMed: 1332070]
37. Dong C, et al. ICOS co-stimulatory receptor is essential for T-cell activation and function. *Nature* 2001;409(6816):97–101. [PubMed: 11343121]
38. McAdam AJ, Schweitzer AN, Sharpe AH. The role of B7 co-stimulation in activation and differentiation of CD4+ and CD8+ T cells. *Immunol Rev* 1998;165:231–47. [PubMed: 9850864]
39. Pan XC, et al. Further study of anti-ICOS immunotherapy for rat cardiac allograft rejection. *Surg Today* 2008;38(9):815–25. [PubMed: 18751947]
40. Guo L, et al. Significant enhancement by anti-ICOS antibody of suboptimal tacrolimus immunosuppression in rat liver transplantation. *Liver Transpl* 2004;10(6):743–7. [PubMed: 15162468]
41. Tai CY, et al. Multiple costimulatory blockade in the peripheral nerve allograft. *Neurol Res* 2010;32(3):332–6. [PubMed: 19570327]
42. Auchincloss H Jr. et al. The role of “indirect” recognition in initiating rejection of skin grafts from major histocompatibility complex class II-deficient mice. *Proc Natl Acad Sci U S A* 1993;90(8):3373–7. [PubMed: 8475083]
43. Phillips NE, et al. Costimulatory blockade induces hyporesponsiveness in T cells that recognize alloantigen via indirect antigen presentation. *Transplantation* 2006;82(8):1085–92. [PubMed: 17060858]
44. Rulifson IC, et al. Inability to induce tolerance through direct antigen presentation. *Am J Transplant* 2002;2(6):510–9. [PubMed: 12118894]
45. Madsen L, et al. Mice lacking all conventional MHC class II genes. *Proc Natl Acad Sci U S A* 1999;96(18):10338–43. [PubMed: 10468609]

Figure 1.

Flow chart illustrating the direct and indirect pathway for alloantigen recognition. (Adapted by permission from *Journal of Neurosurgery*; 2010 In press, Ray et al. Effect of Cold Nerve Allograft Preservation on Antigen Presentation and Rejection) I have attached an updated version

Figure 2.

As demonstrated the presence of the indirect pathway is necessary for costimulatory blockade induced immune hyporesponsiveness. *MHC*^{-/-} mice that received *BalbC* nerve allografts treated with triple costimulatory blockade with or without adoptive transfer of CD8-depleted splenocytes (Groups 7 and 9) demonstrated relatively poor histomorphometric axonal regeneration. Allograft controls (Group 5) treated with costimulatory blockade had significantly lower fiber counts than *Wild-type BalbC* mice (Group 6) that received *MHC*^{-/-} – nerve grafts treated with costimulatory blockade. Isograft controls were not statistically different than *MHC*^{-/-} isografts controls.

Figure 3. Histologic specimens (toluidine blue stain, 400X) of nerve graft or distal to nerve graft showing excellent regeneration in A) untreated isograft group, poor regeneration in B) allograft group, and C) Group 6 illustrates robust regeneration, that requires an intact indirect pathway.

Table 1

Group Number	Treatment	n=
1	Isograft Control	10
2	Allograft Control	10
3	BalbC with MHC ^{-/-} allograft	10
4	MHC ^{-/-} with BalbC allograft	10
5	Allograft control with costimulatory blockade	10
6	BalbC with MHC ^{-/-} allograft and costimulatory blockade	10
7	MHC ^{-/-} with BalbC allograft and costimulatory blockade	10
8	MHC ^{-/-} with MHC ^{-/-} nerve - isograft control	8
9	MHC ^{-/-} with BalbC allograft and costimulatory blockade with adoptive transfer of wild-type CD4 ⁺ T-cells	8

Table 2

Functional muscle testing data

	Maximum Tetanic Force (Fo,mN)	Normalized Maximum Power (nPmax, W/Kg)	Wet Weight of EDL (mg)
Isograft Control (n = 4)	435.68 ± 67	145 ± 25	12.5 ± 1.73
Allograft Control (n=3)	162.65 ± 166	50 ± 65	10.33 ± 1.52
BalbC with MHC ^{-/-} allograft (n = 4)	331.63 ± 35	81 ± 22	11.5 ± 1.29
MHC ^{-/-} with BalbC allograft (n = 3)	299.33 ± 14	106 ± 20	8.33 ± 0.57
BalbC with MHC ^{-/-} allograft and costimulatory blockade (n = 3)	343.68 ± 75	122 ± 4	11.67 ± 0.57
MHC ^{-/-} with BalbC allograft and costimulatory blockade (n = 3)	196.72 ± 121	82 ± 73	8.66 ± 0.58

*Mean values are presented ± Standard deviation