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Multiple costimulatory blockade in the peripheral nerve allograft

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Abstract

Background—In the nerve allograft model, costimulation blockade has permitted good regeneration but is still inferior to the nerve isograft. We hypothesize that a short course of multiple costimulatory pathway blockade will be more effective in inhibiting the redundancy of the immune response and improve nerve regeneration through the nerve allograft.

Methods—The murine sciatic nerve allograft model was used to reconstruct a 1 cm sciatic nerve gap. Treatment consisted of the inhibition of the CD40, CD28/B7 and ICOS pathways and was compared with only single or double costimulation blockade. Assessment methods included quantitative histomorphometry and ELISPOT assay to quantify the host immune response after 3 weeks post-operatively.

Results—Triple costimulation blockade permitted regeneration through the nerve allograft that was equivalent to the nerve isograft. A short course of three doses was more effective than a single dose for all combinations tested. ELISPOT assay demonstrated minimal in vitro immune response with a short course of double or triple pathway-blocking agents.

Conclusion—Costimulation blockade, especially with the simultaneous inhibition of multiple pathways, remains a promising strategy to promote regeneration through the peripheral nerve allograft, and may be uniquely suited to the temporary immunosuppressive requirements of the peripheral nerve allograft.

Keywords

Nerve allograft; nerve transplant; costimulation blockade

Background

Nerve allograft transplantation is now a clinical reality for selected patients who sustained massive nerve injuries that are otherwise unreconstructable¹. During nerve regeneration through the allograft, immunosuppression is required and the patients are subjected to side effects of therapy for up to 2 years. Despite the unique properties of nerve allografts that permit eventual discontinuation of immunosuppression, minimizing the side effects during therapy remains a top priority for patient safety².

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Recent advancements in the current understanding of T cell mechanisms involved in allograft rejection and the role of costimulation has provided multiple targets with therapeutic potential. Costimulatory signals regulate the activation and expansion of alloreactive T cells, and are transmitted by costimulatory molecules on antigen-presenting cells that bind to specific receptors on the T cell. They are necessary in addition to donor antigen binding to the T cell receptor to fully activate the immune response. The CD40 and B7/CD28 pathways were first described and are still considered to be the most significant pathways for immune manipulation to alter the host alloreactive response^{3–5}. The blockade of either pathway will induce a period of donor-specific immune unresponsiveness but not permanent tolerance to the organ allograft^{6–8}. The simultaneous blockade of both pathways has demonstrated synergism in models of organ transplantation^{7,9}, but has not been studied in nerve transplantation. A number of additional costimulatory pathways in both the B7 and the tumor necrosis factor (TNF/CD40) superfamilies have been identified that participate in the alloimmune response to varying degrees and by different mechanisms^{10,11}. The effect of their blockade has seen only modest extension of allograft survival^{12–14}. Again, when combined with the blockade of either the CD40 or B7/CD28 pathways, the effect has usually been additive or synergistic^{15–17}.

We have been interested in investigating the effects of costimulation blockade in a nerve allograft model. We have previously shown that blockade of the CD40 pathway permits regeneration through the nerve allograft but has still been inferior to the nerve isograft^{18–20}. We hypothesize that a short course of multiple costimulatory pathway blockade will be more effective in inhibiting the redundancy of the immune response and improve nerve regeneration through the nerve allograft.

Methods

Animals

An established mouse sciatic nerve model was used. In brief, 1 cm Balb/c donor sciatic nerve segments were transplanted into C57/B6 recipient sciatic nerve gaps in reversed orientation. 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 institutional Animal Studies Committee. At 3 weeks post-transplantation, recipient spleen and grafts were harvested for analysis. All experiments were approved by the Institutional Animal Care and Use Committee.

Experimental design

Incremental costimulatory blockade targets were used to determine the minimal regimen necessary to achieve optimal nerve regeneration. Twenty-seven recipient animals were randomized into five experimental groups. Groups I and V are isograft and allograft controls, respectively. Triple costimulatory blockade using CTLA4-Ig (cytotoxic T lymphocyte antigen 4-immunoglobulin), MR1 (anti-CD40L monoclonal antibody) and anti-ICOS (inducible costimulatory)-ligand (L) monoclonal antibody was studied in single (Group II) and short course of three doses (Group III) of administration regimens. Group IV studied double costimulation blockade using CTLA4-Ig and MR1 in a short course fashion consisting of three doses. Table 1 summarized the experimental groups, the agents and the regimens used.

Immunosuppression

Three costimulatory blockade agents were evaluated: (1) CTLA4-Ig 0.5 mg, targeting the CD28/B7 complex; (2) MR1 1.0 mg, targeting the CD40/CD40L pathway; (3) anti-ICOSL 0.5 mg, targeting the ICOS/ICOSL pathway (Table 1). For all groups receiving three doses of agents, the drugs were administered by intraperitoneal injections on days 0, 2 and 4 post-

operatively. Single dose groups received CTLA4-Ig and MR1 immediately before transplantation, and anti-ICOSL was given on post-operative day 2 based on previous reports of efficacy with delayed administration due to its greater selective effect on activated T cells.

ELISPOT analysis

Enzyme-linked immunospot assay is used to characterize the immune response to nerve grafts used in mice. In mice donor and recipient, spleens were harvested for splenocytes before killing. Splenocytes or lymphocytes from blood samples were added to ImmunoSpot plates coated with capture antibodies IL-4 and interferon (IFN)- γ . Cells and antibody were incubated in quadruplicate for 4 days (IL-4) or 3 days (IFN- γ). Cells were aspirated, and detection antibody is added and incubated for an additional 24 hours. Substrate avidin-HRP was added and plates were developed for spots. The plates were allowed to dry overnight before they were subjected to image analysis on a Series 3 ImmunoSpot Analyser (Cellular Technology) specifically designed for automated evaluation of ELISPOT results.

Histomorphometric analysis

The entire nerve graft was harvested to include both proximal and distal suture lines. The grafts were fixed in 3% glutaraldehyde (Polysciences Inc., Warrington, PA, USA), washed in 0.1M phosphate buffer (pH 7.2) and post-fixed with 1% osmium tetroxide (Fisher Scientific, Pottsville, PA, USA). After dehydration in graded ethanol solutions, the samples were embedded in Araldite 502 (Polysciences Inc.). One micron midgraft sections were stained in toluidine blue for histomorphometric analysis. Qualitative assessment of neural architecture was followed by binary image analysis for multicomponent analysis of peripheral nerve histomorphometry. Stereology via a two-dimensional nucleator probe was applied to electron microscopy for quantitative ultrastructural analysis. Total myelinated fiber counts were measured based on three representative fields at $\times 1000$ magnification. Percent nerve (nerve area/total area), nerve density (number of fibres/mm²), and fiber width (mm) were calculated. All measurements were made by an observer blinded to the experimental groups.

Statistical analysis

The computer program SigmaStat version 3.0 was used to calculate statistics, and the performance of appropriate statistical procedures was based on the distribution frequency of all of the data. An overall analysis of the differences between group means was calculated by a one-way analysis of variance (ANOVA) for multiple comparisons. If the analysis demonstrated significance, then the means of variables from specific groups were compared using the Student–Newman–Keuls test. In all cases, statistical significance was set at $p < 0.05$.

Results

Gross observation

No complications attributable to immunosuppression were observed in the animals. All animals remained healthy and maintained normal diet and activity. There was no directly observable evidence of rejection.

ELISPOT

ELISPOT results showed that with increasing degrees of costimulatory blockade, cytokine production to donor antigen challenge progressively decreased (Figure 1). In response to donor-strain cells, recipient IFN- γ production correlates directly with the strength of the rejection response and was highest in the untreated allograft controls at 2000 ± 450 spots/million cells. A single dose of CTLA4-Ig was able to reduce the response but which still remained quite high with 1600 ± 500 spots/million cells. Both of these groups showed a significantly greater

response ($p < 0.05$) than the other groups. A course of three doses of MR1 (anti-CD40L mAb) produced a dramatic reduction in the host response to 750 ± 325 spots/million cells. The synergistic effect of combining the blockade of the CD40 and CD28/B7 pathways is seen after only a single dose of each agent with the response reduced further to 400 ± 250 spots/million cells. When three doses of each agent are used, the response is minimal at 75 ± 70 spots/million cells. The blockade of multiple pathways (CD40, CD28/B7, ICOS) demonstrated further synergy with essentially no response at 13.75 ± 12 spots/million cells after three doses of each blocking agent. Three doses of either double or triple blocking antibodies significantly reduced the immune response ($p < 0.05$) compared to all other groups.

Histomorphometry

The histomorphometric data are summarized in Figure 2. The isograft and triple blockade $\times 3$ groups showed significantly greater regeneration than the double blockade and untreated allograft groups at $p < 0.05$. Untreated allograft groups showed essentially no regeneration. The isograft group showed excellent axonal regeneration with a total nerve fiber count of 1960 ± 115 (mean $\pm$ SEM) (Figure 3A). Treatment with a short course of triple costimulation-blocking agents permitted regeneration that was equivalent to the isograft group with an axonal count of 2063 ± 322 (Figure 3B). The group that received double costimulatory blockade (MR1, CTLA4-Ig) showed improved regeneration compared with the untreated allograft (Figure 3C) with a fiber count of 825 ± 265 . A single dose of triple costimulation blockade still provided effective regeneration through the allograft (1436 ± 383) that was not as good as three doses of each agent but better than a short course of double costimulation blockade. Only a single dose of single, double or triple costimulation blockade were less effective and correlated with the strength of the immune response as quantified by the ELISPOT assay blockade and the number of doses administered. Anti-CD40 mAb was more effective than CTLA4-Ig when used alone. At least three doses of three different costimulation-blocking agents appeared to be the threshold at which the redundancy of the T cell response is overcome to allow axonal regeneration equivalent to that seen with an isograft.

Discussion

The redundancy of the immune costimulation system is evidenced by the number of different pathways that have now been described in both the CD40 and CD28/B7 families^{10,21}. Although some particular characteristics of each pathway are known, their exact contribution and significance to the alloimmune response for the most part remains unclear. It has been shown that the blockade of either of the two most significant pathways has a marked effect on the immune response and the blockade of both has a synergistic effect to inhibit acute rejection^{6, 8}. However, complete inhibition of the immune response is less consistent. It would follow that the blockade of additional costimulatory pathways would promote further suppression of the immune response. Because of the effect of anti-ICOS on activated T cells, it appears to provide an additional mechanistic action to the blockade of the CD40 and CD28/B7 pathways, and this is borne out in the peripheral nerve allograft model. While the double blockade of the CD40 and CD28/B7 pathways has permitted lasting survival of organ and composite tissue allografts, its effect seemed to be more modest for regeneration through the peripheral nerve allograft. The blockade of a third costimulatory pathway (ICOS) was necessary before axonal regeneration approached that seen with the nerve isograft. A possible explanation is that the immunosuppressive requirement for optimal nerve allograft function is higher than that for allograft survival, presuming that cellular survival *per se* is necessary but not entirely sufficient for optimal nerve function. The organ allograft differs in that function is dependent on the mass effect of a number of homogeneous cellular units, while the reserve of functioning cells in the nerve allograft is significantly less and therefore more sensitive to the immune response.

The blockade of additional costimulatory pathways may also be useful in the reduction of the dosage of the primary blocking agents to further reduce morbidity and risk^{22,23}. Particularly, the role of CD40 in platelet activation is better understood²⁴ and reduction in the dosage of its monoclonal blocking antibody may help to reduce the risk of thromboembolism which has been noted in the non-human primate model²⁵. There is still much that needs to be understood about how immune costimulation can be manipulated in favor of the allograft. It would be logical that the many costimulation-blocking agents available may be used with the same principles that are exploited when developing regimens of conventional pharmacological immunosuppressive medications to further decrease overall morbidity.

An interesting finding is the discrepancy noted between the ELISPOT and the histomorphometric data in regard to immunosuppressive effect. Based on IFN- γ production, a significant reduction in the host immune response is readily seen with double blockade of the CD40 and CD28/B7 pathways. The short regimen of double and triple costimulation blockade appears 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 double blockade regimen permitting only half as many regenerating axons as the triple regimen or the isograft. There are two 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 type 1 T helper cell activation, and unlike that of muscle and bone, which show a type 2 immune deviation that is more favorable to the allograft. While the quantitative ELISPOT assay accurately reflects the status of the immune response, nerve tissue appears to be much more antigenic than thought and may require more profound immunosuppression (like skin) for satisfactory regeneration and function. Both tissue types share an abundance of an immunologically active cell population, namely, the Langerhan cells of skin and Schwann cells in nerves both act as antigen presenting cells which facilitate the immune response. The second explanation is that the costimulation-blocking agents may have some other effect on the neurological system that is yet to be identified and is independent of their immunosuppressive properties. As such, while double costimulation blockade may be equally immunosuppressive to the acute response as triple blockade, the neurological effect may be further enhanced, possibly in a synergistic manner, with the use of multiple agents.

In summary, the blockade of multiple costimulatory pathways appears to be a promising strategy for the peripheral nerve allograft model. The addition of an agent to block the ICOS pathway provides further synergy with the blockade of the CD40 and CD28/B7 pathways, significantly increasing the degree of axonal regeneration to a level equivalent to that seen in an isograft, and while being equally immunosuppressive by *in vitro* assays. The strategy of costimulation blockade is well suited to the temporary immunosuppressive requirements of the nerve allograft as it provides a period of prolonged unresponsiveness that has been shown to be donor-specific and with a duration that extends well beyond the administration of the last dose. Costimulation-blocking agents are currently in clinical use but in combination with conventional pharmacological medications to permit further reduction in medication dosages and to decrease the well-known risks of non-specific immunosuppression.

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Figure 1.

ELISPOT data showing *in vitro* IFN- γ production ($\pm$ SEM) by host cells in response to culture with donor strain cells. Robust response seen by untreated recipient animals, and progressive unresponsiveness with increasing degrees of costimulation blockade. Double and triple blockade $\times 3$ groups showed a significantly reduced response compared to all other groups, while the allograft and CTLA4-Ig groups had significantly higher responses ($p < 0.05$)

Figure 2.

Quantitative histomorphometric data demonstrating total axon count ($\pm$ SEM) among experimental groups. Recipients treated with triple costimulation blockade showed regeneration comparable to the isograft, with less regeneration with fewer doses or fewer agents. Isograft and triple blockade $\times 3$ groups significantly greater than double blockade and untreated allograft at $p < 0.05$

Figure 3.

Histology specimens (toluidine blue, $\times 1000$). (A) Isograft harvested after 3 weeks showing good axonal regeneration. (B) Allograft from recipient of triple costimulation blockade showing axonal regeneration equivalent to isograft group. (C) Untreated allograft showing poor regeneration

Table 1
Summary of experimental groups, agents used and administration protocol

Group	No.	Treatment	Target pathway	Dose (mg)	Regimen
Isograft I	6	None	n/a		
Triple II	5	CTL A4-Ig	CD28/B7	0.5	3 doses each POD 0, 2 and 4
		MR1	CD40/CD40L	1.0	
		Anti-ICOSL	ICOS/ICOSL	0.5	
Triple III	5	CTL A4-Ig	CD28/B7	0.5	1 dose each POD 0
		MR1	CD40/CD40L	1.0	
		Anti-ICOSL	ICOS/ICOSL	0.5	
Double IV	5	CTL A4-Ig	CD28/B7	0.5	3 doses each POD 0, 2 and 4
		MR1	CD40/CD40L	1.0	
		None	n/a		

Table 2
Summary of histomorphometric data from specimens of mid-graft harvested after 3 weeks following surgery

Group	Total no.	Fiber width (μm)	Nerve density(fibers/ mm^2)	Percent fiber
Isograft	1960 $\pm$ 115	2.82 $\pm$ 0.04	12860 $\pm$ 868	10.4 $\pm$ 0.62
Triple $\times$ 3 doses	2063 $\pm$ 322	2.87 $\pm$ 0.12	7236 $\pm$ 1170	6.41 $\pm$ 1.12
Triple $\times$ 1 dose	1436 $\pm$ 383	2.68 $\pm$ 0.19	8722 $\pm$ 2434	7.15 $\pm$ 2.14
Double $\times$ 3 doses	825 $\pm$ 265	2.76 $\pm$ 0.05	9281 $\pm$ 3043	7.14 $\pm$ 2.3
Allograft	209 $\pm$ 116	1.72 $\pm$ 0.57	1616 $\pm$ 997	1.46 $\pm$ 0.96