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# Simultaneous inhibition of B7 and LFA-1 signaling prevents rejection of discordant neural xenografts in mice lacking CD40L

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Abstract: Transplantation of embryonic human neural tissue can restore dopamine neurotransmission and improve neurological function in patients with Parkinson's disease. Logistical and ethical factors limit the availability of human embryonic allogeneic tissue. Embryonic xenogeneic neural tissue from porcine donors is an alternative form of donor tissue, but effective immunomodulatory techniques are warranted for neural xenotransplantation to become clinically feasible. We transplanted embryonic porcine ventral mesencephalic tissue into the brains of adult untreated C57BL/6 mice, untreated CD40L-/–mice and CD40L-/–mice that received injections of anti-LFA-1, CTLA4Ig or both compounds. Double-treated CD40L-/–mice had large grafts with high numbers of dopaminergic neurons 4 wk after transplantation. The grafts were completely devoid of lymphocytes, macrophages and activated microglia. Untreated C57BL/6 mice had rejected their grafts. Untreated CD40L-/–mice and CD40L-/–mice treated with monotherapy of anti-LFA-1 or CTLA4Ig had smaller grafts and more microglial and lymphocytic infiltration than double-treated CD40L-/–mice. We conclude that immunomodulation with concomitant inhibition of LFA-1 and B7 signaling in the perioperative period in CD40L-/–mice prevented the rejection of discordant neural xenografts. The treatment most likely reduced antigen presenting capacity and interfered with the costimulatory signaling needed for T cell activation to occur.

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Key words: CD40 ligand – CTLA4Ig – embryonic – LFA-1 – neural tissue grafts – porcine – Parkinson's disease

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## Introduction

Parkinson's disease is the second most common neurodegenerative disorder causing significant morbidity and cost to society. Although drug treatments are available they provide only symptomatic relief, which, after about a decade, dwindle in efficacy in the majority of patients [1]. Patients who contract the disease at a young age, arbitrarily at age 45 or younger, have an estimated 30–35 yr expected survival time after diagnosis with considerable handicap commonly ensuing within a few years. Embryonic neural tissue can integrate into the host brain, grow axonal nerve fibers and restore neuroneal circuits, with reformation of lost synaptic connectivity [2]. Symptomatic relief, and restoration of dopamine transmission has been demonstrated in animal experiments, in patients with toxin-induced dopamine depletion [3] and in patients with an active Parkinson's disease, the latter for up to 10 yr after grafting [4–6]. In the best cases, complete restoration of the dopaminergic synthesis and normalized synaptic release have been demonstrated in vivo using imaging techniques [7].

Logistical and ethical factors limit the availability of human embryonic allogeneic tissue, which is usually obtained from induced abortions. Alternative sources of donor tissue include various forms of stem or progenitor cells or xenogeneic embryonic tissue [8,9]. Stem cells need to be differentiated into a stable non-dividing, dopaminergic neuroneal phenotype, and to integrate in the complex neuroneal circuitries in the basal ganglia in order to relieve the symptoms of

*LL and MC contributed equally to this study.

Parkinson's disease. So far, this is not possible for human stem cells or precursor cells. Primary embryonic neural tissue is obtained from the ventral part of the mesencephalon, which constitutes the anlage for the substantia nigra in the adult brain, and contains the dopaminergic neurons that degenerate in Parkinson's disease. Embryonic ventral mesencephalic tissue from another species, such as the pig, could provide a readily available source of cells.

Currently, a double blinded, sham surgery controlled study using embryonic porcine donor tissue is ongoing in the US. This study is in part based on the findings from a recently completed first clinical trial with porcine neural tissue grafted into Parkinson's disease patients. Three of the 12 patients in the first study improved significantly and there were no adverse events related to the use of porcine tissue in these patients [10]. No transmission of porcine endogenous retrovirus (PERV) was detected in any of the transplanted patients [11]. Post-mortem analysis of the brain of one deceased patient that died from a pulmonary embolism 7 months after transplantation, revealed 638 surviving transplanted dopamine cells [12]. It is estimated that 80,000 cells is the minimum number required for functional effects to occur [4]. The patient was treated with cyclosporine A as a mono-therapy, and there were infiltrates of T-lymphocytes indicative of a completed or on-going rejection process. It is thus possible that the lack of significant clinical improvement in several patients in the study was due to immunological rejection of the tissue. Effective immunomodulatory techniques are thus warranted for neural xenotransplantation.

The brain is considered to be an immunologically privileged site where immune reactions are more restricted than in the rest of the body [13]. There are several contributing factors, including unconventional lymphatic drainage and possibly less effective antigen presentation in the brain, but grafts placed in the brain parenchyma do immunize recipients. A blood–brain barrier restricts passage across the endothelium to activated lymphocytes only, and restricts the passage of antibodies, cytokines, chemokines, and other effectors of the immune system [13]. Nevertheless, it is clear that manipulations of the host immune system, and possibly the donor tissue, are needed to permit stable neural xenograft survival [14,15]. Several studies have been performed in order to characterize the immune response against discordant neural xenografts, as reviewed in Brevig et al. [16]. It has been shown that xenograft rejection was delayed up to 4 wk in both immunoglobulin- and CD1.1-deficient mice, but eventually occurred in all animals [17,18]. In contrast to organ xenografting, NK cells do not seem to be important for the rejection process against a cellular graft in the brain [18]. CD4- and CD8-positive lymphocytes, microglia and macrophages are found in high numbers in rejecting transplants and are likely to participate in the rejection process. Conventional immunosuppression with cyclosporine A has proven inefficient in the pig-to-rat model of discordant neural xenografting [14,15]. In a meta-analysis, the rate of neural xenograft survival with cyclosporine A monotherapy was around 75%, but most of the models included in the meta-analysis were concordant [9]. Combinations of immunosuppressive drugs are more effective [19], but chronic systemic immunosuppression carries risks of opportunistic infections and tumor development. Since Parkinson's disease is not life threatening and the treatment perspective should be very long, a selective short-term immunotherapy would be desirable.

B7 (CD80/86) – CD28/CTLA4 and CD40-CD40L (CD154) receptor-ligand pairs are independent signaling pathways that are essential to the initiation of immune responses. B7-CD28/CTLA4 provides costimulatory signals for optimal T cell activation and IL-2 production [20]. Interference with the costimulatory pathways may lead to unresponsiveness or anergy [20]. CD40-CD40L not only costimulates T cells, B cells and endothelial cells, but also macrophages and the bone marrow derived equivalent cell type in the brain, microglia [21,22]. There is a reciprocal activation of the effector cell and the stimulator, antigen presenting cell, facilitating the development of induced immune responses.

We have previously shown in highly immunogenic skin graft models that rejection can be mediated by CD8-positive T cells independent of B7 and CD40L blockade [23]. It has also been shown that porcine neurons can stimulate human CD8-positive lymphocytes in vitro [24]. LFA-1, binding to its ligand ICAM-1, is an important adhesion molecule but also functions in costimulation of T-cells. Anti-LFA-1 antibodies have been effective in inhibiting the proliferation as well as the cytotoxicity of CD8-positive T cells, which implies it may be able to synergize in the prevention of xenograft rejection [25]. In a recent study, injections with anti-LFA-1 antibodies were shown to lead to long-term acceptance of islet allografts in a mouse model [26]. The purpose of our current study was to investigate costimulatory

requirements necessary for the rejection of discordant porcine neural xenografts.

## Materials and methods

### Experimental design

Neural xenografts were transplanted into adult C57BL/6 (Möllegaard, Köge, Denmark) (n=6) and CD40*L*-/–mice (C57BL/6 background, Jackson Laboratories, Bar Harbor, Maine, USA) (n=30) that were maintained with free access to fresh water and food. The CD40L-/–mice were divided into four groups of which one remained untreated (n=9) and the other three received i.p. injections of anti-LFA-1 (n=6) (clone M17/5.2, Bio Express Inc., West Lebanon, New Hampshire, USA), CTLA4Ig (n=6) (kind gift from Diane Hollenbaugh at Bristol-Myers, Seattle, Washington, USA) and both compounds (n=9) on the day of transplantation and every second day for the first 10 days after transplantation. The dosages were 0.2 mg of anti-LFA-1 and 0.5 mg of CTLA4Ig. Three mice died in the double-treated group, two directly at the time of i.p. injection, due to misplaced injections, and one 3 wk post-transplant, due to pneumonia. The grafts in these mice were not analyzed. All other mice remained healthy throughout the study.

### Donor tissue and transplantation

The ventral mesencephalon from 26-day-old pig embryos (Swedish Landrace×Yorkshire/Hampshire) was dissected, cut in small pieces and incubated in trypsin 0.1% (Worthington, Lakewood, New Jersey, USA) at 37° for 20 min. The tissue was rinsed, 0.05% DNAse (Sigma, Sweden) was added, and then dissociated using fire polished Pasteur pipettes into a crude cell suspension, containing single cells and small cell aggregates. Dissections and rinses were performed in Hank's balanced salt solution (Gibco, Life Tech, Täby, Sweden) with 3.0 μm tirilazad (Pharmacia & Upjohn, Kalamazoo, Michigan, USA). Each mouse received tissue approximately equivalent to 1/4 ventral mesencephalon. Viability of the cell suspension was >95% at the end of the transplantation session as determined by trypan blue exclusion. Recipients were anesthetized with Rompur-Ketalar® i.p. and put in a stereotactic frame (D. Kopff Industries, Tujunga, CA, USA) with the toothbar set at zero. The skull was exposed and a 1.0-mm-wide hole was drilled. Two μl of the cell suspension were injected in the right striatum using a Hamilton syringe (Hamilton Co., Reno, NV, USA) fitted with a metal canula, outer diameter 0.47 mm.

### Histological processing

The mice were transcardially perfused with saline followed by 75 ml of 4% paraformaldehyde under deep pentobarbital anesthesia. The brains were taken out and placed in paraformaldehyde for an additional 2 h, then put in 20% sucrose (dissolved in 0.1 m phosphate buffer) overnight. They were frozen and coronal sections were cut at 30 μm on a sliding microtome. Immunohistochemical staining was performed as described earlier as free floating specimens [17,18], with rabbit anti-tyrosine hydroxylase (TH, a marker for dopaminergic neurons) 1 : 500 (Pel-Freez, Rogers, Ark., USA), CD4 1 : 250 (KT174, Serotec, Oxford, UK), CD8 1 : 500 (KT15, Serotec) and F4/80 (a marker for mouse microglia cells) 1 : 400 (CI:A3–1, Serotec) as primary antibodies. The secondary antibody for the TH-antibody was biotinylated swine anti-rabbit immunoglobulin (Dakopatts, Copenhagen, Denmark) and for the other primary antibodies biotinylated rabbit anti-rat immunoglobulins (Dakopatts). The sections were then incubated in ABC solution (Vectastain ABC kit, Vector Laboratories, Burlingame, CA, USA) for 1 h and developed in 3,3′-diaminobenzidine and hydrogen peroxide (DAB kit, Sigma). All rinses and dilutions were performed in PBS. The free-floating sections were mounted on glass slides, dehydrated in ethanol, defatted in xylene and coverslipped using DePeX mountant (Kebo Lab, Spanga, Sweden).

### Optical methods

Microglial activation and accumulation was measured using a computer assisted image analysis system described earlier [15,17]. The F4/80 stained sections were digitized using a 1.6x objective lens in a Nikon light microscope (Nikon, Japan), which is connected with a high resolution 3CCD color videocamera module (XC-003P, Sony, Japan), and the software package Image-grabber-34 (Neotech Inc., Naperville, Illinois, USA) in a Power Macintosh 7100/80 computer. In each section, the striatum on both sides was manually delineated on the screen and the optical densities were assessed. The background optical densities were also determined for each glass slide, in two areas – the corpus callosum and in the ventricle – and subtracted in order for all sections to be normalized. The optical density in the transplanted striatum was expressed as a percentage of the optical density in the intact striatum. Light conditions were fixed during the digitizing process. The three sections with the highest optical density in each mouse, located around the center

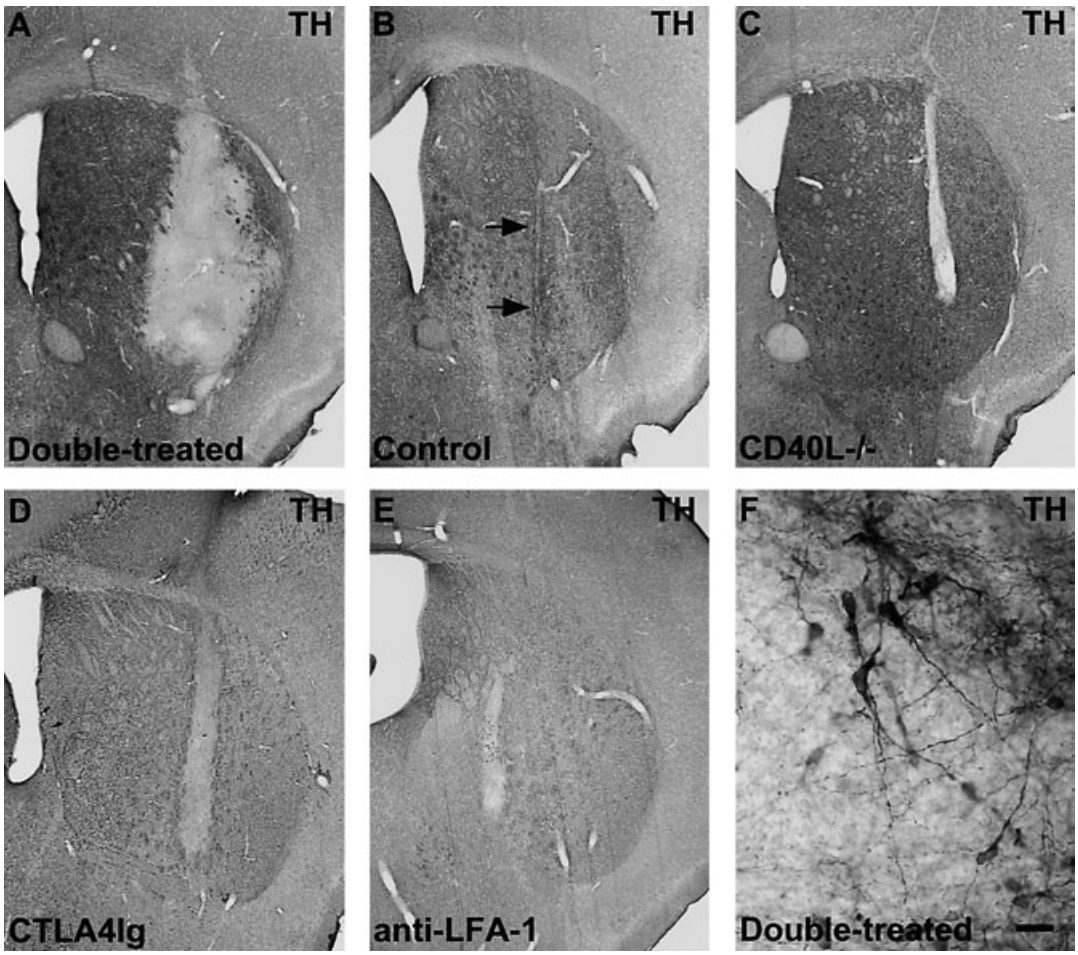


*Fig. 1.* Coronal brain sections stained for tyrosine hydroxylase (TH), showing the porcine neural tissue grafts in the right striata. The striatum surrounding the graft stains positively (dark) for TH since the enzyme is present in the endogenous nerve terminals of the mice. The porcine tissue is white since no such diffuse TH-expression is present, but higher magnification reveals the presence of porcine neurons specifically expressing TH and extending axons. (A) A CD40L-/–mouse which was treated with both CTLA4Ig and anti-LFA-1. (B) The scar in a control C57BL/six mouse, marked by the arrows. (C) An untreated CD40L-/–mouse with a small graft volume. (D) A CD40L-/–mouse that received CTLA4Ig. (E) A CD40L-/–mouse that received the anti-LFA-1 antibody. (F) High magnification of the TH-positive neurons in the transplant in a double-treated CD40L-/–mouse. The fiber extension from the neurons is visible. Scale bar = 20 μm.

of the graft or the needle track, were selected for statistical comparisons.

### Stereological and statistical methods

The graft volumes and the number of TH-positive cells were obtained using a stereological analysis system described earlier [15]. A CAST-Grid software package (Interactivision, Silkeborg, Denmark) was used to obtain cell counts by randomly and systematically placing a counting frame over the delineated transplant. The number of TH-positive cells within the frame was counted and the total number was obtained using a stereological algorithm [27]. The graft volumes were calculated by delineating the graft-host border and measuring the thickness on the TH stained serial sections.

Statistical analyzes were performed using the Statview 4.51 program. In order to allow for pooling of the experiments that were performed on different days with different donor tissue preparations, a two-factor anova was performed to detect any systematic differences between the graft preparations and procedures. No such difference was detected and data from the two sessions were pooled. Kruskall–Wallis anova followed by Mann–Whitney *U*-tests corrected for multiple comparisons were used to study differences in graft size and dopaminergic cell counts, due to the non-parametrical distribution of data with complete rejection of some grafts. The significance

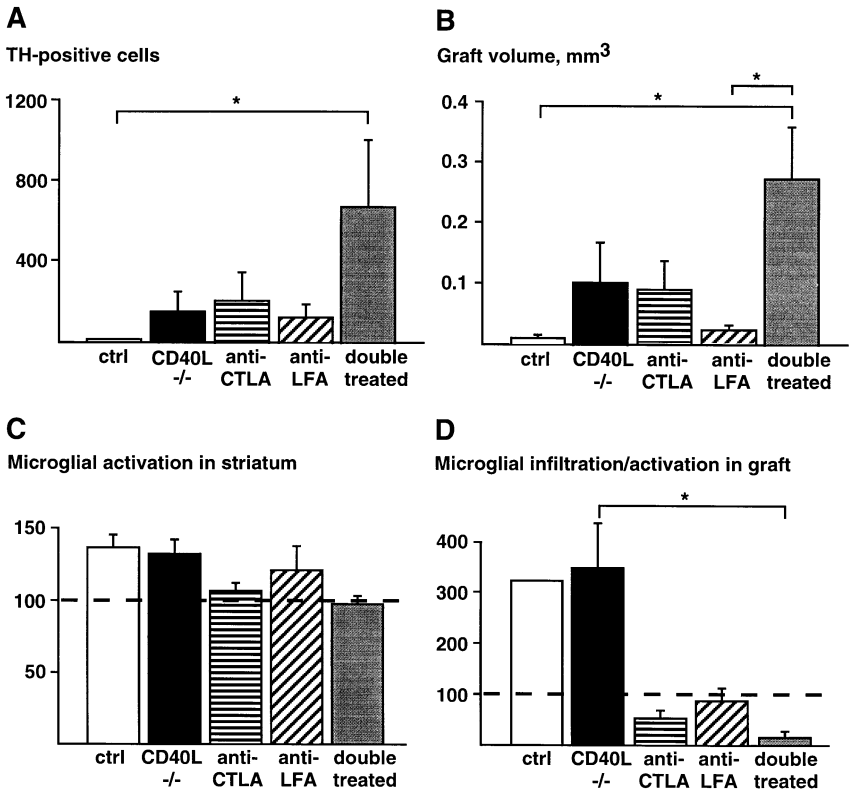


*Fig. 2.* (A) Number of dopaminergic neurons in the porcine neural tissue grafts. Kruskall Wallis anova showed significant differences between the groups for the number of dopaminergic neurons in the grafts ($H_{(4,13.4)}=0.009$, $P<0.05$). Mann–Whitney U-tests corrected for multiple comparisons detected differences between the double-treated CD40L-/-mice and controls ($P=0.002$). (B) Graft volume. There were significant differences between the groups also for graft volume ($H_{(4,15.9)}=0.003$, $P<0.05$, Kruskall-Wallis ANOVA). The grafts in the double-treated CD40L-/–mice were significantly larger than the grafts in the control mice ($P=0.004$, Mann–Whitney U-test) and in the CD40L-/-;mice that received anti-LFA-1 monotherapy ($P=0.004$, Mann–Whitney U-test). (C) Activation of host microglia cells in the striatum surrounding the grafts. There was no activation of microglia in the double-treated group (100% is the background value, i.e. the degree of activation in the striatum on the contralateral side). In the other groups, microglia were activated. (D) Infiltration of host microglia into the porcine grafts. There was significantly less microglial infiltration in the grafts of the double-treated group compared with untreated CD40L-/–mice (One-way ANOVA $F_{3,18}0.05$, followed by Scheffée's posthoc test, $P=0.003$). The infiltration from the surrounding striatum was very low in the double-treated, anti-LFA-1 treated and CTLA4Ig treated groups, which is shown in the diagram by optical density measurements less than 100% of the intact striatum. The untreated C57BL/6 group was excluded from the statistical analysis since only one mouse in this group had a surviving graft.

level for the Mann–Whitney *U*-tests was set at $P=0.005$. The optical density measurements of microglial activation were compared using a parametrical anova, followed by Scheffée's post-hoc test, significance level $P=0.05$.

## Results

### Recipient health and survival

Three recipients died, two due to direct complications of misplaced i.p. injections and one, in the double treated group, due to pneumonia. CD40L/–mice are known to be susceptible to pneumonia caused by pneumocystsis carinii. This susceptibility was most likely increased by the complete interruption of the costimulation to which the recipient mouse was subjected during the treatment period. There may thus be a need for prophylactic antibiotic treatment during the period of costimulation blockade. However, there were no indications of an excessively immunocompromized state in the other recipients in this study.

### Graft survival

All CD40L-/–mice that were double-treated with anti-LFA-1 and CTLA4Ig had large grafts

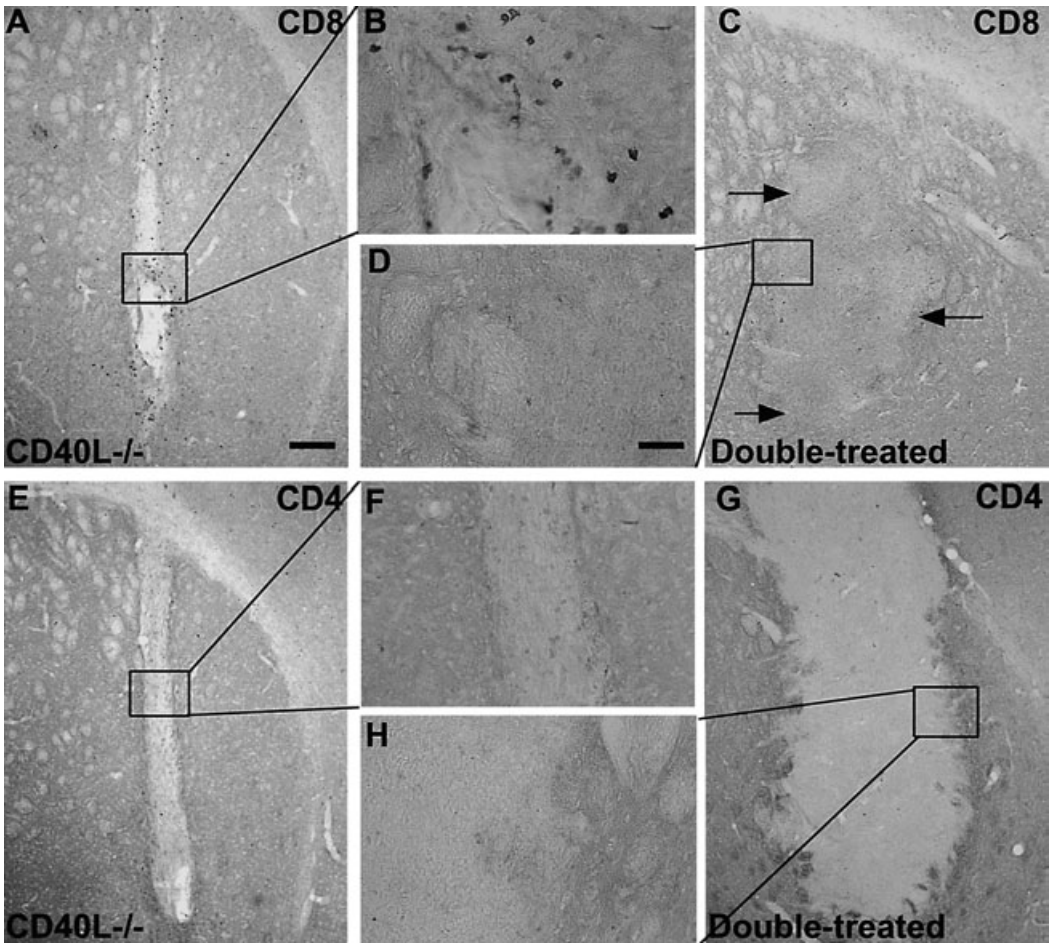


*Fig. 3.* Immunohistochemical staining of mouse CD8 and CD4 expression in the grafts. (A) CD8-positive cells are present in a graft in the CD40L-/–group. Scale bar=200 μm. (B) Higher magnification reveals specific staining on cells with a lymphocytic morphology. (C) No such cells are visible in the grafts (marked by the arrows) in the double-treated group. (D) High magnification of the squared area in (C). Scale bar=50 μm. (E) No CD4-positive cells are present in the CD40L-/–mice. (F) Higher magnification reveals light staining on cells in the graft area, due to remaining endogenous peroxidase activity. However, no specific staining is observed. The cells are likely to be neutrophils. (G) No specific CD4 positive cells are seen in the graft in the double treated CD40L-/–mouse (H) as further shown in high magnification.

($0.27 \pm 0.09$ μl, mean $\pm$ SEM) with high numbers of neurons staining positively for tyrosine hydroxylase [$674 \pm 324$] at 4 wk (Figs 1 and 2). These grafts were fiber rich and had a normal neuroneal architecture, indicative of an uncompromised development of the neurons (Fig. 1A,F). The grafts in the double-treated mice were significantly larger ($P=0.004$, Mann–Whitney's *U*-test) and contained more TH-positive cells ($P=0.002$) than the grafts in the control mice ($0.007 \pm 0.006$ μl). Scars were found in all control mice except one, where a very small graft was present, and none of the control mice had any TH-positive neurons. This is consistent with our previous studies where porcine neural xenografts in untreated wild-type mice have invariably been rejected within 2 wk [17,18]. In the untreated CD40L-/–mice and the CD40L-/–mice that received mono-therapy of either anti-LFA-1 or CTLA4Ig, graft condition was more variable. Grafts containing TH-positive cells were present in the majority of the mice in each of these groups, but scars or very small grafts were present in two mice in each group.

There was a complete absence of infiltrating CD4- or CD8-positive lymphocytes in the grafts of the double-treated CD40L-/–mice (Fig. 3). This is in contrast to our previous studies of porcine neural xenografts in wild-type mice and rats, where large numbers of lymphocytes infiltrated the grafts and were present in cuffs around dilated blood vessels [15,17,18]. No such dilated vessels were observed in this study, except in one untreated control mouse with an ongoing rejection. Other control mice contained scars, indicative of a completed rejection process at 4 wk. There was only minimal infiltration of CD4-positive cells in all untreated CD40L-/–mice and the CD40L-/–mice that received anti-LFA-1 or CTLA4Ig. However, in three out of

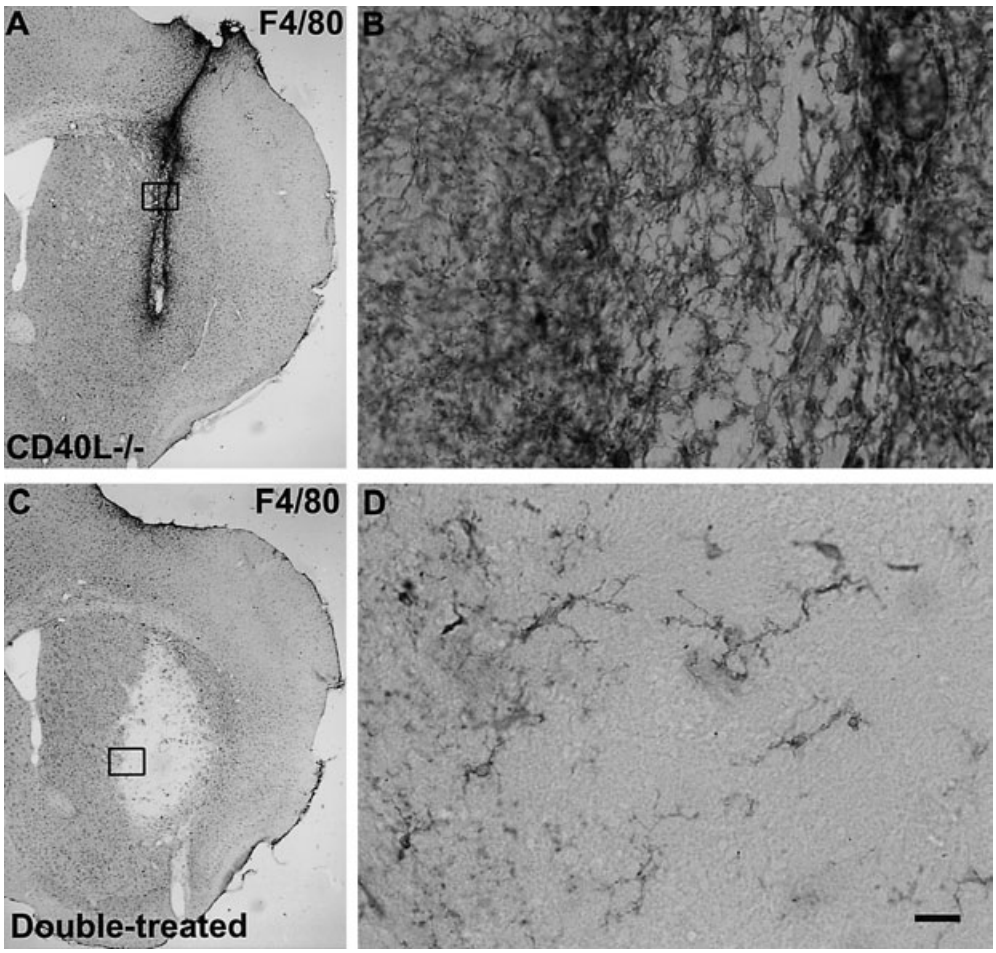


*Fig. 4.* Reactivity of macrophages and microglia in and around the grafts. (A) F4/80 staining of microglia in a CD40L-/– mouse. There is a high degree of microglial activation around the graft. (B) The mouse microglial cells have also migrated into the porcine graft, as seen in higher magnification. (C) Only minimal infiltration of microglia cells in a double-treated CD40L -/– mouse. The porcine graft is markedly lighter than the surrounding striatum. (D) Higher magnification of the squared area in (C) reveals a few microglial cells, weakly positive for F4/80 that have migrated into the graft area. Scale bar=20 μm.

nine untreated CD40L-/–mice, substantial amounts of CD8-positive cells were found to be infiltrating the grafts (Fig. 3A,B).

F4/80 is an immunohistochemical marker, which is expressed on resting mouse microglia in small amounts and is upregulated during activation [28]. Porcine microglia do not express this marker so all F4/80 staining in the grafts is of recipient origin. There was no activation of microglia or macrophages in the striatum surrounding the grafts in the double-treated CD40L-/–mice, as determined by optical density measurements of the F4/80 staining (Fig. 4). This was in contrast to the other groups in the study, where an increased activation of microglia was observed. In addition, there was only very limited infiltration of host microglia into the grafts of the double-treated group (Fig. 4). Graft infiltration was also low in the CD40L-/–mice that received monotherapy of CTLA4Ig or anti-LFA-1, in contrast to the extensive infiltration seen in the untreated CD40L-/–mice and in the rejecting graft in the untreated C57BL/6 mouse. The graft infiltration of microglial cells was significantly lower in the double-treated CD40L-/–mice than in the untreated CD40L-/–mice ($P=0.003$, Scheffée's post hoc test, Fig. 2). The controls could not be included in the comparative tests of microglial graft infiltration, since graft tissue was found in only one control mouse.

## Discussion

We describe the outcome after implantation of embryonic neural tissue from pig ventral mesencephalon into the brains of adult mice, as a means to transplant dopaminergic tissue for repair strategies in Parkinson's disease. In this cellular discordant xenogeneic donor-recipient graft combination, untreated recipients invariably reject their grafts. In the CD40L-/–mice there is a defect signaling in the activation of T cells and B cells,

and antigen presenting cells, but additional costimulatory factors seem to be able to take over when CD40/CD40L signaling is defective and rejection can still occur. Even when both CD40-CD40L and B7(CD80/86), or CD40-CD40L and LFA-1/ICAM-1 are interfered with, there may be xenograft rejection in the brain. When all three costimulatory factors (B7 and LFA-1 by antibody treatment, in CD40L-/–mice) are inhibited there was increased graft volume and the number of cells staining positively for TH was high. Moreover, there was no immunohistochemical evidence of lymphocytic infiltration or of activated microglia in the grafts of the double-treated group. TH, tyrosine hydroxylase, is an enzyme necessary for the production of dopamine, the neurotransmittor that is deficient in Parkinson's disease. We have shown in previous studies that the porcine neural grafts mature over time, with increased TH expression and outgrowth of axons. We have demonstrated that the porcine grafts are able to restore function in the rat model of Parkinson's disease, after a maturation process that takes between 6 and 9 wk [15]. In that study, we immunosuppressed the rats with a high dose of cyclosporine A, and around 50% of the grafts were rejected at 14 wk. Only the rats with good graft survival, that is with a low degree of lymphocytic infiltration and a healthy morphology with high numbers of TH expressing neurons, recovered in the behavioral test. Minimizing the immune responses to the neural xenografts is thus crucial for good graft survival and functional effects. In the current study using costimulation blockade, the mice were sacrificed at 4 wk after grafting and therefore we did not expect to see completely mature grafts. The large grafts in the mice that received the double treatment contained an average of 700 TH expressing neurons, which is a high number compared with our previous studies in mice hosts with a survival time of 4 and 5 wk. In these studies, using immunoglobulin-, CD1d1-deficient and NK-cell depleted mice, the average number of TH expressing neurons has been between 0 and 10 at 4–5 wk [17,18]. However, it is possible that in long-term studies with costimulation blockade, allowing a complete maturation of the porcine grafts, the numbers of TH expressing neurons would be even higher than in the current study. Such long-term studies are ongoing.

The results in this study have implications not only for neural xenograft rejection but for transplantation as a whole. The histological data indicate that the addition of anti-LFA-1, possibly by interfering with the effector phase or the maturation of CD8 cells, to the standard costimulation blockade of CD4 cells (CTLA4Ig+ anti-CD40L) may prolong survival and improve function of allografts and other types of xenografts. We have previously shown that treatment with CTLA4Ig and anti-CD40L only had a moderate effect on the rejection of allogeneic skin grafts in C57BL/six mice [23]. We also showed that this rejection was mediated by CD8-positive T cells that expressed asialo-GM-1 [23]. Recently it was demonstrated that mouse CD8-positive cells could reject porcine islet xenografts in the absence of CD4-positive cells [29]. Furthermore, it has been shown that LCMV (lymphocytic choriomeningitis virus) has the potential to induce a vigorous immune response in CD40L-/–mice and that this response is mediated by CD8-positive T cells [30]. Thus, there may be two major pathways to induce cellular rejection. The first is inhibited by CTLA4Ig and anti-CD40L and is found isolated in C3H mice [31]. The second pathway is mediated by CD8-positive T cells and may be inhibited by the addition of anti-LFA-1. This concept extends not only to neural xenotransplantation, but could potentially be applied to other immunological phenomena such as the optimal signaling requirement for an anti-tumor response or strategies to re-educate a self-destructive immune system.

Demonstration of similar effects as observed in the CD40L-/–animals, by antibody treatment to wild type mice is underway. We have not investigated if the animals have developed tolerance to the implanted porcine tissue. By grafting into an immunologically privileged site, and manipulating the recipient response, a type of peripheral tolerance may develop. Previous examples of such effects include an indefinite survival of concordant neural xenografts with depletion of CD4-positive cells, and specific tolerance induction to neural allografts using antibody injections to the IL-2 receptor [32,33]. If tolerance develops our model will be assessed in future studies.

In conclusion, large grafts with high numbers of dopaminergic neurons were observed at 4 wk post-transplant in CD40L-/–mice after an initial treatment with CTLA4Ig and anti-LFA-1. The grafts did not demonstrate any immunohistochemical evidence of lymphocytes, macrophages or activated microglia. These findings may lead to the development of short-term regimens to selectively inhibit the immune response so that porcine tissue may survive in patients without the risks associated with chronic immunosuppression.

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## References


1. Schapira AHV. Parkinson's disease. BMJ 1999; 318: 311.
2. Dunnett SB, Björklund A. Prospects for new restorative and neuroprotective treatments in Parkinson's disease. Nature 1999; 399: A32.
3. Langston JW, Forno LS, Tetrud J et al. Evidence of active nerve cell degeneration in the substantia nigra of humans years after 1-methyl-4-phenyl-1, 2,3, 6–tetrahydropyridine exposure. Ann Neurol 1999; 46: 598.
4. Björklund A, Lindvall O. Cell replacement therapies for central nervous system disorders. Nat Neurosci 2000; 3: 537.
5. Widner H, Tetrud J, Rehncrona S et al. Bilateral fetal mesencephalic grafting in two patients with severe parkinsonism induced by 1-methyl-4-phenyl-1, 2,3, 6–tetrahydropyridine (MPTP). N Engl J Med 1992; 327: 1556.
6. Kordower JH, Freeman TB, Snow BJ et al. Neuropathological evidence of graft survival and striatal reinnervation after the transplantation of fetal mesencephalic tissue in a patient with Parkinson's disease. N Engl J Med 1995; 332: 1118.
7. Piccini P, Brooks DJ, Bjorklund A et al. Dopamine release from nigral transplants visualized in vivo in a Parkinson's patient. Nat Neurosci 1999; 2: 1137.
8. Gage FH. Mammalian neural stem cells. Science 2000; 287: 1433.
9. Pakzaban P, Isacson O. Neural xenotransplantation: reconstruction of neuronal circuitry across species barriers. Neuroscience 1994; 62: 989.
10. Schumacher JM, Ellias SA, Palmer EP et al. Transplantation of embryonic porcine mesencephalic tissue in patients with PD. Neurology 2000; 54: 1042.
11. Dinsmore JH, Manhart C, Raineri R, Jacoby DB, Moore A. No evidence for infection of human cells with porcine endogenous retrovirus (PERV) after exposure to porcine fetal neuronal cells. Transplantation 2000; 70: 1382.
12. Deacon T, Schumacher J, Dinsmore J et al. Histological evidence of fetal pig neural cell survival after transplantation into a patient with Parkinson's disease. Nat Med 1997; 3: 350.
13. Widner H, Brundin P. Immunological aspects of grafting in the mammalian central nervous system. A review and speculative synthesis. Brain Res Rev 1988; 13: 287.
14. Galpern WR, Burns LH, Deacon TW, Dinsmore J, Isacson O. Xenotransplantation of porcine ventral mesencephalon in a rat model of Parkinson's disease: functional recovery and graft morphology. Exp Neurol 1996; 140: 1.
15. Larsson LC, Czech KA, Brundin P, Widner H. Intrastriatal ventral mesencephalic xenografts of porcine tissue in rats: immune response and functional effects. Cell Transplant 2000; 9: 261.
16. Brevig T, Holgersson J, Widner H. Xenotransplantation for CNS repair: immunological barriers and strategies to overcome them. Trends Neurosci 2000; 23: 337.
17. Larsson LC, Czech KA, Widner H, Korsgren O. Discordant neural tissue xenografts survive longer in immunoglobulin deficient mice. Transplantation 1999; 68: 1153.
18. Larsson LC, Anderson P, Widner H, Korsgren O. Enhanced survival of porcine neural xenografts in mice lacking CD1d1; but no effect of NK1.1 depletion. Cell Transplantation 2001 (in press).
19. Wennberg L, Czech KA, Larsson LC, Mirza B, Bennett W, Song Z, Widner H. Effects of immunosuppressive treatment on host responses against intracerebral porcine neural tissue xenografts in rats. Transplantation 2001 (in press).
20. Schwartz RH. Costimulation of T lymphocytes: the role of CD28, CTLA-4, and B7/BB1 in interleukin-2 production and immunotherapy. Cell 1992; 71: 1065.
21. Grewal IS, Flavell RA. CD40 and CD154 in cell-mediated immunity. Ann Rev Immunol 1998; 16: 111.
22. Tan J, Town T, Paris D et al. Microglial activation resulting from CD40–CD40L interaction after beta-amyloid stimulation. Science 1999; 286: 2352.
23. Trambley J, Bingaman AW, Lin A et al. Asialo GM1 (+) CD8 (+) T cells play a critical role in costimulation blockade-resistant allograft rejection. J Clin Invest 1999; 104: 1715.
24. Brevig T, Kristensen T, Zimmer J. Expression of major histocompatibility complex antigens and induction of human T lymphocyte proliferation by astrocytes and macrophages from porcine fetal brain. Exp Neurol 1999; 159: 474.
25. Deeths MJ, Mescher MF. ICAM-1 and B7–1 provide similar but distinct costimulation for CD8+ T cells, while CD4+ T cells are poorly costimulated by ICAM-1. Eur J Immunol 1999; 29: 45.
26. Nicolls MR, Coulombe M, Yang H, Bolwerk A, Gill RG. Anti-LFA-1 therapy induces long-term islet allograft acceptance in the absence of IFN-gamma or IL-4. J Immunol 2000; 164: 3627.
27. West MJ, Slomianka L, Gundersen HJG. Unbiased stereological estimation of the total number of neurons in the subdivisions of the rat hippocampus using the optical fractionator. Anat Rec 1991; 231: 482.
28. Andersson PB, Perry VH, Gordon S. The kinetics and morphological characteristics of the macrophage-microglial response to kainic acid-induced neuronal degeneration. Neuroscience 1991; 42: 201.
29. Yi S, Feng X, Hawthorne W et al. CD8+ T cells are capable of rejecting pancreatic islet xenografts. Transplantation 2000; 70: 896.
30. Whitmire JK, Slifka MK, Grewal IS, Flavell RA, Ahmed R. CD40 ligand-deficient mice generate a normal primary cytotoxic T-lymphocyte response but a defective humoral response to a viral infection. J Virol 1996; 70: 8375.
31. Larsen CP, Elwood ET, Alexander DZ et al. Long-term acceptance of skin and cardiac allografts after blocking CD40 and CD28 pathways. Nature 1996; 381: 434.
32. Wood MJ, Sloan DJ, Wood KJ, Charlton HM. Indefinite survival of neural xenografts induced with anti-CD4 monoclonal antibodies. Neuroscience 1996; 70: 775.
33. Wood MJA, Sloan DJ, Dallman MJ, Charlton HM. Specific tolerance to neural allografts induced with an antibody to the interleukin 2 receptor. J Exp Med 1992; 117: 597.