

Costimulation Blockade Induces Foxp3⁺ Regulatory T Cells to Human Embryonic Stem Cells

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Abstract

Transplantation of human embryonic stem cells (hESCs), like other allogeneic cellular transplants, require immunomodulation or immunosuppression in order to be maintained in the recipient. Costimulation blockade applied at the time of transplantation inhibits costimulatory signals in the immunological synapse leading to a state of anergy in the donor reactive T-cell population and a state of immunological tolerance in the host. In models of solid organ transplantation, tolerance is maintained by the infiltration of Foxp3⁺ regulatory T cells into the graft. In order to study if regulatory T cells could be generated to hESC transplants, costimulation blockade (CTLA4Ig, anti-CD40L, anti-LFA-1) was administered for the first week after transplantation of two different hESC lines implanted under the kidney capsule of wild-type mice. hESC transplants were maintained indefinitely, and when harvested at long-term follow-up, Foxp3⁺ T-cells were found surrounding the graft, implying the maintenance of tolerance through the induction of regulatory T cells. These results imply that costimulation blockade could be a useful treatment strategy for the induction of tolerance to hESC transplants and may down-modulate immune responses locally around the graft.

Key words: immunology; immunotherapy; monoclonal antibodies; stem cells

Introduction

HUMAN EMBRYONIC STEM CELL (hESC) transplantation is believed to have a therapeutic potential for a number of chronic and degenerative diseases. Although the understanding of the biology of hESCs is growing at an exponential rate, the immunological responses toward these cells necessitates further investigation. Initially it was reported that hESCs would not succumb to immunological rejection or they had decreased immunogenic capacity.¹ It was later demonstrated that this was not the case, and even though hESC could not function as professional antigen-presenting cells their implantation resulted in an immunological response similar to that observed when other somatic cells are transplanted.² The implication of those findings is that if hESCs are to be transplanted into patients, then some sort of manipulation of the immune system will be necessary.³

Costimulation blockade is an emerging therapeutic strategy in transplantation medicine that circumvents the need for life-long chronic immunosuppression by inhibiting the ac-

tivation of the immune system at the time of transplantation. This is accomplished by inhibiting receptor/ligand pairs in the immunological synapse at the time of MHC/TCR binding. Blocking these signaling molecules leads to altered gene transcription in the responding T cells and dendritic cells and yields a state of immunological anergy.⁴ This anergic state is then maintained in the recipient by the development of memory T cells with a regulatory T-cell phenotype.⁵ Initial studies using CTLA4Ig, anti-CD40L, and anti-LFA-1, given for the first week after hESC transplantation, induced Foxp3⁺ regulatory T cells around hESC grafts implanted in the testis. It was also demonstrated that these regulatory T cells had the capacity to inhibit T-cell proliferation specifically toward hESC antigen *in vitro* indicating that immunological tolerance had been achieved.⁶ Later studies demonstrated that costimulation blockade could be used to induce acceptance of induced pluripotent stem cells, mouse embryonic stem cells, and hESCs when implanted into the thigh muscles of wild-type mice.⁷ In these studies regulatory T cells were shown to decrease in the periphery, but their presence in the grafts was not studied

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and the role of regulatory T-cells was not explored. The efficacy of costimulation blockade has recently been compared to standard immunosuppression in a model of myocardial and hind-leg implantation. In these studies it was demonstrated that the combination of CTLA4Ig and anti-LFA-1 was superior to cyclosporine and prednisolone in maintaining hESC transplants in immunocompetent mice. However the histology of these grafts was not explored and the presence of regulatory T cells was not investigated.⁸

In order to study if regulatory T cells are present when hESCs are transplanted outside of the immunologically privileged testis, we implanted hESCs under the kidney capsule while tolerance was induced by 1 week of treatment with costimulation blockade. The results of these experiments could help explain the mechanism of tolerance induction by costimulation blockade and highlights the potential utility of this strategy for clinical hESC transplantation.

Materials and Methods

The hESC lines used were ES181 grown on feeders⁹ or ES360 grown in suspension.¹⁰ ES181 has been shown to maintain expression of alkaline phosphatase, Oct-4, SSEA-4, and TRA-1-60 after 40 weeks of culture and the ability to spontaneously differentiate into spontaneously beating cells and neuron-like cells.⁹ ES360 has been shown to differentiate into neurospheres after being maintained in culture for 20 weeks without the need for feeder cells, making this a potentially interesting hESC for neurological applications.¹¹ Eight-week-old C57bl/6, Balb/C, NOD Scid gamma (NSG), and Scid/beige (Scanbur AB, Sollentuna, Sweden) were used as recipients. The Animal Care Committee of Karolinska University Hospital approved all procedures. The animals were anesthetized with continuous isoflurane and $(3-5) \times 10^6$ cells were injected under the kidney capsule. Recipient mice received 0.5 mg CTLA4Ig, 0.5 mg anti-CD40L, and 0.2 mg anti-LFA-1 (Bioexpress, West Lebanon, NH) as an intraperitoneal injection (costimulation blockade) or 0.5 mg human IgG, 0.5 mg hamster IgG, and 0.2 mg rat IgG2b intraperitoneally (control antibodies) on days 0, 2, 4, and 6 after transplantation. The NSG mice did not receive any treatment.

Kidneys were harvested at 6–12 weeks after transplantation, snap-frozen, and sectioned into 5- μ m sections. Fluorescent *in situ* hybridization (FISH) for human cells (human DNA)⁹ was performed around the injection site (determined by hematoxylin and eosin staining) and/or where deviant structures were found. In order to determine if regulatory T cells were present in or around the transplant, kidneys were stained for FoxP3, a transcription factor specifically expressed in regulatory T cells.¹⁰ Slides were fixed in 4% formaldehyde (art no. 02176; Histolab, Gothenburg, Sweden), boiled in citric buffer pH 6 (Invitrogen, Carlsbad, CA), and blocked with 10% donkey serum (Jackson ImmunoResearch Lab, West Grove, PA) in phosphate-buffered saline (Invitrogen). The primary antibody, rat anti-mouse anti-FoxP3 (clone FJK-16s; eBioscience, San Diego, CA) was applied at a ratio of 1:50 and incubated overnight in a humidified chamber. The secondary antibody, donkey anti-rat IgG (Alexa fluor) was added the next day at a ratio of 1:700, and the slides were incubated for 1 h in a humidified chamber.

For CD4 and CD8 staining, slides were fixed in 4% formaldehyde (Histolab) and blocked with 5% donkey serum (Jack-

son ImmunoResearch Lab) and 5% mouse serum (Dako, Glostrup, Denmark) in Tris-buffered saline. The primary antibody anti-CD4 and anti-CD8 (respectively clone YTS191.1 or clone KT15; Serotec, Oslo, Norway), was applied at 1:50 and incubated in a humidified chamber overnight. The secondary antibody, donkey anti-rat IgG (Alexa fluor) at 1:700, was added the next day and incubated for 1 h in a humidified chamber. The slides were mounted with an anti-fading reagent containing 4, 6-diamidino-2-phenylindole before visualization in a fluorescence microscope (Olympus BX60; Olympus, Hamburg, Germany). Splens from C57bl/6 mice were used as controls.

Results and Discussion

hESC line ES181 was transplanted to NSG mice under the kidney capsule as an initial control to study viability of the cells in this model. Kidneys were harvested 8 weeks later and studied for the presence of hESCs by staining with hematoxylin and eosin and for human DNA by FISH. Large grafts were found under the kidney capsule positive for human DNA and resembled large teratomas (Fig. 1A). Convinced of the utility of the model, we transplanted ES181 to wild-type C57bl/6 and Balb/C mice and treated the animals with costimulation blockade or isotype control antibodies on the day of transplantation and days 2, 4, and 6 after transplantation. Scid/beige mice were also transplanted as a positive control, with or without costimulation blockade.

After 6–8 weeks, kidneys were harvested and stained using FISH and hematoxylin and eosin to study graft survival. In 3 of 12 wild-type mice treated with costimulation blockade, teratoma developed as determined by FISH staining (Fig. 1A.). In the isotype control groups, all grafts were rejected. Kidneys were stained for the expression of Foxp3, which is a transcription factor specific for regulatory T cells (Fig. 1B),¹² and subsequently the most specific marker of these cells. The Foxp3⁺ cells were found on the outskirts of the hESC graft in the kidney parenchyma (Fig. 1B), indicating that they may have a role in down-modulating immune responses locally toward the accepted graft. In order to further define the phenotype of the surrounding lymphocytes, grafts were stained for CD4 and CD8 (Fig. 1B). Most of the cells in the regions that stained positive for Foxp3 were also positive for CD4. Some staining for CD8 could also be found, but CD4 was the dominant cell population.

In order to study if hESC tolerance could be achieved toward another stem cell line, ES360 stem cells were transplanted in the same manner to wild-type C57bl/6 mice, and animals were randomized to the costimulation blockade or isotype control antibody treatment. A similar result was achieved with large intact grafts found in the costimulation blockade-treated group (Fig. 1B), and only scar formation was found in the isotype control treated recipients. Again, the accepted grafts were surrounded by CD4⁺ Foxp3⁺ cells found in the parenchyma of the kidney (Fig. 1B).

Transplantation of Scid/beige mice was performed as a positive control to the transplants performed in wild-type mice to ensure viability of the transplanted cells and to study if the costimulation blockade impeded engraftment. Surprisingly, kidneys of Scid/beige recipients were found to be devoid of hESC transplants, even when treated with the costimulation blockade. This was an unexpected result,

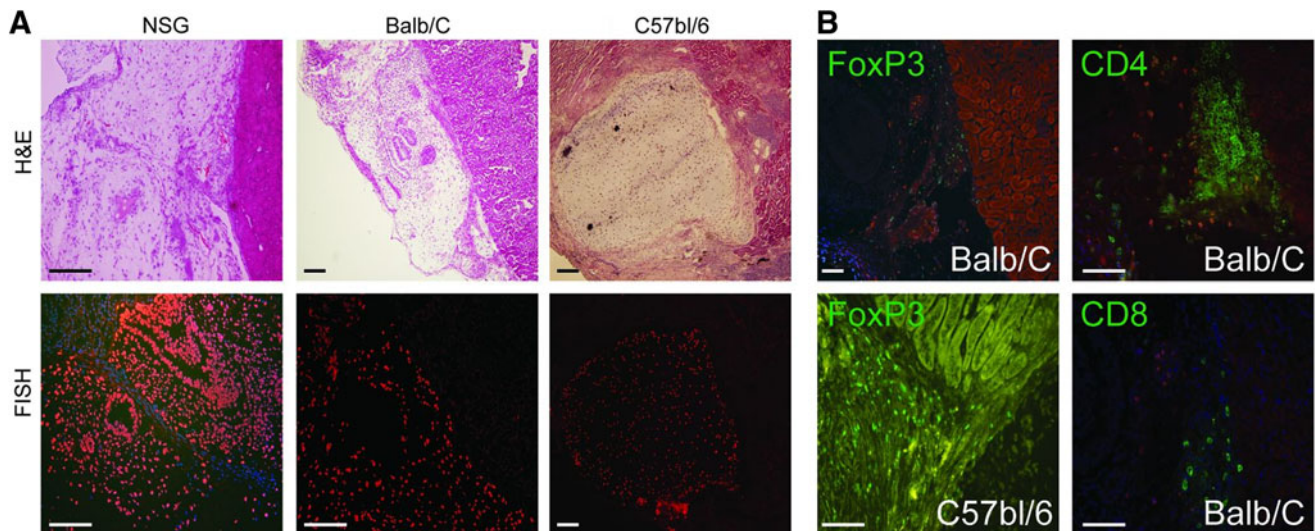


FIG. 1. hESC engraftment. **(A)** H&E staining and FISH from three different animals transplanted with hESC under the kidney capsule. NSG and Balb/c mice received HS181, and C57bl/6 mice received HS360. Balb/c and C57bl/6 mice were treated with costimulation blockade. H&E shows teratoma-like structures (to the left) in NSG and Balb/c. C57bl/6 has an area inside the kidney resembling cartilage. FISH results show human cells as red fluorescent spots which correspond to the teratoma- and cartilage-like structures of the H&E stainings. Scale bars represent 50 μm . **(B)** Immunohistochemistry for FoxP3, CD4, and CD8. FoxP3 images are serial sections from the Balb/c and C57bl/6 recipients presented in **(A)**. The green fluorescent cells are FoxP3+ regulatory T-cells situated at the border between the kidney and xenograft. CD4 and CD8 stainings are serial sections from the same Balb/c mouse as in **(A)**. The green cells are positive for CD4 or CD8. In the area where FoxP3+ cells are abundant, there are many CD4+ cells. CD8+ cells are also present, but not as numerous. Scale bars represent 100 μm . NSG, NOD Scid gamma; H&E, hematoxylin and eosin; FISH, fluorescent *in situ* hybridization.

but prior studies of human cells transplanted to these recipients show the presence of functioning natural killer (NK) cells and the elimination of transplants within 4 weeks.¹³ This indicated that even in the presence of costimulation blockade, hESC transplants were eliminated in this strain indicating NK cell activity autonomous to costimulation through B7, CD40L, and anti-LFA-1. This could indicate that in the absence of regulatory T cells, NK cells lack their immunological restraints and are thereby capable of rejecting hESC transplants in this immunodeficient model.

Many recipients transplanted with the ES181 stem cell line did not show engraftment, even when treated with the costimulation blockade, whereas when the ES360 line was

used, engraftment seems to have been more successful (Table 1). These two stem cell lines are from different donors and are cultured under different conditions. ES181 is cultured on feeders and ES360 is cultured in suspension, which could effect the cells' ability to engraft. Further experiments are required to determine if culture methods affect engraftment.

Prior studies with transplantation of stem cells to the muscles of the hind-leg showed a decrease in the levels of regulatory T cells in the peripheral blood but did not analyze their fate. Here we demonstrate that Foxp3+ regulatory T cells can be found surrounding the hESC transplant when acceptance is long term. In previous studies, we showed that regulatory T cells could inhibit naïve T-cell activation toward hESC antigen.⁵ The ability of costimulation blockade to generate regulatory T cells specific to donor antigen and the presence of CD4+ Foxp3+ around the hESC transplant imply that regulatory T cells are probably acting locally to prevent rejection and that costimulation blockade maybe a promising treatment regime for hESC transplantation.

Disclosure Statement

No competing financial interests exist.

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TABLE 1. HUMAN EMBRYONIC STEM CELL TRANSPLANTS IN DIFFERENT MOUSE STRAINS

	Positive	Negative	Total
HS181 in NSG	2	0	2
HS181 in Scid/beige+costimulation blockade	0	3	3
HS181 in Scid/beige+control antibodies	0	3	3
HS181 in Balb/c, C57bl/6+costimulation blockade	3	9	12
HS181 in Balb/c, C57bl/6+control antibodies	0	12	12
HS360 in C57bl/6+costimulation blockade	2	0	2
HS360 in C57bl/6+control antibodies	0	2	2

NSG, NOD Scid gamma.

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Abbreviations Used

FISH = fluorescent *in situ* hybridization

hESC = human embryonic stem cell

NK = natural killer

NSG = NOD Scid gamma