Skin Allograft Acceptance with Anti-CD154 in a Non-Human Primate Model

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ABSTRACT

Background: *Traditional burn wound management involves application of topical antimicrobial agents with frequent dressing changes for superficial partial thickness burns and early excision and grafting of deep partial or full thickness burns. Combat related injuries tend to affect large areas and treatment has been often limited to split-thickness skin autografts. This approach has been limited by its requirement for an autologous donor graft sites. Allogenic skin transplantation would alleviate many of these problems, but has remained impractical due to graft rejection. To date, no clinically available intervention has been reported to induce long-term primary skin graft survival. However, murine models utilizing either costimulation blockade or costimulatory blockade together with donor specific transfusions (DST) have met with limited success. Previously we have used a humanized monoclonal antibody (hu5C8) directed against CD154 to induce long-term graft survival in a primate renal allograft model without the use of DST. In this work, we have applied these regimens with the addition of DST to a primary skin transplant model.*

Methods: *Ten animals were transplanted with full thickness skin allografts mismatched at both class I and class II major histocompatibility loci. Of these, two were given no treatment, five were treated with anti-CD154 mAb alone, and three received anti-CD154 mAb combined with whole blood DST. All recipients also received autografts.*

Results: *Treatment with both hu5C8 alone and hu5C8 plus DST greatly prolonged allograft survival with mean survival time in the monotherapy group of > 226 days and mean survival time in the DST group of > 263 days.*

Conclusion: ***T****hese results suggest that costimulation blockade with anti-cd154 can attenuate acute rejection of skin allografts and may lead to long-term survival of these grafts without chronic immunosuppression. Further, we noted that dst provided no survival advantage over anti-cd154 monotherapy alone. The ability to improve and simplify burn wound management has significant impact upon the ability to return our operational forces to full duty. Further studies are ongoing to obtain durable graft survival and thereby transition these immunomodulatory strategies into clinical practice.*

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1.0 INTRODUCTION

Skin allografts remain an extremely rigid test of any intervention designed to prevent allograft rejection. Despite considerable success in solid organ transplantation, skin allografts have not been successfully applied clinically, and experimental success has generally required ablative therapy. Current treatment strategies for burns and similar injuries have been limited to skin autografts to provide wound coverage but only limited functional restoration. Significant burns account for roughly ten percent of all combat related casualties and a considerable portion of accidents to active duty service members. Injuries that require coverage for large wound surface areas are limited to split thickness skin grafts (STSG) which provide less than optimal cosmetic and functional outcomes when compared with full thickness grafts or myocutaneous flaps. The infectious risks present in such large wounds as in burn or multi-system trauma patients may preclude standard global immunosuppression strategies. Therefore, a treatment regimen facilitating long-term acceptance of full thickness skin allografts would be adventurous allowing patients to have improved function and cosmesis. Furthermore, immunoregulatory strategies applied to skin allografts would likely be successful for other peripheral injuries (digits, limbs, facial parts) providing for additional improved outcomes for injured patients.

In vivo, T lymphocytes require at least two signals for complete activation. These signals are comprised of an antigen specific signal received through the T cell receptor and a second costimulatory signal, delivered predominantly through CD28 (1) as well as CD154 (2) and their cognate receptors CD80/86 and CD40 on the APC. These signals allow CD4 T cells to produce sufficient IL-2 and other cytokines to allow autocrine-driven clonal expansion. In the absence of a co-signal anergy is often induced.

Long-term skin graft acceptance of allogeneic skin has been previously achieved in a mouse model when costimulation blockers were utilized (3,4). Some have used strategies involving both costimulation blockade and donor specific transfusion (DST) (5,6). Additionally, we and others have demonstrated that blockade of CD154 and CD40 interactions with a humanized anti-CD154 monoclonal antibody (hu5C8) has been shown in both small animal and primate models to induce graft survival in solid organ transplants (7,8). To date, the use of hu5C8 in human clinical trials has been put on hold as a result of reported thrombotic events in autoimmunity patients.

In rodent studies, donor specific transfusions clearly are superior to therapies that use costimulation blockers alone. Several groups have shown prolonged survival of islet, cardiac, and skin allografts with the combination of DST, anti-CD154, and thymectomy (5,9). Although the mechanism by which DST influences the immune response remains unclear, one hypothesis is that it induces activation of alloresponsive CD8+ cells which in turn lead to deletion or anergy of these cells after costimulation blockade(9). We have previously reported the effect of a hu5C8-based treatment regimen previously shown to be effective in renal transplantation to a non-human primate model with and without the use of DSTs and now update those results. (10).

2.0 METHODS

2.1 Skin Allografts. Animals were divided into three groups based on their treatment after transplant (see below). All received both mismatched allografts and autografts. Anesthesia was administered by IM ketamine (10 mg/kg) and xylazine (2 mg/kg) and animals redosed as necessary. Full thickness two by two centimeter abdominal skin grafts were procured under aseptic conditions and sharply defatted in normal saline. Wounds were closed with 4-0 nylon sutures and the animal then placed in the prone position. Skin

ellipses were discarded from the lower back at the level of the iliac crest and 2 mg hu5C8 was injected into the graft base (allografts only). Both allografts and autografts were then secured with simple interrupted 4-0 nylon sutures with hair follicles reversed for later identification. Dressings were placed, changed on postoperative day 3 and sutures removed on postoperative day 10. All experiments were approved by the Naval Medical Research Center IACUC under animal use protocol 97-17.

2.2 Treatment. Group one received no therapy to prevent rejection and served as allograft controls (n=2), group two received anti-CD154 monoclonal antibody alone (hu5C8, Biogen) (n=5), and group three received hu5C8 with DST (n=3). All experimental animals were treated with 2 mg of hu5C8 injected into graft beds prior to transplantation. Anti-CD154 (hu5C8) was given at 20mg/kg IV for the non-DST group. Recipients of DSTs were transfused with 20mg/kg hu5C8, 20 ml donor whole blood, and 100 units heparin given at the time of transplantation. All experimental animals where then placed on a six month dosing regimen of hu5C8 with intravenous injections of 20 mg/kg on days 1, 3, 10, 18, 28, and then monthly. Repeat challenge and third party grafts from MLR high responder donors were placed at day 300 in one matched DST and non-DST treated pair.

2.3 Postoperative monitoring. Skin biopsies were performed on postoperative day 120 from both treatment groups and at the onset of rejection (day 50) in one animal. Dermal elasticity was determined by palpation. Both pre and post-operatively blood was drawn for MLR analysis. Unidirectional MLRs were performed pre-operatively as previously described (7). Briefly, peripheral blood mononuclear cells (PMBC) were isolated by ficoll gradient. The PMBC were washed with PBS then resuspended in RPMI-1640 cell culture media (Life Technologies, Grand Island, NY) supplemented with 10% heat inactivated fetal calf serum, 2 mM L-glutamine, and penicillin/streptomycin. Gamma irradiated (50Gy) donor cells (105/ well) served as stimulators and were co-cultured at a ratio of 1:1 with responder cells. The cultures were incubated at 37°C for 5 days then pulsed with 10 □Ci of H3 thymidine and incubated for an additional 24 h before harvest.

2.4 Histology. Skin biopsies were performed under ketamine and xylazine chemical restraint. Tissues were obtained using full thickness biopsy and closed with interrupted sutures. Tissue samples were embedded in O.C.T. compound (Tissue Tek/Sakura Finetek, Torrance, CA), snap-frozen in a dry ice/ isopentane bath, and stored at -70°C. Six micrometer frozen sections were stained with hematoxylin and eosin using standard histological techniques.

3.0 RESULTS

3.1 Allograft controls. Animals (n=2) that received neither hu5C8 alone nor a combination of hu5C8 and DST demonstrated graft rejection at five and seven days respectively as evidenced by necrosis (table1). Histology obtained during rejection demonstrated epidermal necrosis and lymphocytic infiltration consistent with acute cellular rejection.

3.2 Anti-CD154 monotherapy. Five animals were transplanted using treatment with hu5C8 alone. Mean survival time in this group was > 262 days**.** Approximately seven days after transplantation, all grafts demonstrated erthyema, which was not present in control autografts. These subsequently resolved without further treatment. All grafts demonstrated normal dermal elasticity and the presence of hair growth. Four animals rejected their allografts at 314, 310, 140, and 50 days. The animal that rejected its graft at day 314 underwent repeat challenge and third party grafting at day 300, and promptly rejected these grafts at day 14

and 8, respectively. Rejection was evidenced by graft erthyema, edema, ulceration and eventually loss. Histology at the onset of graft loss was consistent with acute rejection with a large lymphocytic infiltrate in the dermis and pykotic keratinocytes consistent with apoptosis (*figure 2b*). One animal remained without signs of rejection at >500 days post transplant and which point the animal was euthanized (*table 1)*. Histology obtained by skin biopsy in the long surviving grafts on postoperative day 110 showed no epidermal necrosis and mild inflammation when compared with controls.

3.3 Anti-CD154 and DST. Three animals were transplanted using hu5C8 in combination with a single whole blood transfusion. Mean survival time for this group is currently > 247 days. One of these animals remained well with healthy allografts until >500 days following transplantation at which point the animal was euthanized (table 1). Interestingly, one died on postoperative day two from a febrile illness clinically consistent with sepsis. Blood cultures and a detailed necropsy did not reveal a cause of death. The third animal rejected its allograft at 239 days following transplantation (table 1). This animal underwent repeat challenge and third party grafting at day 300 and rejected both of these grafts at one week. As with the previous group, approximately seven days after transplantation all grafts demonstrated the onset of erythema, which subsequently resolved without treatment. All grafts exhibited normal dermal elasticity and the presence of hair growth. Histology from day 110 biopsy showed viable epithelium with low numbers of mixed inflammatory cells, similar to results with ant-CD154 mAb monotherapy.

Treatment	Survival	Comment
Allograft Control	5, 7	Acute rejection (AR)
hu5C8 w/ DST	2**, 239*, >500	*Reject,**POD2 death, others AW
hu5C8 w/o DST	314*, 310*, 140*, 50*, >500	*REJECT, OTHERS AW

Table 1: Summary of all animals

4.0 DISCUSSION

These results demonstrate that costimulation blockade with anti-CD154 mAb greatly delays and, in some cases, prevents rejection of skin allografts. Skin grafts transplanted with this regimen demonstrate hair growth, no wound contracture and normal function. As the mean survival time of DST treated animals in this small series is significantly less than non-DST treated animals, DST seems to provide no survival advantage. However, additional studies will have to be conducted before this conclusion is verified. The ability of a pair of these animals to reject both repeat challenge and third party grafts placed two months after cessation of antibody therapy confirms that tolerance is not achieved in this model. Previously reported results in a murine model showed long term graft acceptance with the addition of thymectomy to treatment regimens combining DST and a two week course of anti-CD154 mAb (50 days vs. greater than 100 with thymectomy) (6). Our results involve the use of a six-month treatment protocol similar to that reported in our renal transplant models (7). The effects of anti-CD154 mAb therapy may be mediated through both the innate immune system by interrupting dendritic/T cell interactions and the acquired immune system directly. These interactions

possibly lead to either clonal deletion or apoptosis of alloreactive T cells which may lead to long lasting antigen specific anergy. (2)

The cause of the postoperative day two death in the DST treated animal remains unclear. Although the clinical picture resembled sepsis both necropsy and laboratory evaluation did not reveal a source. Other possibilities include systemic inflammatory response syndrome (SIRS) from cytokine release as seen with IL-2 treatment for renal cell carcinoma or possibly a thromboembolic phenomenon related to antibody crosslinking with platelets. However, we have in vitro data suggesting that neither cytokine release nor platelet crosslinking occurs as a result of anti-CD154 mAb treatment. The etiology of this death remains speculative, future use of DST will need to be closely monitored.

The erythema seen in all transplanted animals during the first week may represent the same process of early lymphocytic infiltration reported in renal allografts treated with anti-CD154 mAb, which we were unable to be associated with any functional abnormalities (7). The episodes of delayed rejection were preceded by a picture similar to the erythema seen initially and biopsy at that time showed a large diffuse infiltrate, comparable to that seen in rejecting renal allografts (7). This may represent reemergence of alloreactive CD8+ T cells which then participate in graft rejection.

The success of an anti-CD154 based treatment regimen in a non-human primate model, will hopefully allow for the clinical application of costimulation blockade with this antibody to prevent rejection of human skin transplant recipients. The applicability of anti-CD154 to composite tissue allotransplantation is another appealing aspect of the results presented. Treatment both with and without DSTs does not necessitate the use of complex protocols involving radiation and/or thymectomy. Simple intravenous infusion of the antibody with or without a single DST is all that is required for treatment. Postoperative evaluation for rejection is not complex either, simple graft surveillance will suffice. While the possible pro-thrombotic effects of hu5C8 are currently under investigation, there remains a clear role for costimulation blockade in the development of clinically relevant treatment protocols. Costimulation blockade with anti-CD154 mAb may provide for functional and cosmetically acceptable of full thickness skin transplantation which can be applied to military and civilian casualties.

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