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14. ABSTRACT

We have undertaken studies of the immune mechanisms contributing to rejection of vascularized composite allografts (VCA) in murine models, and how these may be overcome to promote long-term allograft survival. We have now firmly established orthotopic hindlimb and forelimb VCA models in our lab and using these have shown that 3 distinct protocols, namely CD154 monoclonal antibody plus 4 weeks of rapamycin (RPM), or CTLA4Ig plus 4 weeks of RPM, or TCR mAb plus RPM, can each achieve long-term VCA survival without maintenance immunosuppression. We have now shown that the efficacy of both protocols is dependent upon a radiation-sensitive donor bone marrow (BM) cell component, namely CXCR4+ Foxp3+ donor Treg cells. In addition, some limited prolongation on VCA survival was detected using anti-CXCR3 mAb; adoptive transfer of Foxp3+ Tregs; or using a pan-HDAC inhibitor (Trichostatin-A) plus RPM. The most important findings of our work are the potential for long-term engraftment using any one of the 3 clinically applicable therapeutic protocols noted above as a result of limited peri-transplant immunotherapy.

15. SUBJECT TERMS
allograft rejection, tolerance, costimulation blockade

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1. **INTRODUCTION:**

   Our study sought to analyze the immune mechanisms contributing to rejection of vascularized composite allografts (VCA) in murine models, and how to overcome these responses and promote long-term VCA survival. The work was wide-ranging in nature, encompassing studies of chemokines and chemokine receptors, costimulation blockade and related immunomodulatory strategies, and application of Foxp3+ T-regulatory (Treg) cells, either by expansion or by pharmacologic modulation with histone/protein deacetylase inhibitors (HDACi).

2. **KEYWORDS:** Provide a brief list of keywords (limit to 20 words).

   vascularized composite allotransplantation, allograft rejection, tolerance, costimulation blockade, T-regulatory cells, HDAC inhibitors, Foxp3

3. **ACCOMPLISHMENTS:** The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction.

   What were the major goals of the project?
   List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

   The important activities and readouts for our studies are summarized in the Table on the next page of this report.

Aims, Tasks and Subtasks

Specific Aim 1: Investigate the mechanisms of VCA rejection in murine models of hindlimb VCA, and contrast those responsible for solid organ allograft rejection	**Months**	**Progress**
Major Task 1: Obtain regulatory approval and establish murine hindlimb models		
1.1.1 Subtask 1: Seek IACUC & ACURO approvals for murine limb allograft studies	1-3	Achieved
Milestone(s) Achieved: Obtain IACUC and ACURO approvals	3	Achieved
1.1.2 Subtask 2: Establish heterotopic hindlimb allograft model.	3-6	Achieved
1.1.3 Subtask 3: Establish orthotopic hindlimb allograft model	3-9	Achieved
Milestone(s) Achieved: Established heterotopic and orthotopic limb allograft models in mice	9	Achieved
Major Task 2: Test whether targeting of chemokine/chemokine receptor pathways will allow long-term VCA survival without development of chronic injury		
1.2.1 Subtask 1: Target the CXCR3 pathway using blocking anti-CXCR3 mAb and/or CXCR3 KO mice as VCA recipients.	5-12	Achieved
1.2.2 Subtask 2: Target the CCR5 pathway using blocking anti-CCR5 mAb and/or CCR5 KO mice as VCA recipients	13-18	Achieved
1.2.3 Subtask 2: Test efficacy of dual CXCR3/CCR5 targeting	19-24	Achieved
1.2.4 Subtask 2: If and as implicated by mechanistic studies, test efficacy of targeting additional chemokine pathways	13-24	Achieved
Milestone(s) Achieved: Limited efficacy of chemokine/receptor targeting on VCA survival	24	Achieved
Major Task 3: Test whether peri-transplant strategies involving costimulatory blockade will allow long-term VCA survival without development of chronic injury		
1.3.1 Subtask 1: Target the CD40/CD40L (CD154) pathway, using blocking anti-CD154 mAb ± brief rapamycin therapy	13-18	Achieved
1.3.2 Subtask 2: Target the CD28/B7 pathway, using blocking CTLA4-Ig ± brief rapamycin therapy in VCA recipients	19-24	Achieved
1.3.3 Subtask 2: Test efficacy of dual CD40L/CD28 targeting, by combining anti-CD154 mAb and CTLA4-Ig in VCA recipients	25-27	Achieved
1.3.4 Subtask 2: Test efficacy of ICOS/B7RP-1 targeting using blocking mAbs and/or KO mice as VCA recipients	28-36	Achieved
1.3.5 Subtask 2: Test efficacy of targeting further costimulatory or co-inhibitory pathways implicated by mechanistic studies	19-36	Achieved
Milestone Achieved: Key data on the efficacy of HDACi therapy on VCA survival	36	Achieved
Major Task 4: Test the ability of Treg-directed therapies to promote VCA outcomes		
1.4.1 Subtask 1: Test effects of pan-HDACi ± low dose rapamycin	13-18	Achieved
1.4.2 Subtask 2: Test effects of a class I or class II HDACi ± low dose rapamycin	19-24	Achieved
1.4.3 Subtask 3: Test effects of individual HDAC deletion based on success in tasks 1.4.1 or 1.4.2	25-36	Achieved
1.4.4 Subtask 4: Test efficacy of Treg cell therapy after Treg ex vivo expansion and/or activation and their reintroduction	37-40	Achieved
Milestone(s) Achieved: Key data on whether beneficial effects of HDACi on prolongation of VCA survival are critically Treg-dependent	40	Achieved
Major Task 5: Test efficacy of optimal combination therapies to promote VCA outcomes		
1.5.1 Subtask 1: Assess optimal combinations of therapies suggested by data generated	37-48	Achieved
Milestone(s) Achieved: 1-2 papers in review or accepted for publication	48	Achieved

We have undertaken of the listed Tasks and Subtasks and achieved the listed milestones.

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

Major Task 1: Obtain regulatory approval and establish murine hindlimb models

Local (CHOP) and ACURO IACUC approvals were obtained for the work, and heterotopic and orthotopic hindlimb allograft models were established; examples of the orthotopic grafts are shown in **Fig. 1**.

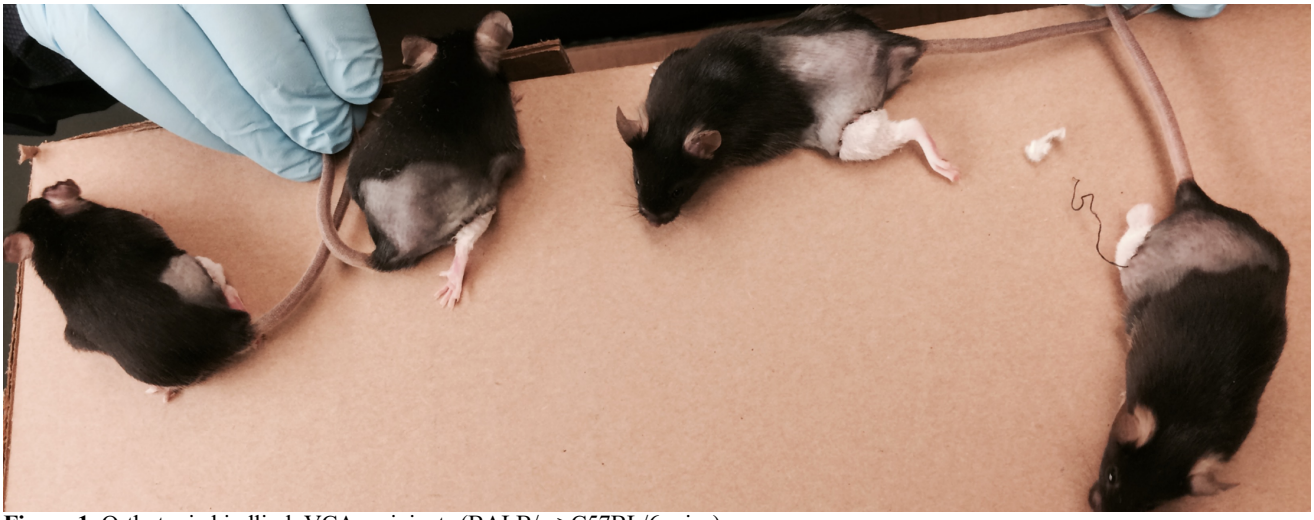


Figure 1. Orthotopic hindlimb VCA recipients (BALB/c->C57BL/6 mice).

Major Task 2: Test whether targeting of chemokine/chemokine receptor pathways will allow long-term VCA survival without development of chronic injury

Chemokine and chemokine receptor mRNA expression within isografts and allografts harvested at days 3, 5 and 7 post-transplantation were analyzed by qPCR; changes in mononuclear cell-associated CXCR3 and CCR5 pathways were especially prominent in allograft samples, with increases by day 3 and peaking at day 5 (histologically, day 7 samples had typically begun to show severe, end-stage injury with neutrophilic infiltrates). Accordingly, we tested the effects of a hamster anti-CXCR3 mAb (CXCR3-173) we had generated and previously shown highly effective in prolonging cardiac and islet allograft survival, especially when used with a sub-therapeutic regimen of rapamycin (RPM) (1). VCA recipients were treated with 4 doses of anti-CXCR3 mAb 200 µg/day, qod, from the day of transplantation, plus 0.2 mg/kg/day of RPM, i.p., for a total of 14 days (**Fig. 2**). This regimen prolonged VCA survival ($p<0.05$) but was far less effective than anticipated. In our previous studies, optimal effects (i.e. permanent allograft survival) after brief peri-transplant therapy) were seen using 500 µg qod along with 0.1 mg/kg/day of RPM for 14

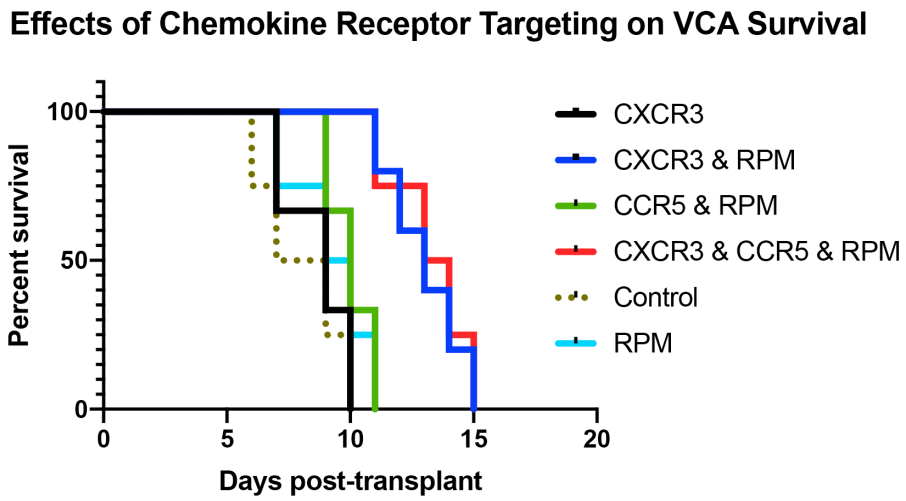


***Figure 2**. CXCR3 mAb plus low dose RPM prolonged VCA ($p<0.05$) compared to recipients treated with control IgG or RPM alone. In contrast, CCR5 mAb had no significant effect in this model, even with low dose RPM or when combined with CXCR3 mAb plus RPM. Data from 4-6 allografts/group & representative of 2 separate experiments.*

days from the time of transplantation (1). Moreover, use of a rat anti-mouse CCR5 mAb (C34–3448) that we had previously shown to prolong murine cardiac allograft survival (2) had no effect on the tempo of VCA rejection, and combined therapy with mAbs to CXCR3 and CCR5 had no added benefit over the prolongation seen with CXCR3 mAb alone (**Figure 1**). ••• ***These findings led us to conclude that chemokine receptor targeting was not able to markedly prolong VCA survival***.

Major Task 3: Test whether peri-transplant strategies involving costimulatory blockade will allow long-term VCA survival without development of chronic injury

Costimulation blockade and heterotopic allografts - Previous studies from our group showed that use of MR-1 mAb directed against CD40L (CD154) was highly effective at prolonging cardiac allograft survival in mice, especially when coupled with donor splenocyte transfusion (DST) at the time of transplantation (3, 4). We have also shown that therapy with CTLA4-Ig was effective at prolonging cardiac allograft survival. Lastly, and remarkably, the combination of these 2 approaches, i.e. CD154 mAb and CTLA4Ig, was reported by others to promote long-term skin allograft survival (5). We therefore tested these approaches with the heterotopic VCA hind-limb model in mice (**Fig. 3**). Combined use of CD154 mAb (MR-1) and CTLA4Ig only increased allograft survival by about 2-fold (**Fig. 3a**). These data were surprising to us. We had anticipated that combined costimulatory blockade (CD154 mAb/CTLA4Ig) would be much more effective in achieving long-term VCA survival than was observed (**Fig. 3a**), given the published effects of this protocol on skin allograft survival in this same strain combination (BALB/c->C57BL/6) (5), and our assumption that the skin would be the most immunogenic component of the limb allograft. In contrast to combined CD154 mAb (MR-1) and CTLA4Ig, use of a single peri-transplant injection of CD154 mAb plus DST was more effective than multiple injections of CD154 and CTLA4Ig, resulting in a tripling of allograft survival (**Fig. 3b**). The latter therapy results in intravenous trafficking of donor cells to the spleen where they are largely trapped and provide a pulse of donor MHC to the recipient immune system. The concomitant administration of CD154 mAb is thought to prevent generation of a second signal and render the host alloreactive T cells anergic (3, 4). These considerations led us to consider the testing, in future studies, of the effects of multiple rounds of CD154/DST, e.g. every 3 weeks, and also to consider that donor MHC+ cells within donor bone marrow might provide an ongoing stimulus for rejection (this was explored below). Lastly, the best effects to date were seen by adding 14 days of RPM (2 mg/kg/d, i.p.) to the CD154 mAb/DST protocol, with engraftment of >100 days (**Fig. 3c**) and healthy appearance. ••• ***Anti-CD40L/DST/RPM therapy led to >100 days of VCA survival without maintenance immunosuppression***.

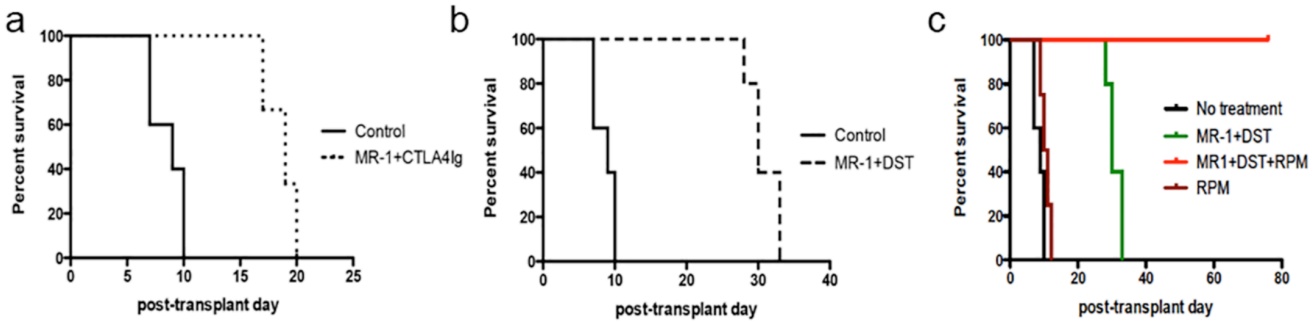


Figure 3. *Data from the heterotopic allograft model. (a) VCA recipients were treated with 6 doses of MR-1 mAb, directed against CD40L (CD154), plus murine CTLA4Ig, using 500 µg of each, qod from transplantation, resulting in only a doubling of allograft survival compared to IgG-treated controls ($p<0.05$). (b) VCA recipients were treated with MR-1 (CD154) mAb (200 µg) plus donor splenocyte transfusion (DST, 5 x 10^6 cells, i.v.) at the time of transplantation, leading to a tripling of allograft survival ($p<0.01$). (c) Addition of 2 weeks of rapamycin (RPM, 2 mg/kg/d, i.p.) to MR1/DST protocol led to long-term VCA survival (>100 days).*

RPM and tolerance induction - Using CD40L/DST/RPM therapy, we tested the extent of allograft tolerance induction in a series of allograft recipients (4-6 mice/group). Using heterotopic hindlimb VCA recipients that had survived for 100 days or more post-Tx (BALB/c->C57BL/6) as a result of peri-transplant CD40L/DST/RPM therapy, third party cardiac allografts (C3H) were performed. To our surprise, third-party cardiac allografts were maintained (>30 days so far) whereas the previously accepted limb allografts were now rejected within 1-2 weeks. This result is in some ways like the phenomenon termed "split tolerance" and could reflect differences in: (i) the antigenicity of cardiac vs. limb allografts, or (ii) the mechanisms required for maintenance of graft acceptance. We

have previously reported that donor-specific cardiac allograft tolerance in this stringent strain combination could be achieved by CD40L/DST therapy, and that the ability to accept second donor cardiac grafts but to reject third party cardiac allografts was Treg-dependent (6). This is not a ready answer to the current data, since if long-term VCA survival was dependent upon Treg function of this type, one would have expected rejection of the third-party grafts but maintenance of the VCA tissues. A similar outcome would be envisaged if the VCA grafts had now lost most or all of their immunogenicity. Thus, the data are most consistent with an active regulatory component being present but that immune responses to VCA components (likely skin) remain near threshold rejection levels and events associated with the second surgery, e.g. cytokine production, overcame Treg function and/or upregulated donor MHC, increased VCA immunogenicity and led to rejection. By contrast, residual Treg function was able to suppress alloresponses to the far less immunogenic cardiac allografts. However, this result differs from the donor-specific tolerance noted above with cardiac allografts in mice treated with CD40L/DST (but not with RPM as well).

To explore this point further, we undertook cardiac allografts (BALB/c->C57BL/6) in mice treated with CD40L/DST (as in our previous cardiac allograft tolerance work) or CD40L/DST/RPM (as per our best VCA therapy). At 30 days post-Tx, we challenged recipients with second donor grafts or third party (C3H) cardiac allografts. In mice given CD40L/DST, second donor allografts were accepted long-term (currently >50 days), whereas third party allografts were acutely rejected (7-10 days). However, when RPM was included (i.e. allograft recipients received CD40L/DST/RPM), both second donor and third-party allografts were accepted. ••• ***Hence addition of RPM to our CD40L/DST protocol, while necessary to achieve long-term VCA survival, leads to hyporesponsiveness or anergy rather than active tolerance***.

Immunogenicity of orthotopic allografts - In baseline studies, use of the calcineurin inhibitor (CNI), FK506 (Tacrolimus) increased heterotopic VCA allograft survival from 7-8 days in untreated mice to 50% survival of about 4 weeks ($p<0.05$) when using Alzet pump delivery of 2 mg/kg/d for 14 days (**Fig. 4**). When maintenance immunosuppression was increased to 28 days, at the same dosing, heterotopic VCA survival was markedly increased, with 50% survival to 92 days ($p<0.01$) (**Fig. 4**). By contrast, the same 4 weeks of FK506 led to only a doubling of orthotopic VCA survival, with all grafts rejected by 37 days post-Tx (**Fig. 4**). Though we did not explore use of higher doses, 4 weeks of therapy was already accompanied by >50% rejection such that longer courses of therapy, at least at the current dosing, is unlikely to be useful. Clearly, it is harder to prolong survival of orthotopic vs. heterotopic VCA hindlimb grafts using maintenance immunosuppression. We propose this is because of the markedly increased amount of tissue grafted when using the orthotopic model, though other factors may contribute. Rather than pursuing this point, we moved on to explore the effects of peri-transplant immunomodulation, with the knowledge that the potential barrier to graft acceptance was likely be considerably higher than that faced using the heterotopic model.

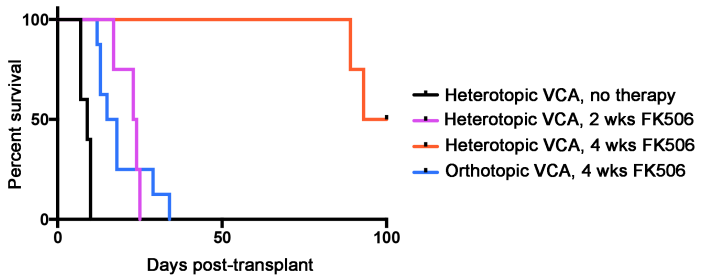


Figure 4. *VCA recipients were treated with FK506, delivered by Alzet pumps at a dose of 2 mg/kg/d for the periods specified. In untreated recipients, times to rejection of orthotopic VCA were comparable to that of heterotopic VCA.*

Despite the relatively poor efficacy of FK506 in prolonging orthotopic vs. heterotopic VCA grafts, excellent results were seen with CD154 mAb-based costimulation blockade. Thus, a single intravenous injection of CD154 mAb (MR1, 200 µg) and 5 x10^6 donor splenocytes (DST), along with 4 weeks of rapamycin (RPM, 2 mg/kg/d, via Alzet pumps), led to 75% long-term orthotopic VCA survival (**Fig. 5**) ($p<0.01$ vs. untreated controls or controls given CD154/DST or RPM alone). ••• ***In subsequent studies with orthotopic allografts, this peri-transplant regimen of CD154 mAb/DST/ RPM led to >100 days of orthotopic VCA survival***.

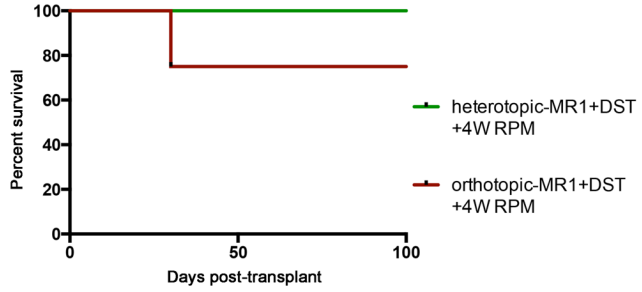


Figure 5. *(a) VCA recipients were injected once, i.v., with CD154 mAb (MR1) plus donor splenocytes, at the time of transplantation, and received RPM for 4 weeks thereafter via Alzet pumps; VCA groups are as shown.*

FACTORS CONTRIBUTING TO EFFICACY OF CD154/DST/RPM

i) Donor bone-associated tissues: Complete removal of the limb bone from the graft prior to transplantation shortened VCA survival compared to survival of intact grafts in recipients treated with a single injection of CD154 mAb (MR-1)/DST.

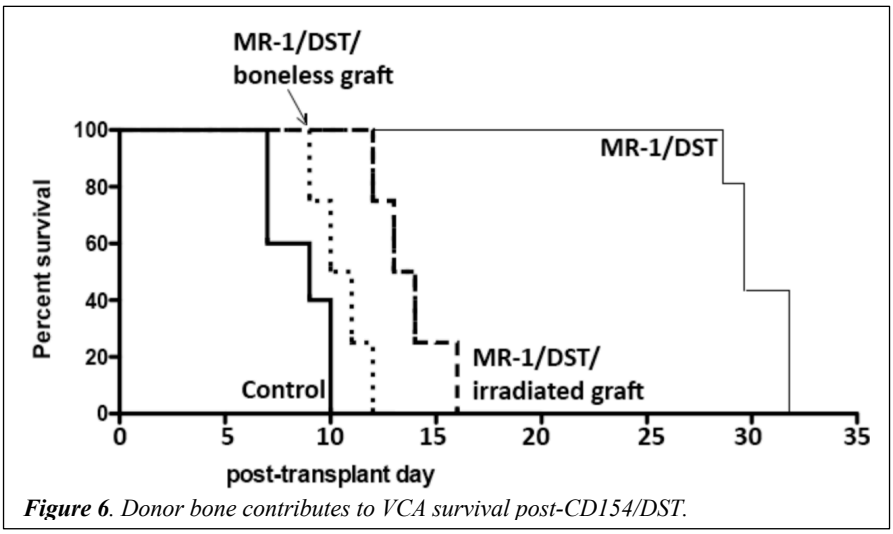


***Figure 6**. Donor bone contributes to VCA survival post-CD154/DST.*

ii) Donor component is radiation sensitive: Gamma-irradiation of donor allografts pre-transplant significantly shortened survival of heterotopic allografts in mice treated with a single injection of CD154 mAb (MR-1)/DST ($p<0.05$). Moreover, while we achieve >100 days of heterotopic hind limb allograft survival with 1 injection of CD40L mAb (MR-1) plus a 5 million donor splenocyte transfusion (DST), followed by 14 days of therapy with rapamycin (RPM), the effects of CD40L/DST/RPM are abrogated if BALB/c mice are subjected to whole-body irradiated prior to their use as limb transplant donors (**Fig. 7**). Irradiation was not a non-specific cause of the graft injury since limbs from irradiated C57BL/6 (B6) mice were maintained long-term without any therapy (**Fig. 7**).

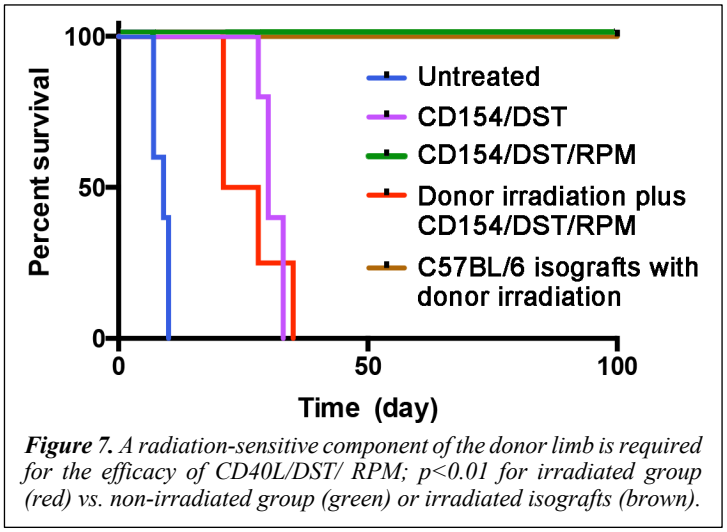


***Figure 7.** A radiation-sensitive component of the donor limb is required for the efficacy of CD40L/DST/ RPM; $p<0.01$ for irradiated group (red) vs. non-irradiated group (green) or irradiated isografts (brown).*

iii) Radiosensitivity is not be overcome by i.v. BM cells: We reasoned that this dose of radiation might be affecting a BM cell component required for long-term allograft survival using CD154/DST/RPM, such that peripheral injection of donor BM, at the time of transplantation, might restore efficacy of this protocol despite donor irradiation. However, as seen in **Fig. 8**, irradiated donor limbs were rejected despite use of costimulation blockade plus RPM (CD154 mAb/ DST/RPM, shown in blue) or in conjunction with peripheral iv injection of 30 million donor BM cells (shown in green); the latter number approximates the numbers of BM cells that can be flushed from donor limbs pre-Tx. ••• ***Hence, there is a radiation-sensitive component of the donor limb that is required for long-term VCA survival using CD154/DST/ RPM, and this donor component cannot be restored by injection of donor BM cells in the periphery at the time of transplantation***.

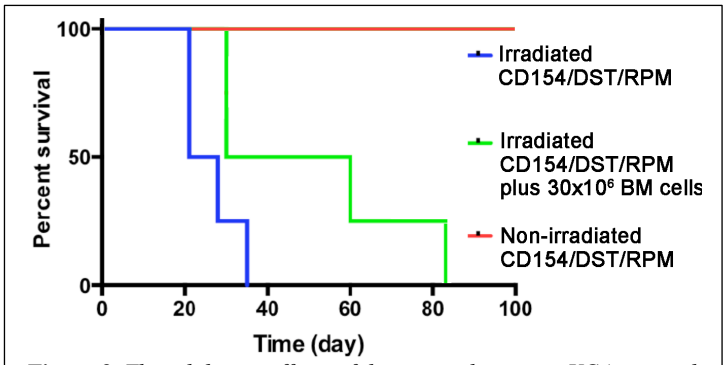


***Figure 8**. The inhibitory effects of donor irradiation on VCA survival, despite recipient treatment with CD154/DST/RPM, cannot be overcome by intravenous injection of normal (non-irradiated) donor BM cells at the time or engraftment; $p<0.01$ for either irradiated group vs. non-irradiated group.*

iv) Requirement of Rag1+ and CXCR4+ BM cells: Rag1-/- mice lack mature T and B cells (7). As seen in **Fig. 9a**, whereas all 6 WT donor limbs were accepted long-term (>100 days) in recipients treated with CD154mAb/DST/RPM, 50% of the recipients of Rag1-/- limbs rejected their grafts by 3 weeks and all 6 were

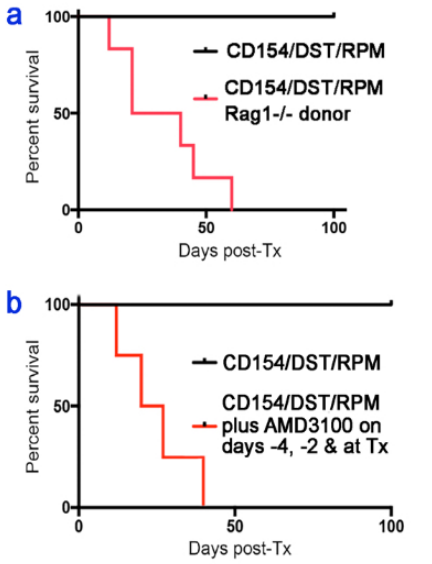


Figure 9. *The efficacy of CD154 mAb/DST/RPM (a) requires Rag1+ cells, and (b) is abrogated by pre-transplant donor therapy with AMD3100 (CXCR4i).*

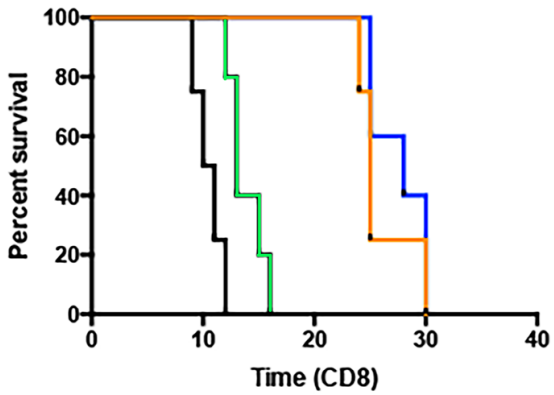


Figure 10*. CD8 T cells do not play a dominant role in VCA rejection in mice receiving CD154 (MR1)/DST costimulation blockade.*

rejected by 60 days ($p<0.01$). In parallel studies, we tested the effects of donor treatment with AMD3100 (Plerixafor), a clinically approved CXCR4 inhibitor (CXCR4i) used to mobilize lineage-negative BM hematopoietic stem (HSC) cells (8). Consistent with the literature, flow cytometry studies (not shown) indicated that CXCR4i mobilized multiple cell types including T and B cells (9), and myeloid cells (10) from donor BM. We found that injection of the donor with CXCR4i on days -5, -3 and -1, followed by harvest of the donor limb for transplantation on day 0, blocked the efficacy of CD154/DST/RPM ($p<0.01$) (**Fig. 9b**). • ***These data indicate disruption of events in donor BM by CXCR4i also disrupts long-term VCA survival, and this is likely to be a T or B cell based on the lack of efficacy when using Rag1-/- donors***.

v) CD8 T cells are not required: Therapy with CD40L mAb (MR1)/DST is thought to act primarily by immunomodulatory effects on the CD4 T cell population, and so we questioned whether CD8 T cells might be promoting "breakthrough" rejection of limb VCA in this context. However, mAb depletion of CD8 T cells did not significantly alter the tempo of rejection in recipients treated with CD40L/DST, nor in recipients treated with RPM alone (**Fig. 10**). • ***Hence, VCA rejection in our studies does not appear to involve a dominant role for CD8 T cells.***

vi) Preservation of donor BM cells: Flow cytometric analysis of BM cells recovered at 3, 5, and 7 days post-Tx from untreated recipients or mice treated with CD40L mAb/DST/RPM (data from 2/group at each time-point are shown). As seen in Fig. 11, donor CD4 and CD8 (H-2K^b-negative) were progressively depleted in serial grafts, with only very small numbers cells remaining at day 7, whereas use of costimulation blockade (COB)/RPM led to preservation of these cells. • ***Beneficial effects of CD154 mAb/DST/RPM are associated with preservation of donor BM cells***.

At this point we decided to investigate whether the biology we were unveiling was unique to the CD154 mAb-based protocol or whether it might also occur with other forms of costimulation blockade, especially that involving CTLA4Ig, which is used in clinical transplantation as Belatacept.

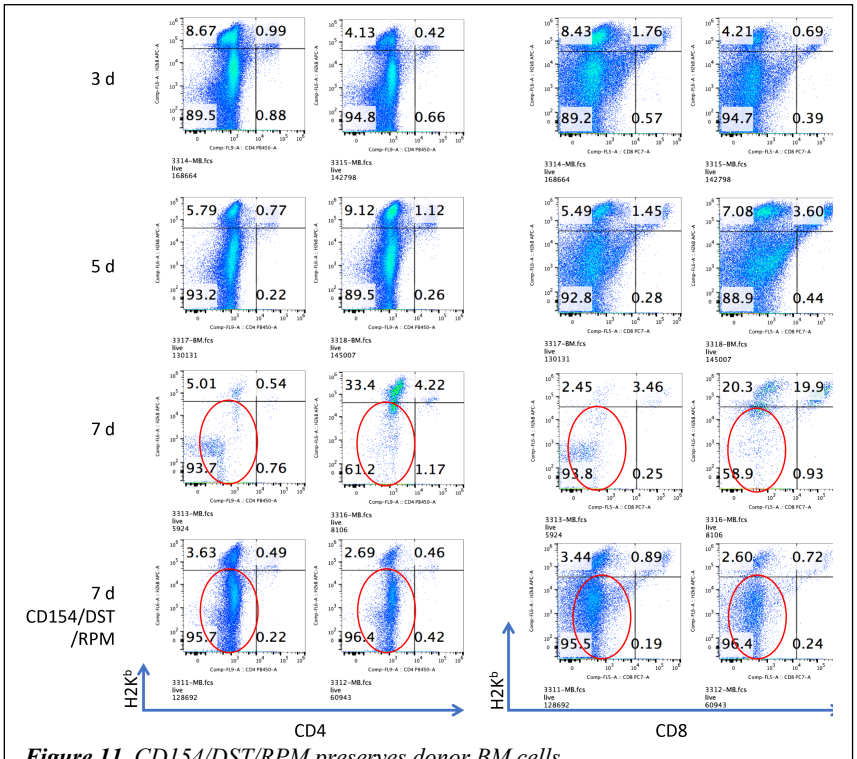


Figure 11*. CD154/DST/RPM preserves donor BM cells.*

CTLA4Ig as the basis for a second form of costimulation blockade in VCA - After exploring various CTLA4Ig-based protocols, optimal efficacy was achieved using CTLA4Ig 500 µg, i.p., days 0, 2 & 4 post-Tx and RPM 2 mg/kg/d (4-wk Alzet pump) (**Fig. 12**). As with CD154/DST/RPM, long-term survival was prevented by donor irradiation, use of Rag1-/- donors, and donor pre-treatment with CXCR4i. The evidence that comparable factors blocked the efficacy of the 2 protocols that are highly effective in prolong orthotopic VCA survival, CD154/DST/RPM and CTLA4Ig/RPM, led us to focus on which T or B cell population might be required. Our quarterly reports had shown that that murine BM contains a major population of Foxp3+ T-regulatory (Treg) cells, and humans share this quality, too, with the bone marrow containing a large pool of Foxp3+ Treg cells. Thus, as in the mouse, there is a higher frequency of CD4+CD25+ Foxp3+ Treg cells in human BM than in any other secondary lymphoid organs (11). In addition, BM strongly expresses functional stromal-derived factor (CXCL12), the ligand for CXCR4, and murine and human Tregs traffic to, and are retained in, BM through CXCR4/CXCL12 signals (11). There is also evidence from hemopoietic stem cell transplant models that the BM may be an immunologically privileged site, whereby donor Tregs can protect allogeneic cells from immune destruction (12). Such considerations are of direct relevance to our findings, such that we undertook studies of Tregs present in donor limb BM samples pre-Tx.

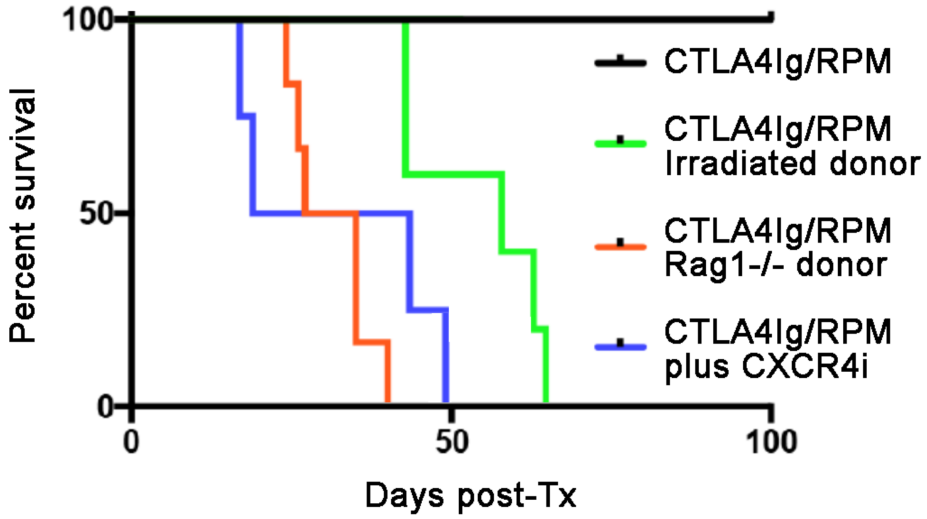
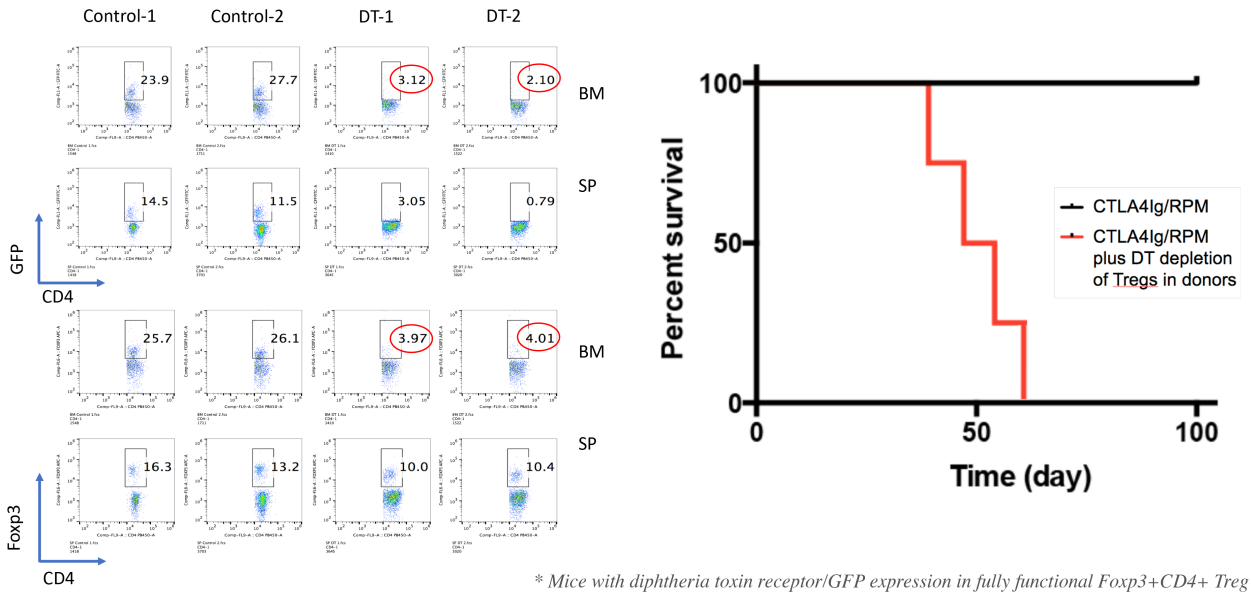


***Figure 12**. Peritransplant CTLA4Ig/RPM induces long-term VCA survival.*

Donor Foxp3+ Treg depletion induced by DT in DEREG mice blocks the ability of CTLA4Ig/RPM to induce long-term VCA survival - DEREG mice are engineered to express the diphtheria toxin receptor plus a green fluorescent protein (DTR-eGFP) within fully functional Foxp3+CD4+ Treg cells (13). Administration of diphtheria toxin (DT) markedly reduced Foxp3+ Tregs in donor BM pre-Tx (**Fig. 13, left**) and use of these treated donors blocked efficacy of CTLA4Ig/RPM in promoting long-term survival of orthotopic hind-limb VCA (**Fig. 13, right).**



** Mice with diphtheria toxin receptor/GFP expression in fully functional Foxp3+CD4+ Treg cells*

***Figure 13**. Donor BM Tregs are essential to the efficacy of CTLA4Ig-based therapy in limb transplantation. Left, WT (control) or DEREG mice were treated pre-Tx with DT and the levels of residual GFP+ and Foxp3+ Tregs assessed in donor BM and spleen (SP) samples. Right, DT-treated mice were then used as donors of hind-limbs in VCA ($p<0.01$, DT-treated vs. control VCA recipients).*

CXCR4 and Treg biology - These data were very provocative and led us consider whether the donor Treg requirement could be complemented by use of one of more additional approaches that do not involve targeting with diphtheria toxin (!). Hence, we proceeded to test the effects of CXCR4 deletion in donor BM Tregs. We conditionally deleted CXCR4 within Foxp3+ Treg cells by crossing Foxp3cre and floxed CXCR4 mice. This led to a 3 to 5-fold reduction in Tregs resident within the BM (**Fig. 14**). CXCR4 deletion also led to derepression of IL-2 and IFN-g production in Foxp3+ Treg cells, and these Tregs had decreased suppressive function *in vitro*, and when used as limb donors, led to failure of the CTLA4Ig/RPM protocol to induce long-term VCA survival (**Fig. 14**). CXCR4-/- Tregs also showed downregulation of PTEN and increased Akt-induced phosphorylated Foxo1 (**Fig. 15**). Phosphorylation of Foxo1 leads to its export from the nucleus, followed by ubiquitination and degradation, whereas nuclear Foxo1 is essential for optimal Treg development and function (14-16). • ***CXCR12/CXCR4 signals are key to the BM localization and maintenance of Treg function in donor BM***.

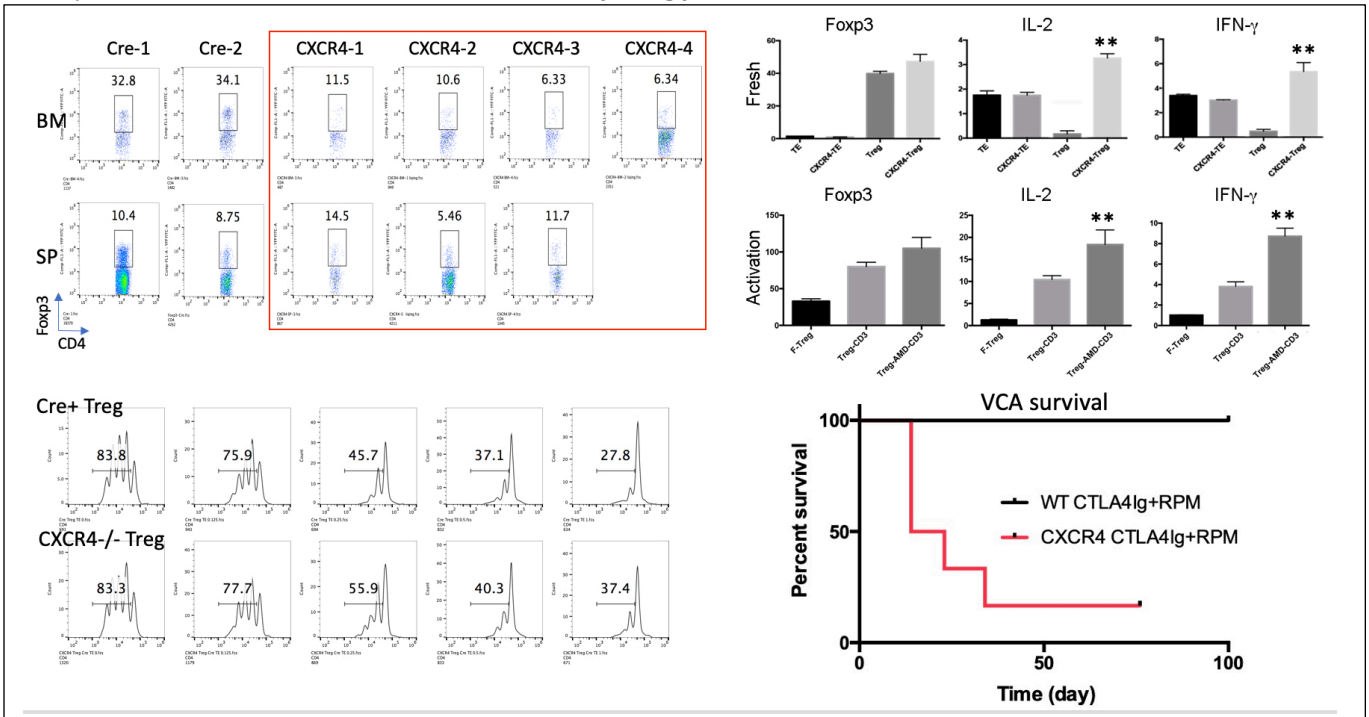


Figure 14. *Effects of CXCR4 deletion on Treg numbers, gene expression and function in vitro and in vivo.*

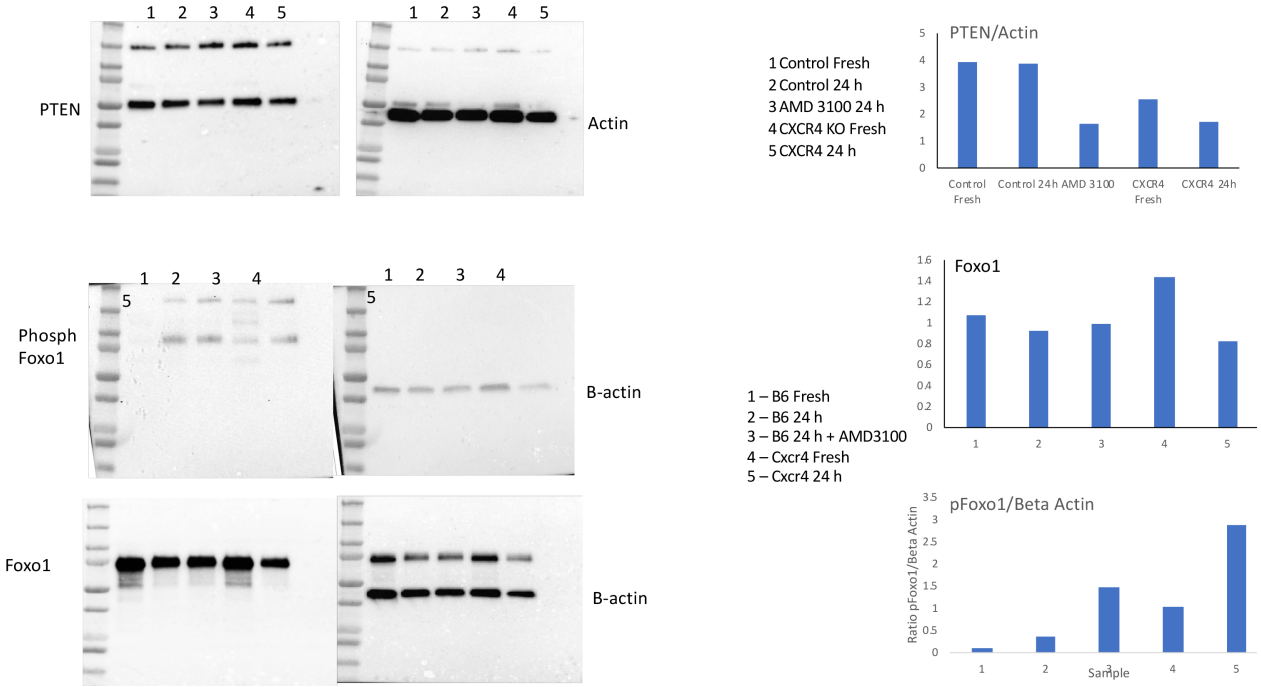


Figure 15. *Without tonic CXCR4 signaling, Tregs undergo downregulation of PTEN and increased Akt-mediated pFoxo1 generation*

In summary of our costimulation blockade work - Peritransplant costimulation blockade-based protocols can promote long-term orthotopic limb allograft survival in murine models. Efficacy is dependent upon a radiation-sensitive, CXCR4+ Treg population resident within donor bone marrow, based on studies of donor (i) DEREG mice and (ii) Foxp3^{cre}CXCR4^{flfl} mice. Tonic SDF1/CXCL12 signals via CXCR4 expressed by Foxp3+ Tregs maintain high levels of PTEN; when CXCR4 signals are blocked (AMD3100, CXCR4 deletion), PTEN levels fall, Akt and multiple downstream pathways are activated, including phosphorylation of Foxo1, leading to its export to the cytoplasm. Nuclear Foxo1 is required for stable expression of Foxp3. • ***These studies show how and why donor BM Tregs play a key role in facilitating long-term of limb allografts***.

RELATED WORK INVOLVING USE OF TCR MAB

Effects of peri-transplant T cell depletion - Induction therapy with polyclonal or monoclonal Abs is widely used in clinical transplantation. Two anti-thymocyte globulin (ATG) preparations are licensed for clinical use in the US for treatment of acute renal allograft rejection and are used as induction agents before and/or during kidney transplantation. ATG drastically reduces the number of circulating T cells, preventing acute cellular rejection of transplanted organs. Other transplant groups use CD25 (anti-IL-2R) mAb for induction therapy, given its safety profile and specificity for activated T cells. Given this clinical rationale, we have tested the efficacy of a depleting anti-TCR mAb (H57-597) that we have previously shown was efficacious in murine cardiac allograft recipients (17). Anti-TCR mAb depleted circulating T cells (CD4 and CD8) as shown by flow cytometric analysis of blood samples collected at day 14 post-Tx, whereas the proportions of CD4+Foxp3+ Treg cells were comparable (**Fig. 16**). Comparable depletion was still apparent at 36 days post-Tx (data not shown).

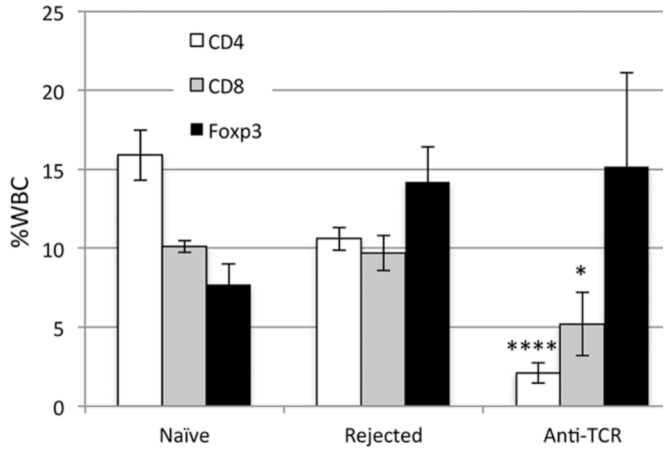


Figure 16*. Anti-TCR mAb therapy decreased circulating CD4 and CD8 cells compared to untreated VCA recipients undergoing rejection, or normal controls; graph shows analysis of % of circulating WBC at day 14 post-Tx for CD4+ and CD8+ cells (*p<0.05, ****p<0.001 vs. rejection group). Data for Foxp3 refer to proportion of Foxp3+ cells within the CD4 population in each case.*

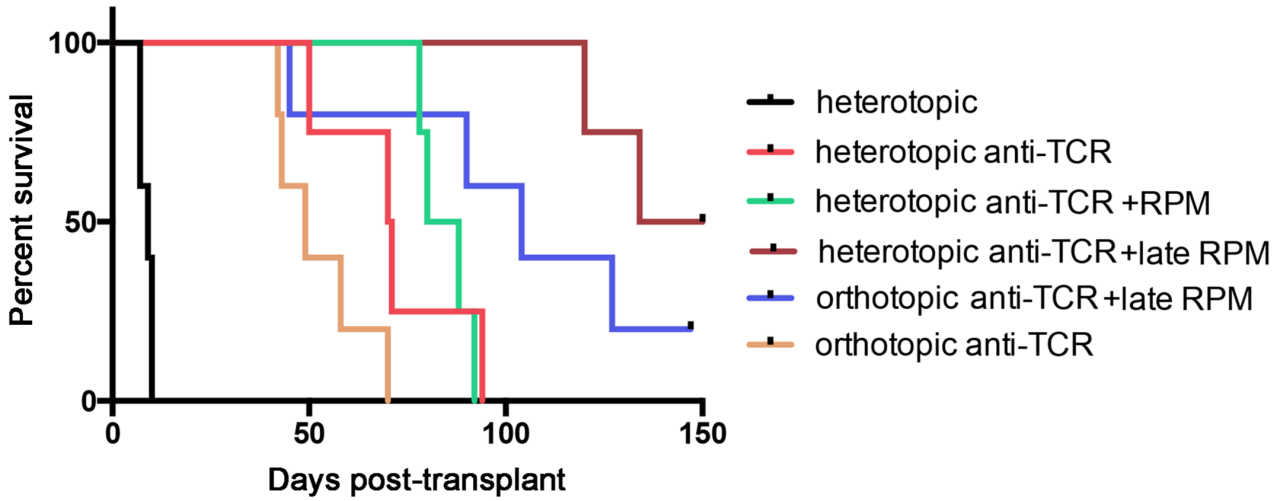


Figure 17*. Effects of anti-TCR mAb (± peri-transplant versus "late" RPM) on heterotopic vs. orthotopic hindlimb VCA survival.*

Anti-TCR mAb alone (100 µg, qod), given for 2 weeks from the time of engraftment and then stopped, prolonged heterotopic VCA survival for ~70 days (50% survival data, **red line in Fig. 17**). The addition of 4 weeks of RPM therapy (2 mg/kg/d, Alzet pumps), beginning at the time of transplantation, had no additional useful benefit on heterotopic VCA survival (p>0.05) (**green line in Fig. 17**), whereas of the 4 week course of RPM was delayed until 30 days post-Tx, 100% of heterotopic VCA survived for >100 days (**brown line in Fig. 17**). As with FK606 or, to a lesser extent, CD154/DST/RPM therapy, anti-TCR mAb was less effective in prolong VCA survival in orthotopic

vs. heterotopic allograft models. However, 50% of orthotopic VCA recipients that also received 4 weeks of RPM from day 30 post-Tx onwards (i.e. "late" RPM) maintained their grafts for >100 days (**purple line in Fig. 17**) ($p<0.01$ vs. untreated recipients or those receiving orthotopic VCA and either TCR mAb alone, or TCR plus RPM from the time of engraftment). ••• ***A brief period of T cell depletion and 4 weeks of RPM (from 30 days onwards) produced long-term (>100 d) survival in >60% of orthotopic VCA recipients. Such effects in this stringent strain combination are encouraging, and, as with the costimulation blockade-based projects, have translational potential, given the widespread use of "induction" therapy in organ transplant recipients.***

Major Task 4: Test the ability of Treg-directed therapies to promote VCA outcomes

Tregs can prolong VCA survival - We set up a model to test aspects of Treg adoptive cell therapy. Immuno-deficient (Rag1-/-) B6 mice were engrafted with BALB/c donor limbs and mice were reconstituted with $1x10^6$ recipient conventional T cells (T-effector) cells ± varying ratios of B6 Treg cells. As seen in **Fig. 18**, use of T-effector (TE) cells alone led to rejection of 50% of grafts by 12-13 days post-Tx. The addition of 0.5 million Tregs did not significantly enhance VCA survival ($p>0.05$). In contrast, addition of 1 million Tregs (i.e. use of a 1:1 ratio to TE:Treg cells) prolonged VCA survival to about 40 days ($p<0.01$). Additional studies (data not shown) indicated that this beneficial effect of Tregs (1:1 ratio) was associated with preservation of donor ($H\text{-}2K^d$-positive) Foxp3+ Tregs as well (i.e. initially present within donor BM and thereafter able to be detected within recipient lymphoid tissues post-Tx). • ***Hence, in a reductionist model, Tregs can promote VCA survival***.

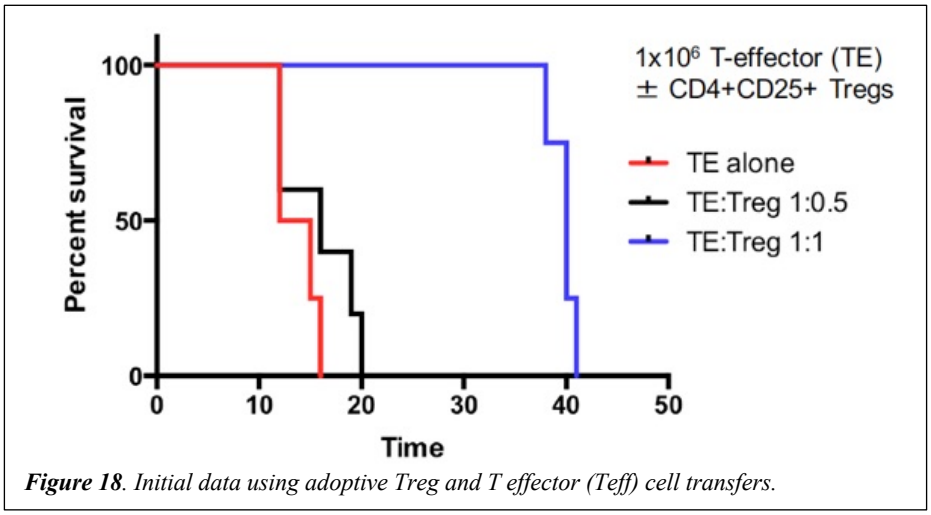


Figure 18. *Initial data using adoptive Treg and T effector (Teff) cell transfers.*

HDACi use can prolong VCA survival - With regard to Task 4 and Treg-oriented studies, we began by testing the effects of the pan-HDAC inhibitor, Trichostatin A (TsA) in conjunction with RPM in VCA recipients. This combination was previously shown to be remarkably effective in promoting donor-specific cardiac allograft tolerance, in a Treg-dependent manner, in this strain combination (18). As shown in **Fig. 19**, the combination of TsA (1 mg/kg/d, 14 d) and RPM (2 mg/kg/d, 28 d) prolonged allograft survival ($p<0.05$), but with 50% survival of 3 weeks, the results were not very impressive. Subsequent testing of an HDAC6i ± RPM did not prolong VCA survival over that seen using RPM alone, suggesting the need to target multiple HDACs to achieve therapeutic efficacy in this model. • ***Accordingly we did not pursue HDACi use further but rather turned to an alternate method of Treg expansion in vivo (IL-2 complex therapy).***

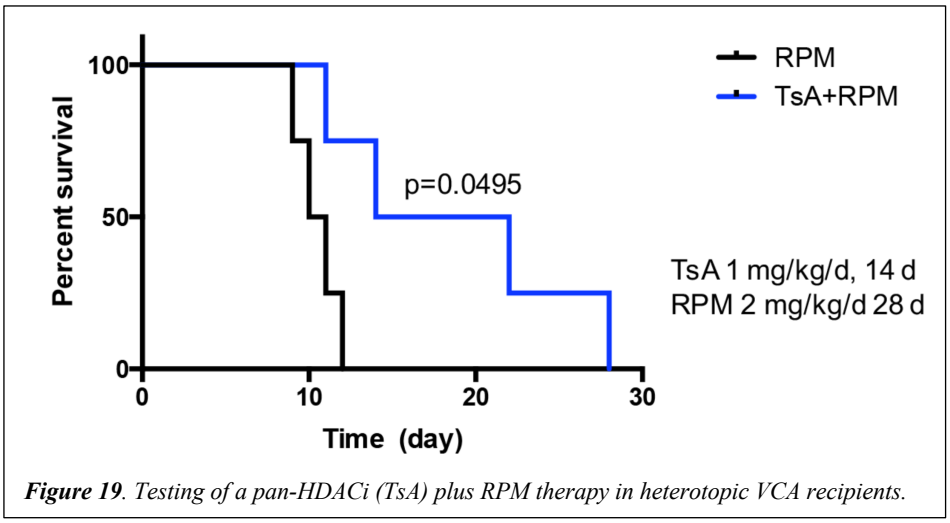


Figure 19. *Testing of a pan-HDACi (TsA) plus RPM therapy in heterotopic VCA recipients.*

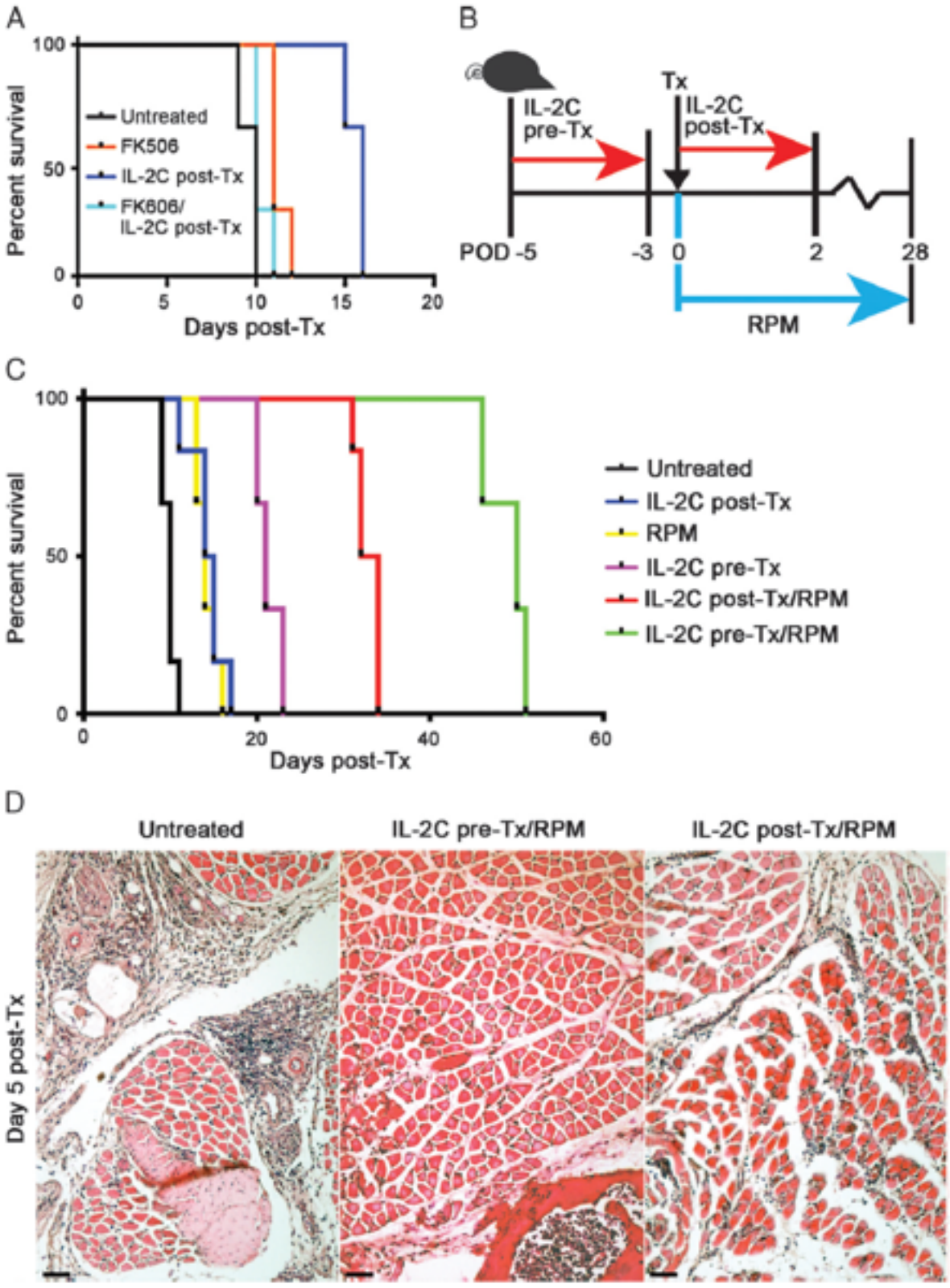


Figure 20. *Both pre- and post-Tx IL-2C treatment prolonged murine forelimb VCA survival. A, Post-Tx IL-2C therapy alone was significantly better ($P < 0.01$) at extending VCA survival than FK506 alone (1 mg/kg per day, i.p. for 14 days), or FK506 plus IL-2C therapy. B, Diagram summarizing use of pre-Tx and post-Tx IL-2C administration, as well as use of RPM in a subset of recipients. C, Kaplan-Meier plots of forelimb VCA survival for the different experimental groups (n = 6 allografts/group). D, Representative histopathology at day 5 post-Tx (H&E-stained paraffin sections, scale bar = 100 μ). H&E, hematoxylin and eosin.*

Use of IL-2 complex to expand host Tregs *in vivo* - We used a fully MHC-disparate (BALB/c-> C57BL/6) murine orthotopic forelimb Tx model to explore the benefits of pre- and post-Tx IL-2/anti-IL-2 mAb complex (IL-2C) administration as a means to expand the host Treg population and thereby attempt to promote Treg-dependent VCA survival. The results of this work were published (19). Expansion of the Foxp3+ Treg population was documented in the paper, and effects on VCA survival reported. In initial studies (**Fig. 20A**) we tested the effects of combining post-Tx IL-2C therapy with administration of FK506 (1 mg/kg/d, i.p.) for 14 days from the time of transplantation. We found that post-Tx IL-2C therapy alone significantly prolonged VCA survival compared to the 3 other treatment groups ($p<0.01$); i.e. FK506 at this dose was ineffective in prolonging survival compared to untreated controls, and its combination with IL-2C therapy revoked the efficacy of the IL-2C regimen. In subsequent studies, we tested the effects of IL-2C therapy alone or in conjunction with RPM therapy (2 mg/kg/d) delivered via 28 d Alzet pumps that were implanted beginning at the time of VCA engraftment. The experimental design is summarized in **Fig. 20B**, and comparisons between groups were undertaken at day 5 post-Tx. This point was selected given the onset of limb swelling and erythema by day 5 in untreated recipients.

Rejection occurred by 10 days post-Tx in 50% of untreated recipients, and all allografts were rejected by day 12 post-Tx (**Fig. 20C**). Administration of IL-2C alone prolonged VCA survival, compared to untreated recipients, using both pre- and post-Tx protocols ($p<0.05$) (Figure 14C), and administration of IL-2C post-Tx for longer periods, e.g. 5 days rather than 3 days had no additional benefit on VCA survival. Use of RPM monotherapy was about as effective as post-Tx IL-2C in prolonging survival ($p<0.05$, **Fig. 20C**). Co-administration of IL-2C and post-Tx RPM had additional benefits, with pre-Tx IL-C plus RPM causing a 5-fold increase in survival, and post-Tx IL-2C plus RPM causing a 3-fold increase in survival, compared to untreated VCA recipients (**Fig. 20C**). Comparison of intragraft events at day 5 post-Tx showed dense mononuclear cell infiltrates within the skin and muscle of grafts from untreated controls, along with areas of focal muscle necrosis (grade III rejection, **Fig. 20D**). Infiltrates were absent in recipients receiving pre-Tx IL-2C plus post-Tx RPM (grade 0, **Fig. 20D**), and were mainly confined to perivascular areas, without epidermal involvement or muscle necrosis, in recipients treated with post-Tx IL-2C plus RPM (grade I, **Fig. 20D**). The results of statistical comparisons of survival data for the various groups are shown in Table 1. We conclude from these data that while each therapy tested had some benefit for graft survival, combinations of IL-2C plus RPM therapy were better, and pre-Tx IL-2C plus RPM resulted in the best overall prolongation of VCA survival and initial preservation of graft histology.

Post-Tx IL-2C therapy was considerably less effective than pre-Tx therapy, with a 2.5-fold prolongation of survival, when coupled with post-RPM, compared with use of RPM alone. In addition to increasing the Foxp3+ Treg cell population, post-Tx therapy was accompanied by expansion of host T cells, especially CD8+ effector T cells producing IFN-γ and granzyme B. CD8 responses in mice receiving post-Tx IL-2C were greater than in untreated controls, indicating effects of IL-2C beyond simply targeting Treg cells. This difference with pre-Tx therapy may reflect the point that IL-2C formation with JES6-1 mAb is not completely selective for Treg cells over conventional T cells (20), or that as T cells undergo alloactivation, they upregulate CD25 and can thereby be rapidly expanded by concomitant IL-2C therapy. An inability of IL-2C therapy to control already-developing T-cell immune responses was noted in other contexts, too, including in the NOD mouse model of type I diabetes, and in experimental allergic encephalomyelitis (21, 22). Efforts to further increase the efficacy of post-Tx IL-2C therapy by extending the duration of IL-2C treatment did not improve outcomes, consistent with data from other models (21, 23). Efforts to promote the efficacy of post-Tx IL-2C therapy by co-administration of FK506 revoked the effects of IL-2C and led to rejection with a tempo similar to that of untreated recipients or those treated with FK506 alone. This finding is consistent with knowledge that calcineurin inhibitor use impairs calcineurin activation and translocation of NFAT, and that NFAT translocation is required for the optimal development and function of Foxp3+ Treg cells (24). • ***Our studies show that pre-Tx expansion of host Treg cell numbers can significantly prolong VCA survival, especially if coupled with post-Tx RPM. Such short-term use of IL-2C therapy is unlikely to increase risk of infections or cancer and appears to warrant further investigation in VCA models in which Treg cell-dependent immunoregulation has considerable potential***.

Major Task 5: Test efficacy of optimal combination therapies to promote VCA outcomes

The studies described above have identified 3 independent approaches by which peri-transplant therapy can induce long-term VCA survival: CD154 mAb/DST/RPM, CTLA4Ig/RPM and TCR mAb/RPM. Prolongation of VCA survival was also possible using Treg expansion with IL-2C, with adoptive transfer of Tregs, with HDACi therapy and with anti-CXCR3 mAb/RPM, though efficacy with each of these 4 methods was of lesser potency. • ***We conclude that the 3 ways to promote long-term engraftment with limited peritransplant therapy each have potential therapeutic application. We plan to publish the data described and proceed to testing using additional animal models and with further evaluation of the extent of restoration of graft function***.

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Nothing to Report.

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Nothing to Report.

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

Nothing to Report.

4. **IMPACT:** Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research

in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

The studies described above have identified 3 independent approaches by which peri-transplant therapy can induce long-term VCA survival: CD154 mAb/DST/RPM, CTLA4Ig/RPM and TCR mAb/RPM.

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Nothing to Report.

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:
- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to Report.

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:
- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to Report.

5. CHANGES/PROBLEMS: The Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

Changes in approach and reasons for change
Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

Nothing to Report.

Actual or anticipated problems or delays and actions or plans to resolve them

Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

Nothing to Report.

Changes that had a significant impact on expenditures
Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to Report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Nothing to Report.

Significant changes in use or care of vertebrate animals.

Nothing to Report.

Significant changes in use of biohazards and/or select agents

Nothing to Report.

6. **PRODUCTS:** List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

  Xu H, Dahiya S, Wang L, Akimova T, Han R, Zhang T, Zhang Y, Qin L, Levine MH, Hancock WW, Levin LS. Utility of IL-2 complexes in promoting the survival of murine orthotopic forelimb vascularized composite allografts. Transplantation 102, 2018, 70-78

  1-2 additional papers are in preparation.

  Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or*

series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: Author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).

Nothing to Report.

Other publications, conference papers, and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Presentations by Dr. Hancock

12/2016 "Novel Immunomodulatory Strategies for VCA"
Department of Defense
Fort Detrick, MD

7/2017 Xu H, Dahiya S, Wang L, Akimova T, Han R, Levine MH, **Hancock WW**, Levin LS.
Utility of IL-2 complexes in promoting vascularized composite allograft survival.
American Transplant Congress
Chicago, IL

7/2017 "An Update on Novel Immunomodulatory Therapies for VCA"
Department of Surgery, Duke University
Durham, NC

10/2017 Wang L, Wang Z, Han R, Ge G, Levin LS, Levine MH, **Hancock WW**.
Foxp3+ Treg cells resident within donor bone marrow are essential for costimulation blockade-induced long-term survival of murine limb transplants.
13th Congress of the International Soc of Vascularized Composite Allotransplantation
Salzburg, Austria

6/2018 Wang L, Wang Z, Han R, Ge G, Levin LS, Levine MH, **Hancock WW**.
Donor bone marrow CXCR4+ Foxp3+ Treg cells are essential for costimulation blockade-induced long-term survival of murine limb transplants.
American Transplant Congress (Plenary session)
Seattle, WA

6/2018 Xu H, Chen Z, **Hancock WW**, Levin LS, Zhang Y.
Rapamycin therapy impairs Treg expression of CXCR3 and limits Treg-dependent survival of vascularized composite allotransplants.
American Transplant Congress
Seattle, WA

6/2018 Wang L, Wang Z, Han R, Ge G, Levin LS, Levine MH, **Hancock WW**.
CXCR4+ Foxp3+ Treg cells resident within donor bone marrow are essential for costimulation blockade-induced long-term survival of murine limb transplants.
International Congress of The Transplantation Society
Madrid, Spain

11/2018 **Hancock WW**, Wang L, Levin LS, Levine MH.
CXCR4+ Foxp3+ Treg cells resident within donor bone marrow are essential for costimulation blockade-induced long-term survival of murine limb transplants.
American Society for Reconstructive Surgery
Chicago, IL

- **Website(s) or other Internet site(s)**
List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to Report.

- **Technologies or techniques**
Identify technologies or techniques that resulted from the research activities. In addition to a description of the technologies or techniques, describe how they will be shared.

Nothing to Report.

- **Inventions, patent applications, and/or licenses**
Identify inventions, patent applications with date, and/or licenses that have resulted from the research. State whether an application is provisional or non-provisional and indicate the application number. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to Report.

- **Other Products**
Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment, and/or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:
    - *data or databases;*
    - *biospecimen collections;*
    - *audio or video products;*
    - *software;*
    - *models;*
    - *educational aids or curricula;*
    - *instruments or equipment;*
    - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
    - *clinical interventions;*
    - *new business creation; and*
    - *other.*

Nothing to Report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?
Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change."

Wayne Hancock, MD, PhD
No change

Liqing Wang, MD, PhD
No change

L. Scott Levin, MD
No change

Matthew Levine, MD, PhD
No change

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing to Report.

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.
Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*

- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

QUAD CHARTS:
Attached (next page)

9. APPENDICES:

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Positioning Vascularized Composite Allotransplantation in the Spectrum of Transplantation

CRMRP-JPC8, "Novel Immunomodulatory Therapies for Vascularized Composite Allotransplantation" MR120023P3

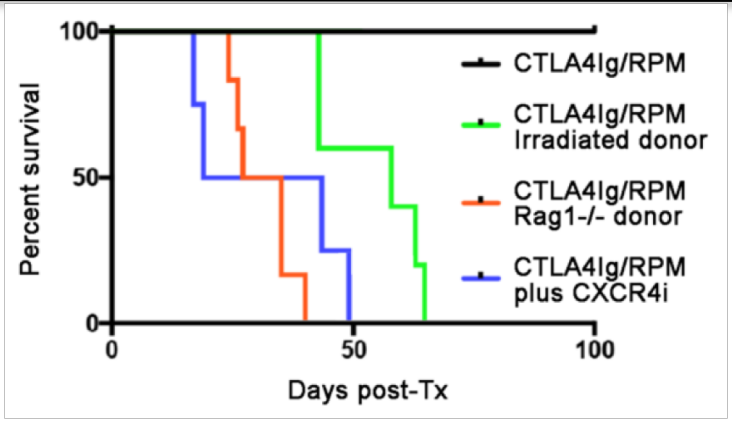
PI: Wayne W. Hancock Org: Children's Hospital of Philadelphia & University of Pennsylvania Award Amount: $1,996,875

Study Aims

- Establish murine hindlimb transplant models
- Target chemokine/chemokine receptor pathways promoting VCA rejection
- Test if costimulation blockade will promote long-term VCA survival
- Test if Foxp3+ Treg-directed therapies will promote long-term VCA survival
- Test optimal combinations of therapies so as achieve VCA engraftment and function, as well as preventing development of chronic injury

Approach

Our combined group recognizes that the long-term effects of chronic immunosuppressive therapies, including increased rates of nephrotoxicity, atherosclerotic disease, diabetes and tumor formation, outweigh their usefulness in VCA recipients. To identify less toxic and more suitable therapies for management of VCA, the group will undertake basic science studies in murine models to elucidate the mechanisms of immune rejection of VCA, and test the efficacy of novel strategies to achieve long-term engraftment without use of maintenance immunosuppressive therapy.



- Long-term orthotopic hind-limb (stringent BALB/c->C57BL/6 model) survival can be achieved with a clinically relevant protocol.
- Efficacy depends on a CXCR4+ donor BM marrow cell that is radiation-sensitive and is likely a T or B cells (efficacy lost using Rag1-/- donors).
- We then showed this is a donor BM Foxp3+ Treg cell population.

Timeline and Total Costs (includes direct & indirect costs)

Activities	2014	2015	2016	2017
Task 1. Obtain regulatory approval and establish murine hindlimb models at CHOP				
Task 2. Target key chemokine/chemokine receptor pathways promoting VCA rejection.				
Task 3. Test if peri-transplant costimulatory blockade will allow long-term VCA survival without development of chronic injury.				
Task 4. Test ability of T-regulatory (Treg) based therapies to promote VCA outcomes.				
Task 5. Test optimal combinations of therapies based on data generated above.				
Estimated Budget (total $K)	511,875	495,000	495,000	495,000

Updated: January 22, 2019

Goals/Milestones

- We undertook studies of the mechanisms of rejection of VCA in murine models, and how these may be overcome to promote long-term allograft survival. Using orthotopic hindlimb and forelimb VCA models, we showed that 3 distinct protocols, i) CD154 monoclonal antibody plus 4 weeks of rapamycin (RPM), ii) CTLA4-Ig plus 4 weeks of RPM, or iii) TCR mAb plus RPM, can each achieve long-term VCA survival without maintenance immuno-suppression. The efficacy of these protocols is dependent upon a radiation-sensitive donor bone marrow (BM) cell component, namely CXCR4+ Foxp3+ donor Treg cells. In addition, some limited prolongation of VCA survival was achieved using anti-CXCR3 mAb; adoptive transfer of Foxp3+ Tregs or their expansion using IL-2 complex; or using a pan-HDAC inhibitor (Trichostatin-A) plus RPM.

Comments/Challenges/Issues/Concerns

- Our work shows the potential for long-term engraftment, using any one of the 3 clinically applicable therapeutic protocols listed above, as a result of limited peri-transplant immunotherapy.

Budget Expenditure to Date

Projected Expenditure: As budgeted
Actual Expenditure: As budgeted