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Peritransplant Treg-Based Immunomodulation to Improve VCA Outcomes

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14. ABSTRACT

We have undertaken studies of how Treg cells, or pharmacologic modulators of Treg function called HDAC inhibitors, may be used to enhance outcomes in VCA recipients, using murine models of orthotopic limb transplantation. Using this model, we have shown that expansion of Tregs can be used to significantly prolong orthotopic limb allograft survival. In addition, we have shown that Treg infusion or use of an HDAC inhibitor can be used to promote long-term orthotopic VCA survival.

These data are highly encouraging and are of potentially important translational significance.

15. SUBJECT TERMS
allograft rejection, tolerance, costimulation blockade, T-regulatory cells

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TABLE OF CONTENTS

1. **INTRODUCTION:** Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

   This project uses murine models of orthotopic limb transplantation (Tx) to assess whether Treg-based cell therapies (Aim 1) or pharmacologic HDAC inhibitors (HDACi) that enhance Treg numbers and/or suppressive functions (Aim 2) can promote vascularized composite allotransplantation (VCA) survival.

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

   vascularized composite allotransplantation, 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.

Specific Aim 1: Can Treg cell therapy causes long-term orthotopic limb allograft survival?	**Months**	**Progress**
Major Task 1: Characterize impact of WT vs. HDAC-/- Tregs on orthotopic VCA survival		
Subtask 1: Seek IACUC & ACURO approvals for Treg-based therapy in limb allograft model	1-4	Achieved
Milestone(s) Achieved: Obtain ACURO approval	4	Achieved
Subtask 2: Undertake Treg expansion, including characterization of suppressive function, and assessment of TSDR demethylation.	5-12	Achieved
Subtask 3: Perform orthotopic limb allografts in conjunction with TCR mAb and/or WT or HDAC-/- Treg cell administration	6-12	Achieved
Milestone(s) Achieved: Efficacy of polyclonal WT vs. HDAC-/- Tregs on VCA survival	12	Achieved
Major Task 2: Effects of donor-specific WT vs. HDAC-/- Tregs on orthotopic VCA survival		Achieved
Subtask 1: Undertake donor-specific Treg expansion in vitro, prior to their infusion in vivo, including characterization of suppressive function, and assessment of TSDR demethylation for each population (WT Tregs, HDAC6-/- Tregs, HDAC11-/- Tregs).	10-12	Achieved
Subtask 2: Perform orthotopic limb allografts in conjunction with TCR mAb and donor-specific WT or HDAC-/- Treg cell administration	10-12	Achieved
Milestone(s) Achieved: Efficacy of donor-specific WT vs. HDAC-/- Tregs on VCA survival	12	Achieved
Specific Aim 2: Can HDACi-based modulation of Tregs cause long-term VCA survival?		
Major Task 1: Efficacy of TCR mAb vs. TCR plus HDAC6i or HDAC11i on VCA survival		Achieved
Subtask 1: Test TCR vs TCR plus HDAC6i	13-18	Achieved
Subtask 2: TCR vs TCR plus HDAC11i	13-18	Achieved
Milestone Achieved: Key data on the efficacy of HDACi therapy on VCA survival	18	Achieved
Major Task 2: Are effects of HDACi Treg dependent?		

Subtask 1: Test effects of Treg targeting (CD25 mAb or p300i) on the survival of otherwise well-functioning VCA in recipients previously treated with TCR mAb and HDAC6i or HDAC11i;	19-22	Achieved
Milestone(s) Achieved: Key data on whether beneficial effects of HDACi on prolongation of VCA survival are critically Treg-dependent	22	Achieved
Major Task 3: Publish the results of our studies and plan future trial(s)		Achieved
Subtask 1: Publish 1-2 papers describing the data and conclusions of our work; plan new trials.	12-24	Achieved
Milestone(s) Achieved: 1-2 papers in review or accepted for publication	24	Achieved

We have undertaken the Tasks of Specific Aims 1 and 2 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.

Specific Aim 1: Can Treg cell therapy causes long-term orthotopic limb allograft survival?

Major Task 1
Characterize impact of WT vs. HDAC-/- Tregs on orthotopic VCA survival

• Aim 1/Major Task 1/Subtask 1: Seek IACUC & ACURO approvals for Treg-based therapy in limb allograft model.

•••• Milestones Achieved: Approvals by local IACUC and ACURO were achieved.

• Aim 1/Major Task 1/Subtask 2: Undertake Treg expansion, including characterization of suppressive function, and assessment of TSDR demethylation.

••• Milestones Achieved: Efficacy of polyclonal WT vs. HDAC-/- Tregs on VCA survival (see data summarized in next subtask, too).

We began by using the strategy of IL-2 complex (IL-2C) administration as a way to boost Foxp3+ Treg numbers. **Fig. 1** shows how IL-2C administration increased Treg numbers in C57BL/6 mice. (A) IL-2C significantly increased the percentage of Foxp3+ Treg cells in the splenic CD4+ T fraction, and total Foxp3+ Treg cell numbers (x10^6 cells/spleen); data (mean ± SD) with 4 animals/group/time-point, **p<0.01, ***p<0.005. A representative flow plot is shown at right with percentage of Foxp3+CD4+ Treg cells indicated. (B) IL-2C administration on days 0, 1 and 2 led to a peak in the percentage of Foxp3+CD4+ Tregs on day 5, with a decline thereafter towards baseline; data (mean ± SD) with 4 animals/group/time-point, *p<0.01, **p<0.01. (C) IL-2C administration did not affect Treg suppressive function as assessed *in vitro* assays (mean ± SD, n=4/group) using cells analyzed at day 5. (D) Western blots of Foxp3 protein expression in Tregs from mice treated with IL-2C or PBS (representative of 3 experiments).

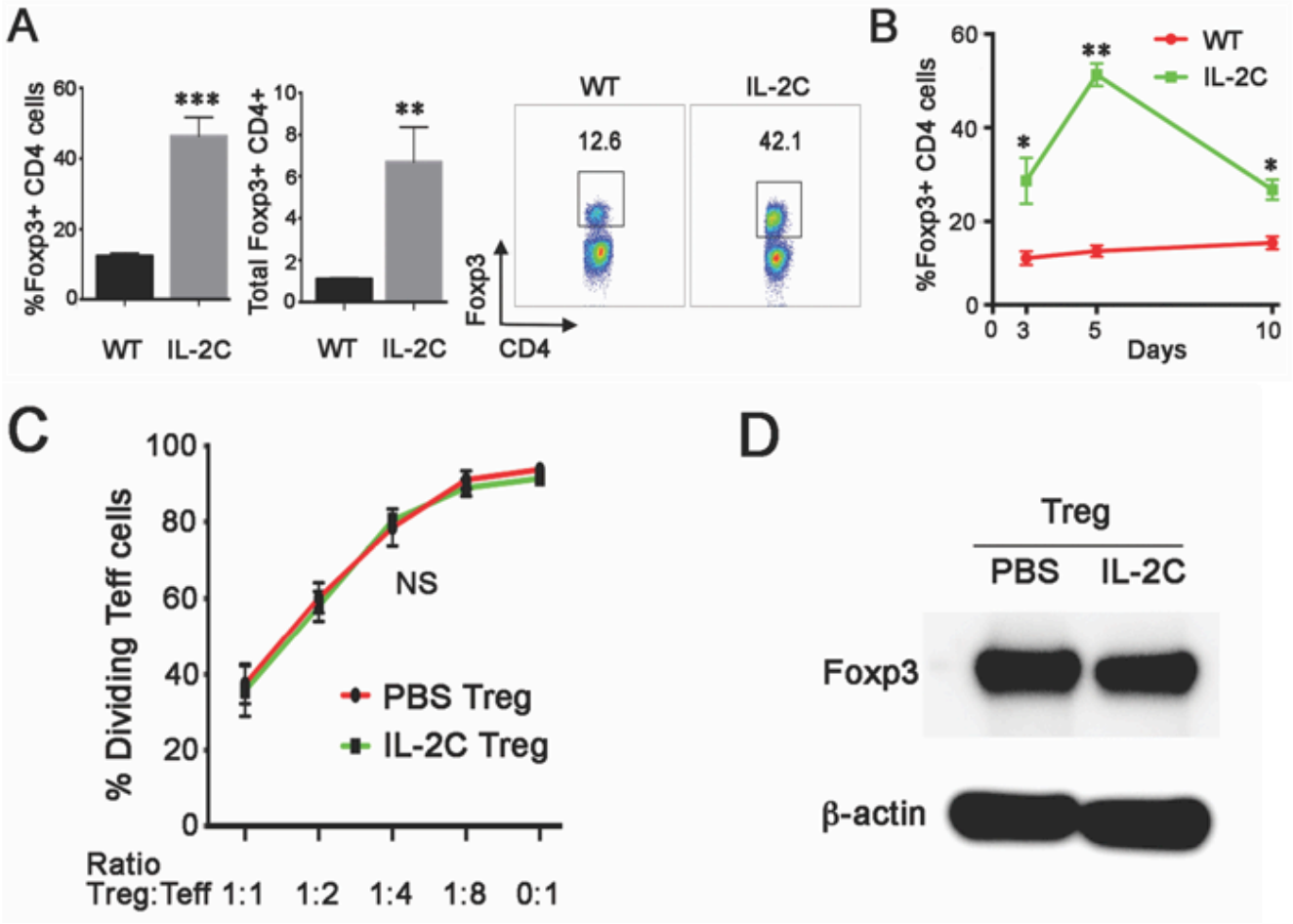


Fig. 1. Expansion and function of Foxp3+ Tregs.

We undertook assessment of Treg-specific demethylation region (TSDR) within the Foxp3 locus, by isolating Tregs and conventional T cells (Teff) from untreated and IL-2C treated B6 mice and undertaking bisulphite conversion, cloning and sequencing. WT Tregs were largely demethylated at the TSDR site (open circles, **Fig. 2**), whereas Teff cells were fully methylated (black circles) at the same site. Analysis of corresponding cells from IL-2C treated mice (day 5) showed comparable demethylation in Tregs but methylation in Teff cells. • ***Hence, IL-2C results in expansion of thymic-derived Tregs in the periphery of IL-2C treated mice, whereas on a per cell basis, Treg suppressive function is comparable to, but not greater than, that of untreated Foxp3+ Treg cells***.

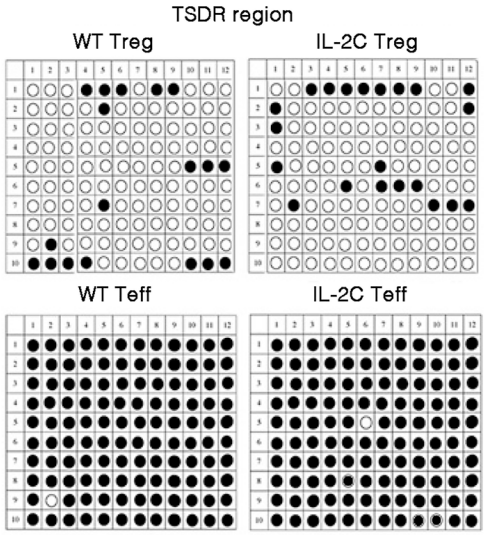


Fig. 2 Demethylation at the TSDR site.

We next tested effects of Treg expansion on VCA survival (**Fig. 3**). In initial studies (Fig. 3A) 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. 3B, 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. 3C). Administration of IL-2C alone prolonged VCA survival, compared to untreated recipients, using both pre- and post-Tx protocols ($p<0.05$) (Fig. 3C), 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. 3C).

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. 3). 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. 3D). Infiltrates were absent in recipients receiving pre-Tx IL-2C plus post-Tx RPM (grade 0, Fig. 3D), 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. 3D). 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 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***.

Table 1. Kaplan-Meier analysis of allograft survival in the various experimental groups[1]

Group	Control	Post-Tx IL-2C	Post-Tx IL-2C+RPM
RPM alone	P<0.001	P=0.502	P<0.001
Post-Tx IL-2C	P=0.002	N/A	P<0.001
Post-Tx & IL-2C+RPM	P<0.001	P<0.001	N/A
Pre-Tx IL-2C	P<0.001	P=0.010	P=0.002
Pre-Tx IL-2C+RPM	P<0.001	P<0.001	P=0.010

[1] Comparison of survival curves (log-rank test, P value) using 6-8 allografts/group.

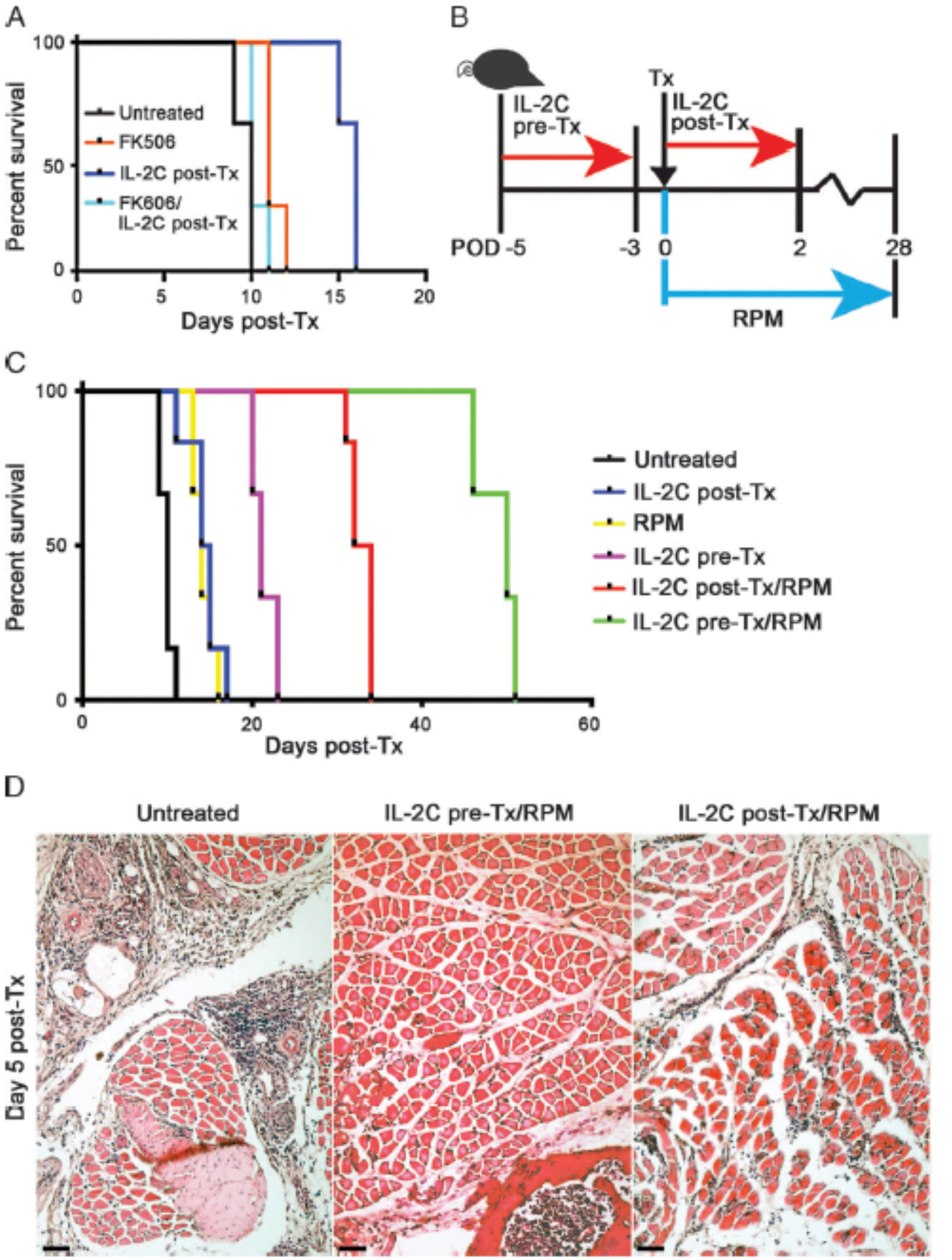


Fig. 3 Effects of Treg expansion on VCA survival.

We next assessed the effects of IL-2C therapy on host Treg and Teff cells (**Fig. 4**). At day 5 post-Tx, the proportions of Foxp3+ CD4+ Treg cells in recipients treated with IL-2C alone, or IL-2C plus RPM, were about 4-fold higher than in untreated allograft recipients ($p<0.05$), and about 2-fold higher than in mice treated with RPM alone (Fig. 4A). Mice treated pre-Tx with IL-2C ($p<0.05$) ± post-Tx RPM ($p>0.05$ vs. RPM alone) had lesser increases in Treg cells (Fig. 4A). However, at day 5 post-Tx, Tregs isolated from all 6 groups of engrafted mice showed comparable levels of IL-10 (Fi. 4B), GITR, ICOS and TGF-β, and comparable levels of cell proliferation (Ki67 expression) (Fig. 4C).

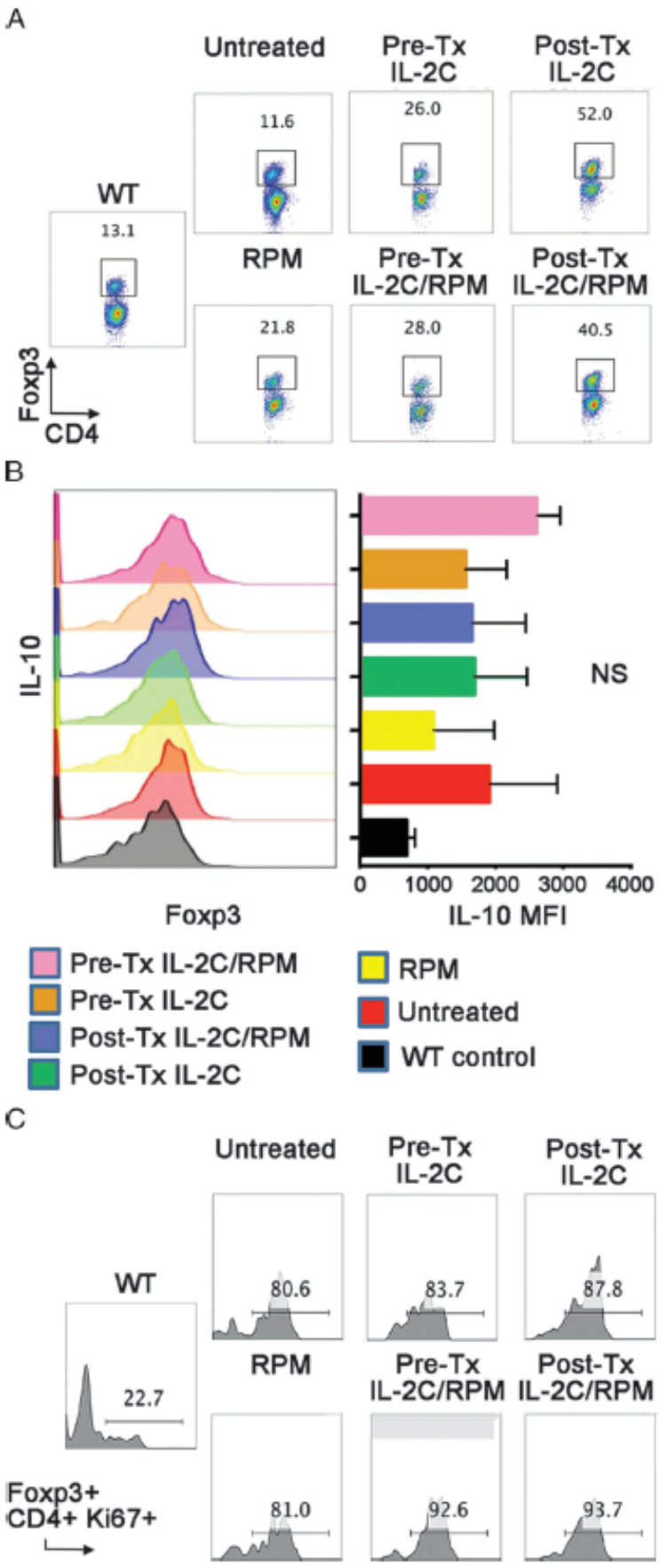


Fig. 4 Effects of IL-2C on Tregs vs. Teff cells (day 5 post-Tx)

Flow cytometric analysis of conventional CD4 and CD8 T cells, at day 5 post-Tx (**Fig. 5**), showed comparable proportions of CD4 cells in untreated recipients and those receiving pre-Tx IL-2C ± RPM (Fig. 5A). However, allograft recipients receiving post-Tx IL-2C ± RPM showed a 3 to 4-fold expansion of the CD8 population (Fig. 5A). Analysis of Ki67 expression showed increased proliferation of CD4 (Fig. 5B) and especially CD8 T cells (Fig. 5C) in all allograft groups compared with WT controls. This increase in proliferating CD8 T cells was most marked in recipients receiving post-Tx IL-2C, and in contrast to the other groups receiving RPM, was not diminished by post-Tx RPM therapy (Fig. 5C). Analysis of IFN-γ production by CD4 (Fig. 5D) and CD8 T cells (Fig. 5E) showed increases in all groups compared to WT controls, but was greatest in the case of recipients receiving post-Tx IL-2C and was diminished but not abolished by concomitant RPM therapy (Fig. 5E). Flow cytometric comparisons of the ratios of proliferating Tregs to CD4 or CD8 T cells at day 5 post-Tx (Fig. 5F) showed that the pre-Tx IL-2C/RPM protocol was especially effective at facilitating Treg expansion while curtailing CD4 and CD8 alloproliferation. In contrast, the groups receiving post-Tx IL-2C ± RPM showed particularly low Treg to CD8 T cell ratios.

• ***These data indicate that there are important differences in the levels of alloreactive CD8 T cells in VCA recipients receiving the post-Tx IL-2C, regardless of added RPM therapy, compared to pre-Tx IL-2C usage.***

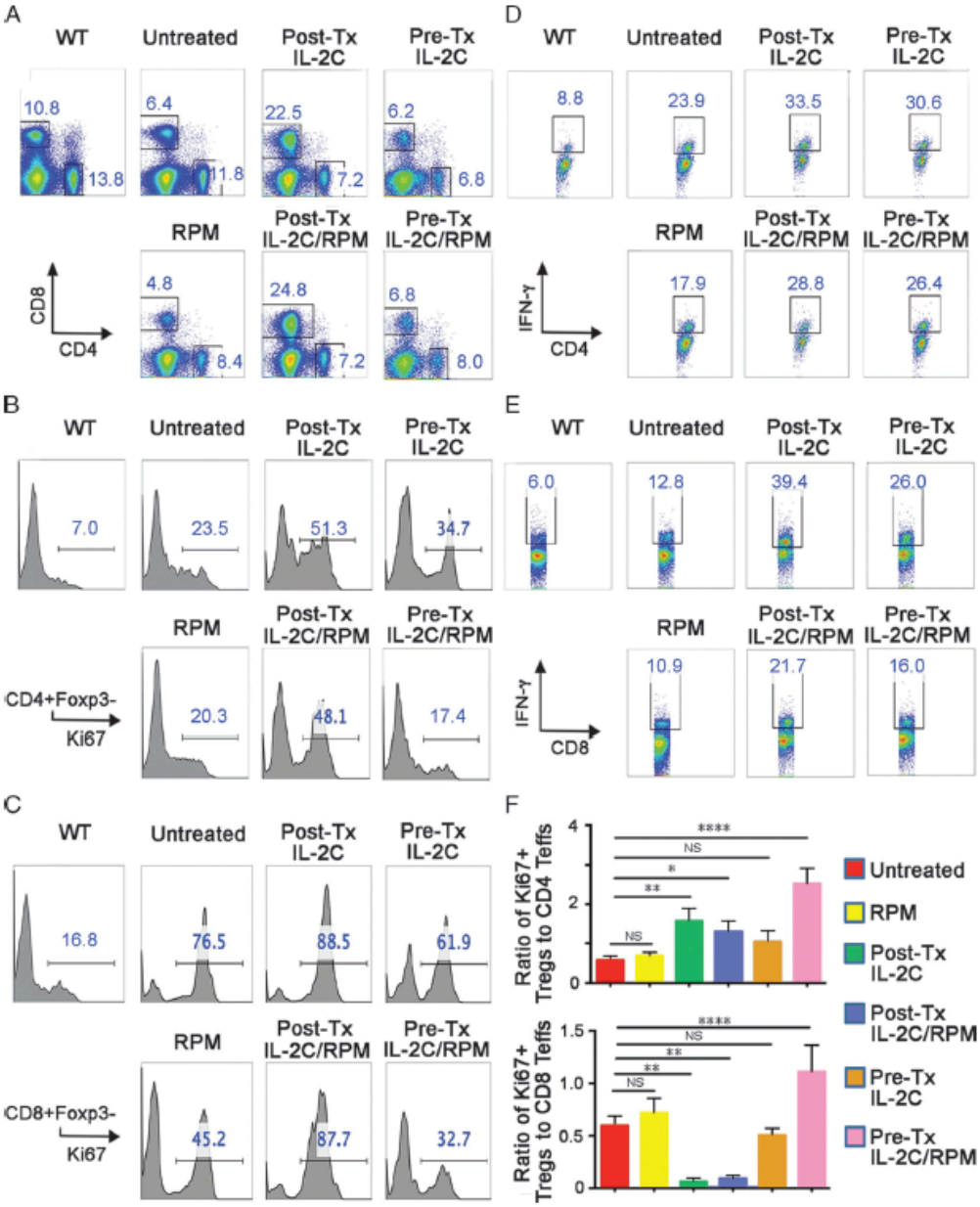


Fig. 5 Effects of IL-2 on non-Treg cells at day 5 post-Tx.

Analysis of intragraft gene expression at day 5 post-Tx showed that, compared to pre-Tx IL-2C therapy, post-Tx IL-2C usage increased intragraft CD8, IFN-γ and granzyme B expression (**Fig. 6**). Addition of RPM decreased expression of CD8, IFN-γ and granzyme B in the post-Tx IL-C group, but was especially effective in decreasing expression of these genes in recipients treated with IL-2C in the pre-Tx period. Foxp3 and IL-10 gene expression were increased in all groups receiving IL-2C therapy, and levels were only modestly decreased by RPM therapy.

• ***These data suggest that at the level of the graft, as with events in secondary lymphoid tissues, post-Tx IL-2C therapy was less effective than pre-Tx therapy in controlling alloreactive CD8 T cell responses***.

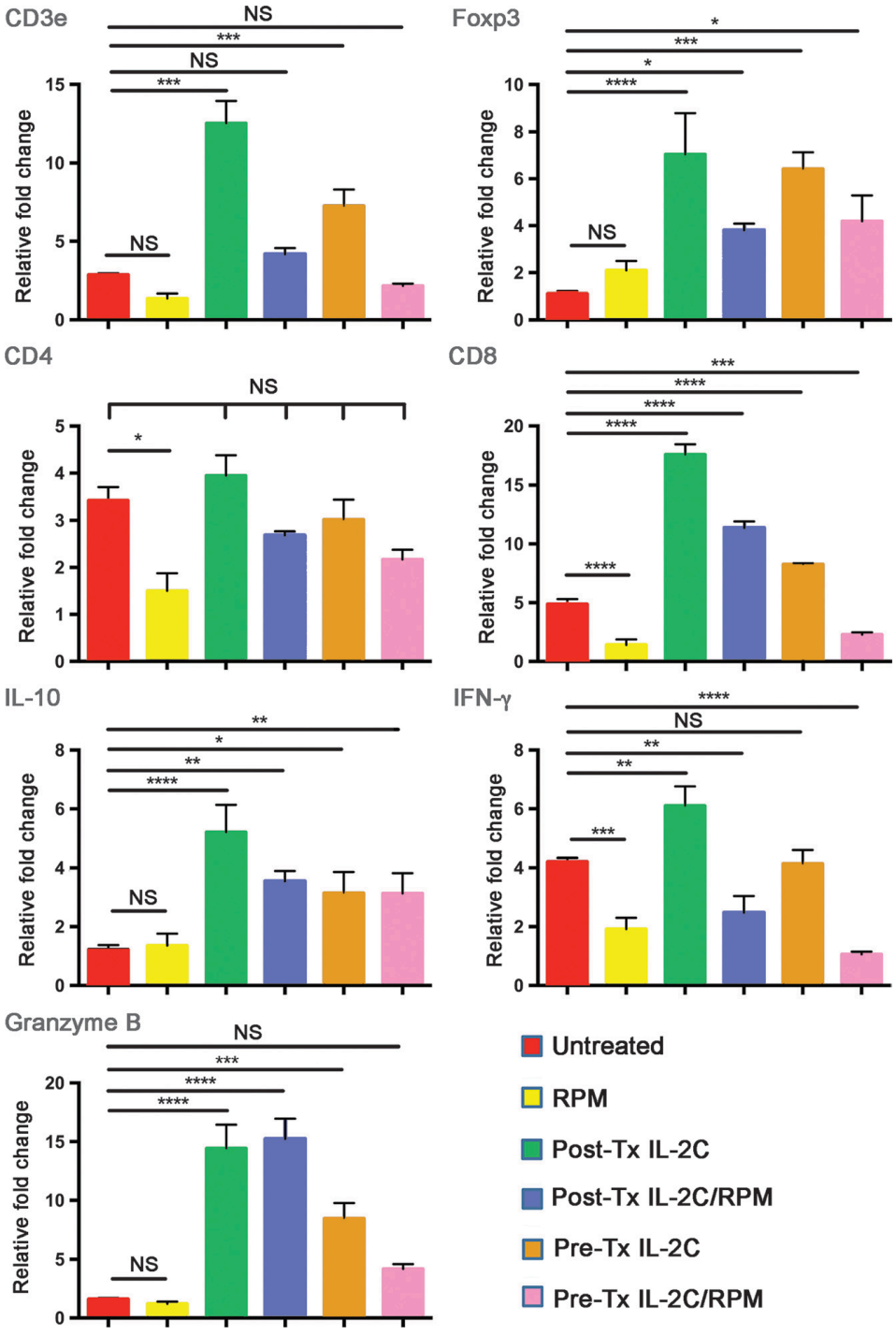


Fig. 6. Real qPCR analysis of intragraft gene expression (day 5 post-Tx, 4/ group).

With regard to successfully achieving Major Task 1, subtask 2, the data shown in Figures 1-6 show that polyclonal Treg expansion can, indeed, be used to significantly prolong VCA survival.

• Aim 1/Major Task 1/Subtask 3: Perform orthotopic limb allografts in conjunction with TCR mAb and/or WT or HDAC-/- Treg cell administration.

••• Milestones achieved: Efficacy of TCR mAb and WT vs. HDAC-/- Tregs on VCA survival was shown.

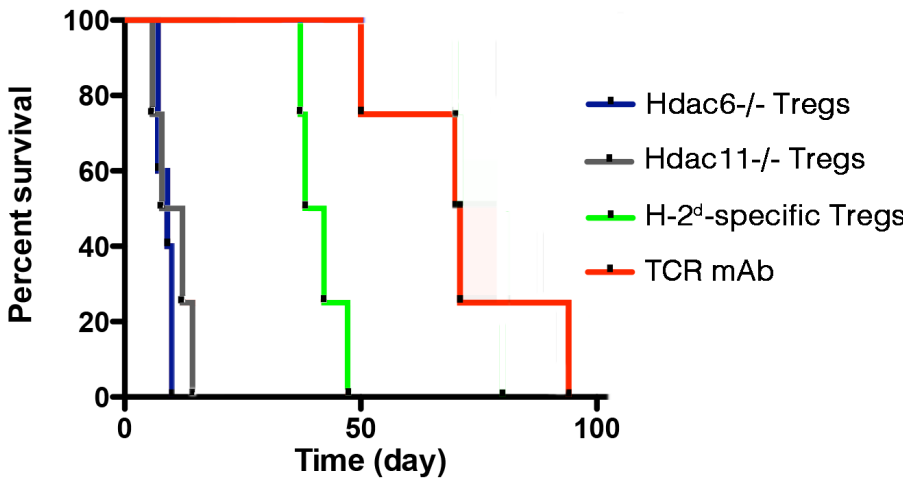


Fig. 7. Effects of infusion of donor-specific or Hdac-/- Treg cells ($5x10^6$, i.v., at engraftment), or use of TCR mAb (hamster anti-mouse TCRβ mAb, H57-597, using 100 µg, i.p., qod until day 14 post-Tx (i.e. 8 doses), on VCA survival (BALB/c->C57BL/6, H-2^d->H-2^b). Effects of donor-specific Tregs and TCR mAb were each significant compared to other groups ($p<0.01$), and TCR mAb was superior to donor-specific Tregs ($p<0.05$).

With regard to Subtask 3, infusion of $5x10^6$ Hdac6-/- Tregs or Hdac11-/- Tregs at the time of transplantation had no effect on the tempo of VCA rejection, whereas TCR mAb therapy transiently depleted recipient T cells and led to 50% survival of about 70 days ($p<0.01$) (**Fig. 7**). Combinations of these approaches are described in the next section (Figures 8-10).

Specific Aim 1
Major Task 2
Effects of donor-specific WT vs. HDAC-/- Tregs on orthotopic VCA survival

• Aim 1/Major Task 2/Subtask 1: Undertake donor-specific Treg expansion in vitro, prior to their infusion in vivo, including characterization of suppressive function, and assessment of TSDR demethylation for each population (WT Tregs, HDAC6-/- Tregs, HDAC11-/- Tregs).

Donor-specific Tregs were generated using FACS-purified YFP+ Foxp3+ B6 (H-2^b) Tregs that were cultured at $5x10^5$ cells/ml and stimulated with Dynal beads coated with anti-CD3/CD28 (4:1 bead: cell ratio) for 7-14 d plus IL-2 (500 IU/ml) and donor APC ($5x10^5$/ml) of BALB/c (H-2^d) or third party C3H (H-2^k) origin.

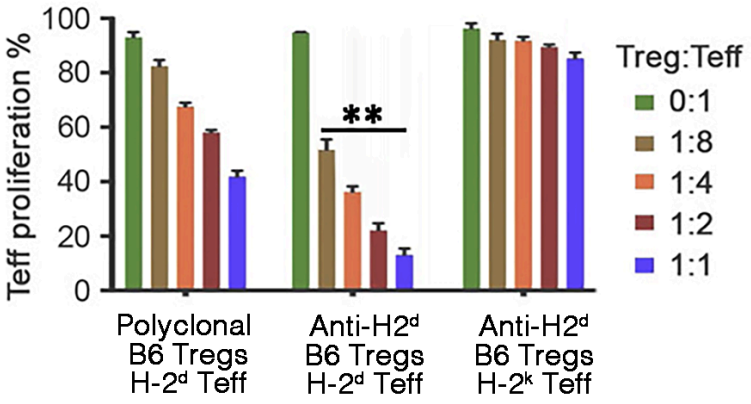


Fig. 8. Treg suppression assays using WT B6 Tregs (polyclonal) and BALB/c (H-2d) Teff cells (left); donor-specific B6 Tregs and donor Teff (H-2^d) cells (middle); or third party (H-2^k) T eff cells (right). Data are shown as area-under-curve (AUC) and 3 samples/group; **$p<0.01$ vs. either WT or third-party responses.

Treg suppressive function post-culture was used to compare suppression against donor (H-2^d) vs. third party (e.g. H-2^k) Teff cells. As seen in **Fig. 8**, using H-2^d Teff cells, donor-specific (anti-H-2^d) Tregs showed enhanced suppressive function, whereas these cells showed minimal suppressive function when tested against third party H-2^k Teff cells. Hence, these Tregs are significantly more suppressive than naïve C57BL/6 Treg cells (AUC analysis) against donor but not third party (C3H, H-2^k) cells.

• Aim 1/Major Task 2/Subtask 2: Undertake VCA in conjunction with donor-specific WT or HDAC-/- Treg administration.

We undertook BALB/c-> B6 orthotopic VCA and infused recipients at the time of engraftment with polyclonal B6 Tregs, donor-specific Tregs, or Tregs specific for third party MHC. As seen in **Fig. 9**, donor-specific Tregs were significantly more effective ($p<0.01$) than WT or third-party Tregs at prolonging VCA survival.

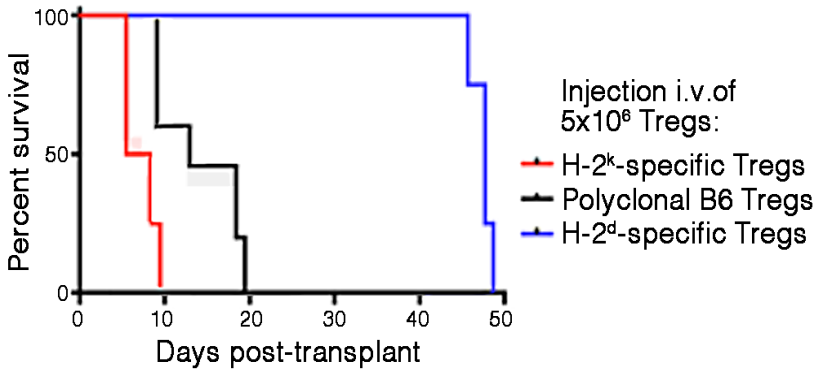


Fig. 9. Effects of infusion of polyclonal, donor-specific or third-party specific Treg infusion on VCA survival (BALB/c->C57BL/6, H-2^d-> H-2^b).

We next evaluated TCR mAb plus donor-specific WT or HDAC-/- Treg cell administration. Use of $5x10^6$ donor-specific Tregs (i.e. specific for donor MHC, H-2^d) led to 50% orthotopic VCA survival of about 45 days ($p<0.01$ vs. Hdac6-/- or Hdac11-/- Tregs) (**Fig. 7**). This was inferior to the effects of TCR mAb ($p<0.05$). However, in mice treated with TCR mAb, recipient T cells began to reappear from about 30 days. Hence, we reasoned that administration of Tregs at that 30 days point might be worth exploring. Indeed, when TCR mAb therapy was combined with Treg therapy (**Fig. 8**), with Treg infusions being undertaken at day 30 post-Tx, use of Hdac6-/- or Hdac11-/- Tregs had no additional benefit, whereas co-administration of donor-specific Tregs at day 30 post-transplant led to long-term (>100 d) orthotopic VCA survival ($p<0.01$ vs. TCR mAb alone).

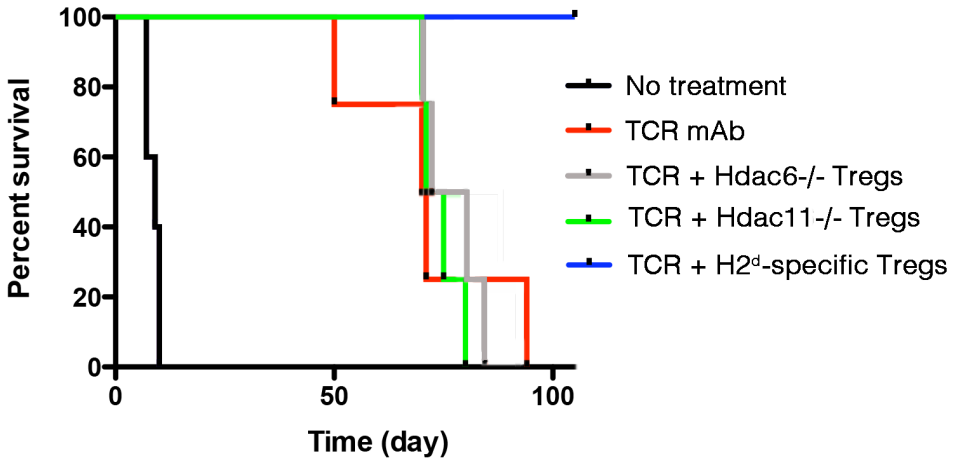


Fig. 10. Effects of infusion of donor-specific or Hdac-/- Treg cells ($5x10^6$, i.v., at day 30 post-Tx, and/or use of TCR mAb (hamster anti-mouse TCRβ mAb, H57-597, using 100 µg, i.p., qod until day 14 post-Tx (i.e. 8 doses), on VCA survival (BALB/c->C57BL/6, H-2^d->H-2^b). The effects of donor-specific Tregs plus TCR mAb were significant compared to all other groups ($p<0.01$).

••• Milestones achieved: These findings are highly encouraging that use of Treg therapy can significantly impact VCA survival.

Specific Aim 2: Can HDACi-based modulation of Tregs cause long-term VCA survival?

Major Task 1
Efficacy of TCR mAb vs. TCR plus HDAC6i or HDAC11i on VCA survival

• Aim 2/Major Task 1/Subtask 1: Test TCR vs TCR plus HDAC6i - We tested whether Hdac6i inhibitor (Hdac6i) therapy could potentiate the effects of TCR mAb in orthotopic VCA recipients. We used 40 mg/kg/d beginning at day 30 post-Tx. As seen in Fig. 9, this had no additional benefit over TCR mAb alone. This dose was selected based upon our previous experience with cardiac allografts (de Zoeten EF, Wang L, Butler K, Beier UH, Akimova T, Sai H, Bradner JE, Mazitschek R, Kozikowski AP, Matthias P, Hancock WW. Histone deacetylase 6 and heat shock protein 90 control the functions of Foxp3+ T-regulatory cells. Molecular and Cellular Biology 31, 2011, 2066-2078). **• Hdac6 targeting does not improve the efficacy of TCR mAb in promoting VCA survival.**

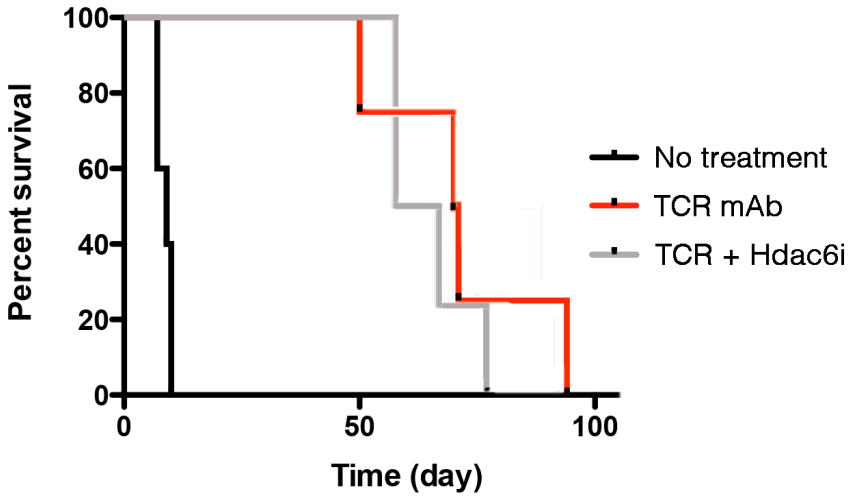


Fig. 9. Lack of efficacy of Hdac6i begun at day 30 (40 mg/kg/d, 14 d, i.p.) on TCR mAb-dependent VCA survival (p>0/05). TCR mAb (H57-597) was administered at 100 µg, i.p., qod until day 14 post-Tx (i.e. 8 doses); BALB/c->C57BL/6 orthotopic limb allograft model.

• Aim 2/Major Task 1/Subtask 2: TCR vs TCR plus HDAC11i - We tested whether Hdac11 inhibitor (Hdac11i) therapy could potentiate the effects of TCR mAb in orthotopic VCA recipients. We used 10 mg/kg/d beginning at day 30 post-Tx. As seen in Fig. 10, this had no additional benefit over TCR mAb alone. This dose was selected based upon our previous experience with cardiac allografts (Histone/protein deacetylase 11 targeting promotes Foxp3+ Treg function. Huang J, Wang L, Dahiya S, Beier UH, Han R, Samanta A, Bergman J, Sotomayor EM, Seto E, Kozikowski AP, Hancock WW. Scientific Reports 2017, 7(1):8626). Likewise, comparable testing of this approach using the Hdac11i at 40 mg/kg/d had no benefit on allograft survival. **• Hdac11 targeting does not improve the efficacy of TCR mAb in promoting VCA survival.**

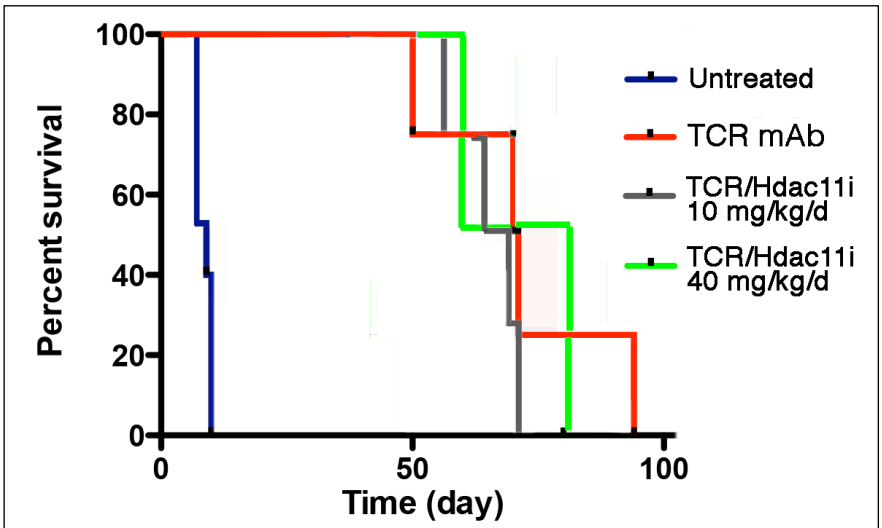


Fig. 10 - Use of an Hdac11 inhibitor, beginning at day 30 post-Tx, did not significantly improve VCA survival over that achieved using anti-TCR mAb alone.

In additional studies with relevance to Specific Aim 2 (both subtasks 1 and 2), we tested whether targeting of multiple Hdac enzymes might be more efficacious than targeting a single one (i.e. Hdac6 or Hdac11). Accordingly, we tested the effects of the combined therapy of anti-TCR mAb and a pan-Hdac inhibitor, Trichostatin-A (TsA, 1 mg/kg/day, 14 days, beginning at day 30) or with the class I Hdac inhibitor, MS-275 (10 mg/kg/day, 14 days, beginning at day 30). These doses were selected based upon our previous experience with cardiac allografts (Deacetylase inhibition promotes the generation and function of regulatory T cells. Tao R, de Zoeten E, Ozkaynak E, Chen C, Wang L, Porrett P, Li B, Turka L, Olson EN, Greene MI, Wells A, Hancock WW. Nat Med. 2007, 13:1299-307). As shown in Fig. 11, the addition of TsA to the base anti-TCR mAb therapy led to long-term survival in orthotopic VCA recipients, whereas use of MS-275 had no additional benefit over anti-TCR mAb alone. In addition, the long-term survival achieved with anti-TCR mAb/TsA depended upon the actions of Foxp3+ Treg cells, since Treg depletion, using CD25 mAb as described in footnote 2, at day 60 post-Tx led to the prompt rejection of all VCA grafts (**Fig. 11**).

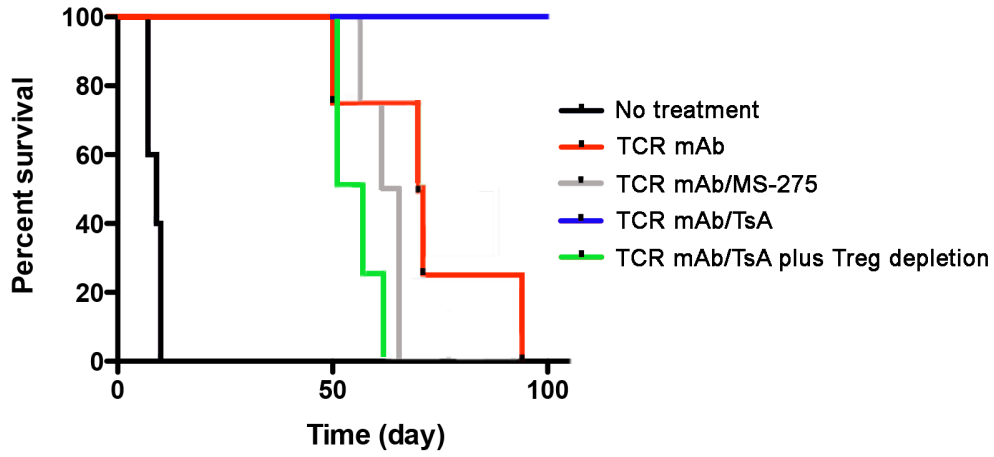


Fig. 11 - The addition of TsA to the TCR mAb protocol led to indefinite VCA survival ($p<0.01$ vs. TCR mAb alone), and was Treg-dependent as shown by the lack of efficacy when Treg cells were depleted. In contrast to the pan-HDAC inhibitor (TsA), addition of the class I Hdac inhibitor (MS-275) had no additional beneficial effect on VCA survival over that seen using TCR mAb alone.

••• **Milestones Achieved**: Key data on the efficacy of HDACi therapy on VCA survival - Collectively, the contrast between lack of significant benefit of additional targeting Hdac6 or Hdac11 individually over TCR mAb therapy alone, versus TCR mAb and use of a pan-Hdaci, suggests that 2 or more Hdacs need to be inhibited to enhance the effects of TCR mAb. These Hdacs are likely not class I in nature (Hdac1, Hdac2, Hdac3, Hdac8) since these Hdacs are inhibited by MS-275. These data are consistent with our previous studies that Hdac6, 10 and 11 targeting can significantly improve Treg function. Class IIa Hdacs are not inhibited by TsA and hence are likely not implicated by this work.

Major Task 2
Are effects of HDACi Treg-dependent?

Aim 2/Major Task 2/Subtask 1: Test effects of Treg targeting (CD25 mAb or p300i) on the survival of otherwise well-functioning VCA in recipients previously treated with TCR mAb and HDAC6i or HDAC11i.

As shown in **Fig. 11**, the long-term survival achieved with anti-TCR mAb/TsA depended upon the actions of Foxp3+ Treg cells, since Treg depletion, using CD25 mAb at day 60 post-Tx led to the prompt rejection of all VCA grafts. These findings led us to test the effects of a p300 inhibitor (p300i) beginning at day 0 or at day 50 (i.e. at the time of transplantation or at a week after cessation of TsA therapy, respectively).

The p300i used (C646) was previously validated, for its inhibitory effects on Treg cells while sparing conventional T cells, by us in allograft and tumor models (Liu Y, Wang L, Predina J, Han R, Beier UH, Wang LC, Kapoor V, Bhatti TR, Akimova T, Singhal S, Brindle PK, Cole PA, Albelda SM, Hancock WW. Inhibition of p300 impairs Foxp3+ T-regulatory cell function and promotes anti-tumor immunity. Nature Medicine 19, 2013, 1173-1177). As seen in Figure 12, use of either a low (1 mg/kg/d) or a high dose (10 mg/kg/d) of the p300i from day 50 post-Tx led to acute rejection, as did use of the p300i (10 mg/kg/d) from the time of allografting (day 0).

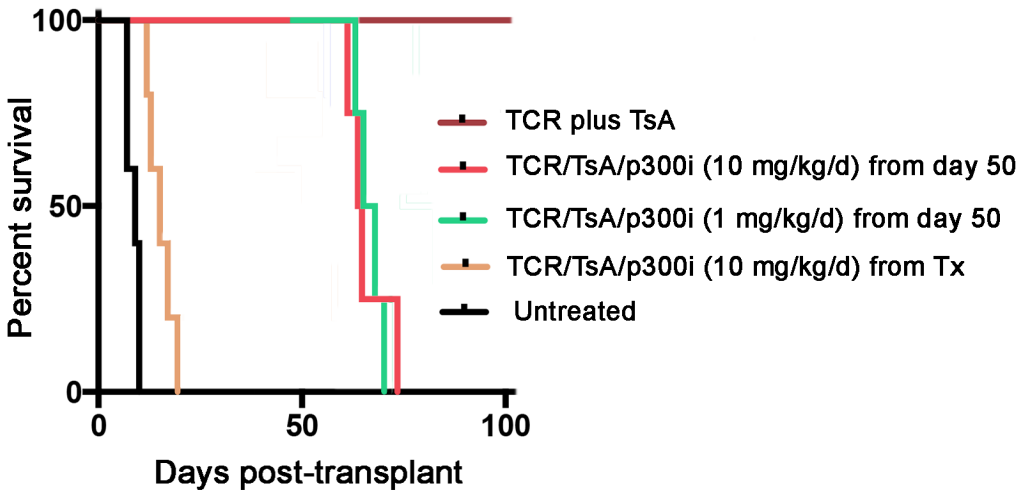


Fig. 12 – Effects of Treg targeting using a p300i (C646) at 1 or 10 mg/kg/d, as shown, on the efficacy of the TCR/TsA protocol that normally induces long-term (>100 d) orthotopic VCA survival.

••• **Milestones Achieved:** Key data showing the beneficial effects of HDACi on prolongation of VCA survival are critically Treg-dependent. These data show that the efficacy of TCR/TsA is critically dependent upon Foxp3+ Treg cells for both the induction and maintenance of VCA survival.

Major Task 3
Publish the results of our studies and plan future trial(s)

Aim 2/Major Task 2/Subtask 3: Publish 1-2 papers describing the data and conclusions of our work; plan new trials.

To date we have published 1 paper from this work and expect to publish an additional 1-2 papers based on the data summarized in this report.

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

••• **Milestone(s) Achieved**: 1-papers in review or accepted for publication.

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).

Nothing to Report.

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 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: For collaborative awards, independent reports are required from BOTH the Initiating PI and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to **https://ers.amedd.army.mil** for each unique award.

QUAD CHARTS: If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.

Attached (next page)

9. APPENDICES: Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

Peritransplant Treg-Based Immunomodulation to Improve VCA Outcomes

DoD Idea Discovery Award W81XWH-16-1-0755

RT150100

PI: Wayne W. Hancock **Org:** Children's Hospital of Philadelphia **Award Amount:** $450,000.00

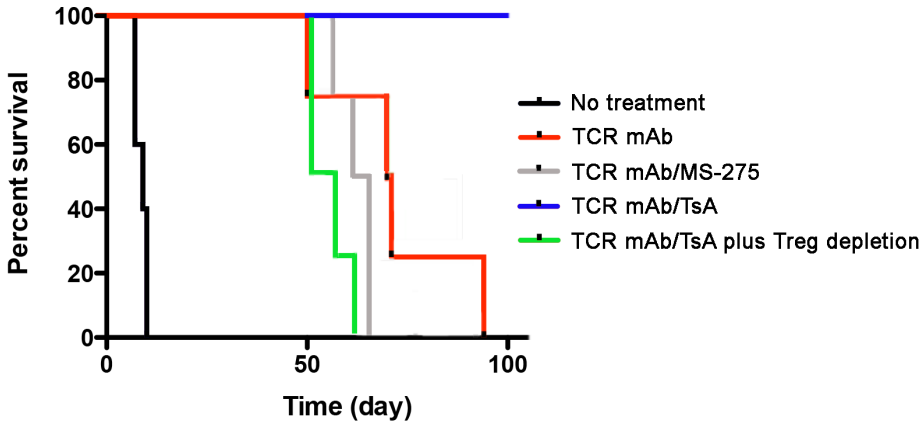
Study/Product Aim(s)

- **Aim 1** - Determine if Foxp3+ T-regulatory (Treg)-based cell therapy can promote long-term murine limb vascularized composite allotransplantation (VCA) survival.

- **Aim 2** - Determine if histone/protein deacetylase (HDAC) inhibitor -based pharmacologic modulation of Tregs will cause long-term VCA survival.

Approach

We propose proof-of-principle studies in murine VCA models with wild-type Treg cells or with Treg cells that have enhanced suppressive function as a result of specific deletion of one or more histone/protein deacetylase (HDAC) enzymes, followed by translational studies testing the effects of one or more courses of therapy with pharmacologic inhibitors of the corresponding HDACs in wild-type (WT) VCA recipients.



We showed limited therapy with TCR mAb plus donor-specific Tregs (not shown) or TCR mAb plus an HDACi (as above), induce permanent survival in a fully orthotopic MHC-mismatched murine limb VCA model (BALB/c-> C57BL/6), and induction and maintenance of survival is Treg-dependent.

Timeline and Cost

Activities CY	16	17	18	
Regulatory approval & begin VCA				
Test effects of Treg cell therapy				
Test effects of HDACi therapy				
Publish results				
Estimated Budget ($K)	**$000**	**$225K**	**$225K**	

Goals/Milestones (Example)

CY16 Goal – Obtain regulatory approval and establish VCA model

☑ IACUC and ACURO approval

CY17 Goals – Test effects of Treg cell therapy on VCA survival

☑Investigate effects of WT & HDAC-/- Tregs on VCA survival

☑Investigate effects of donor-specific WT & HDAC-/- Tregs on VCA survival

CY18 Goal –Test effects of HDACi therapy on VCA survival

☑Test effects of TCR mAb treatment ± HDAC6i or HDAC11i therapy on VCA survival

☑Determine if the benefits of HDACi therapy are Treg dependent

☑Publish the results of our studies

Comments/Challenges/Issues/Concerns

- No concerns.
- Spending on track

Budget Expenditure to Date

Projected Expenditure: $450,000

Actual Expenditure: = $450,000

Updated: January 9, 2019