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14. ABSTRACT <p>We have continued our 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 either of 2 protocols, namely costimulation blockade (CD154 monoclonal antibody plus 4 weeks of rapamycin, RPM), or CTLA4lg plus 4 weeks of 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 type that is of T or B cell origin and is CXCR4+. The latest data indicate that this is a Foxp3+ donor Treg population resident in donor BM. In addition, we have found that Treg therapy can significantly prolong VCA survival.</p> <p>This work is continuing in the form of an approved 1 year no-cost extension.</p>						
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1. INTRODUCTION

The point of our study is to analyze immune mechanisms contributing to rejection of vascularized composite allografts (VCA) in murine models, and to overcome these responses and promote long-term VCA survival.

2. KEYWORDS

Vascularized composite allografts, allograft rejection, tolerance, costimulation blockade

3. OVERALL PROJECT SUMMARY

Our goals for **CY16** were to finalize Tasks 3, 4 and 5; Task 3 is to test if peri-transplant immunotherapy will allow long-term VCA survival without development of chronic injury; Task 4 involves testing the ability of Treg-based therapies to promote VCA outcomes, and Task 5 is to undertake final studies using optimal combinations. Important progress on all 3 was achieved. Note, in the studies summarized below, at least 6 hind-limb orthotopic transplants/group (BALB/c->C57BL/6) were performed.

TASK 3: COSTIMULATION BLOCKADE

3.1 Requirements for the efficacy of peritransplant CD154/DST/RPM to induce long-term VCA survival.

We explored some of the requirements for CD154/DST/RPM-induced long-term VCA survival (Fig. 1).

CD154 mAb (CD40L, MR-1) 200 µg, i.v. and 5×10^6 donor splenocytes (DST) at Tx
RPM 2 mg/kg/d via a 4-week Alzet pump

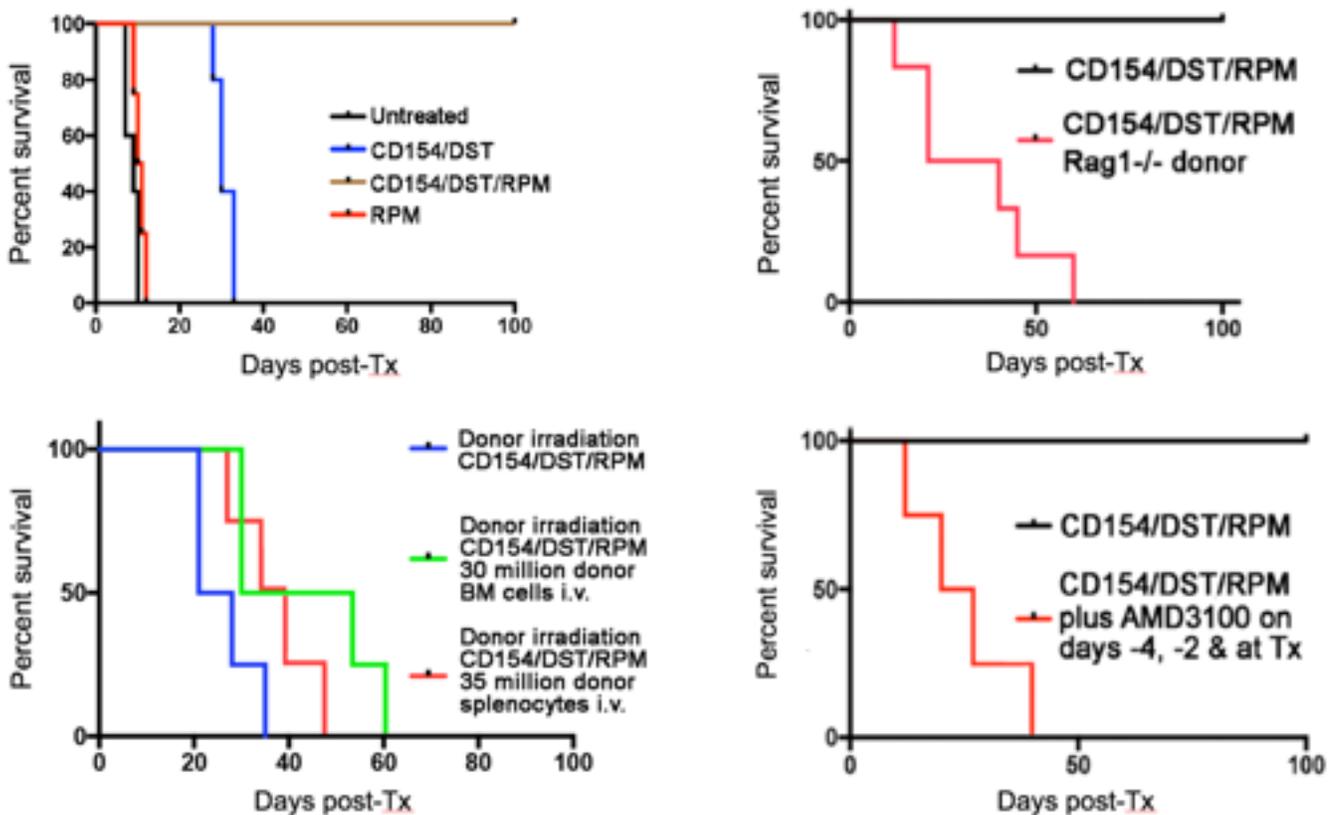


Fig. 1. Both therapeutic components (CD154/DST and RPM) are required (top left). In addition, a radiation-sensitive population (bottom left), that is likely a T or B cell (top right), and can be mobilized by CXCR4 inhibitor therapy is required for efficacy ($p < 0.01$ for CD154/DST/RPM vs. all other groups).

3.2 CD154/DST/RPM preserves donor BM cells (histology).

Histologic analysis of VCA samples at day 7 post-Tx showed that CD154 (MR1)/DST/RPM preserved donor tri-lineage BM cells (**Fig. 2**), in contrast to the destruction of donor BM cells seen in grafts from untreated mice.

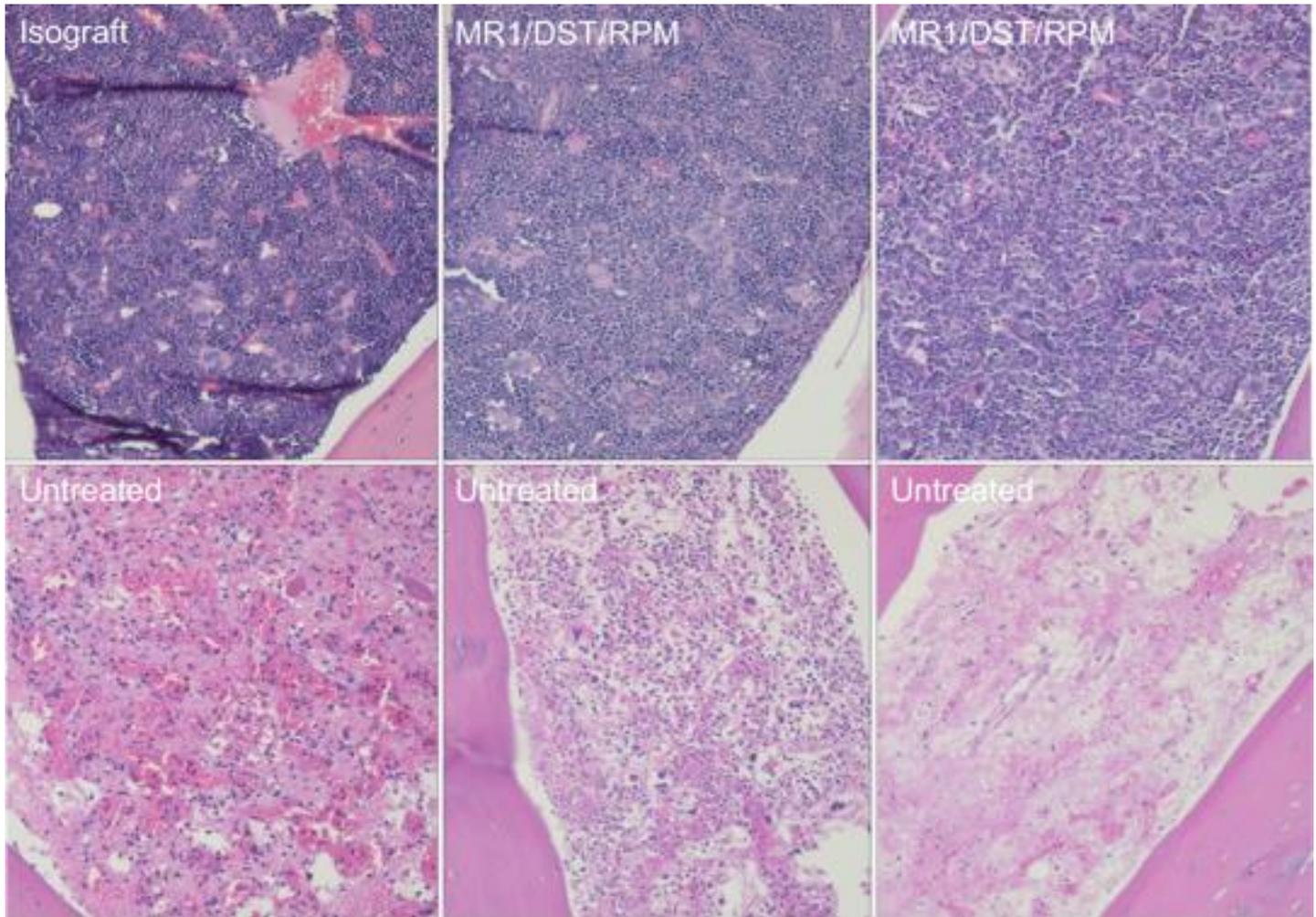


Fig. 2. Preservation of donor BM cells within hind-limb orthotopic hind-limb transplants in recipients treated with CD154/DST/RPM (day 7 samples, H&E-stained paraffin sections, x100 original magnifications).

3.3 CD154/DST/RPM preserves donor BM cells (flow cytometry).

At 7 days post-Tx, VCA limbs were harvested and the BM cells obtained by flushing were analyzed by flow cytometry (**Fig. 3**). We stained the samples with standard CD4 and CD8 monoclonal antibodies (mAbs) plus a mAb to recipient MHC (C57BL/6 mice are H-2^b). Hence, in flow plots with staining for H-2^b on the y-axis, BM cells from healthy, untransplanted BALB/c donors (H-2^d) are unstained, as seen in the panel at top left. Serial analysis of 2 mice (representative of 6/group) showed progressive depletion of donor BM cells in untreated mice (see paucity of cells by day 7 post-Tx), whereas large numbers of H-2^b-negative cells (i.e. donor cells) were presented at day 7 in samples from VCA recipients treated with CD154/DST/RPM.

These findings are consistent with the histologic data (Fig. 2), and show how donor BM is destroyed in conjunction with other components of the limb in untreated recipients, whereas the costimulation blockade-based protocol perseveres these cells.

At this point, we were uncertain of the significance of this preservation and whether it might be unique to the CD154/DST/RPM protocol. This led us to test another, more clinically relevant protocol of CTLA4lg/RPM might have comparable requirements for efficacy.

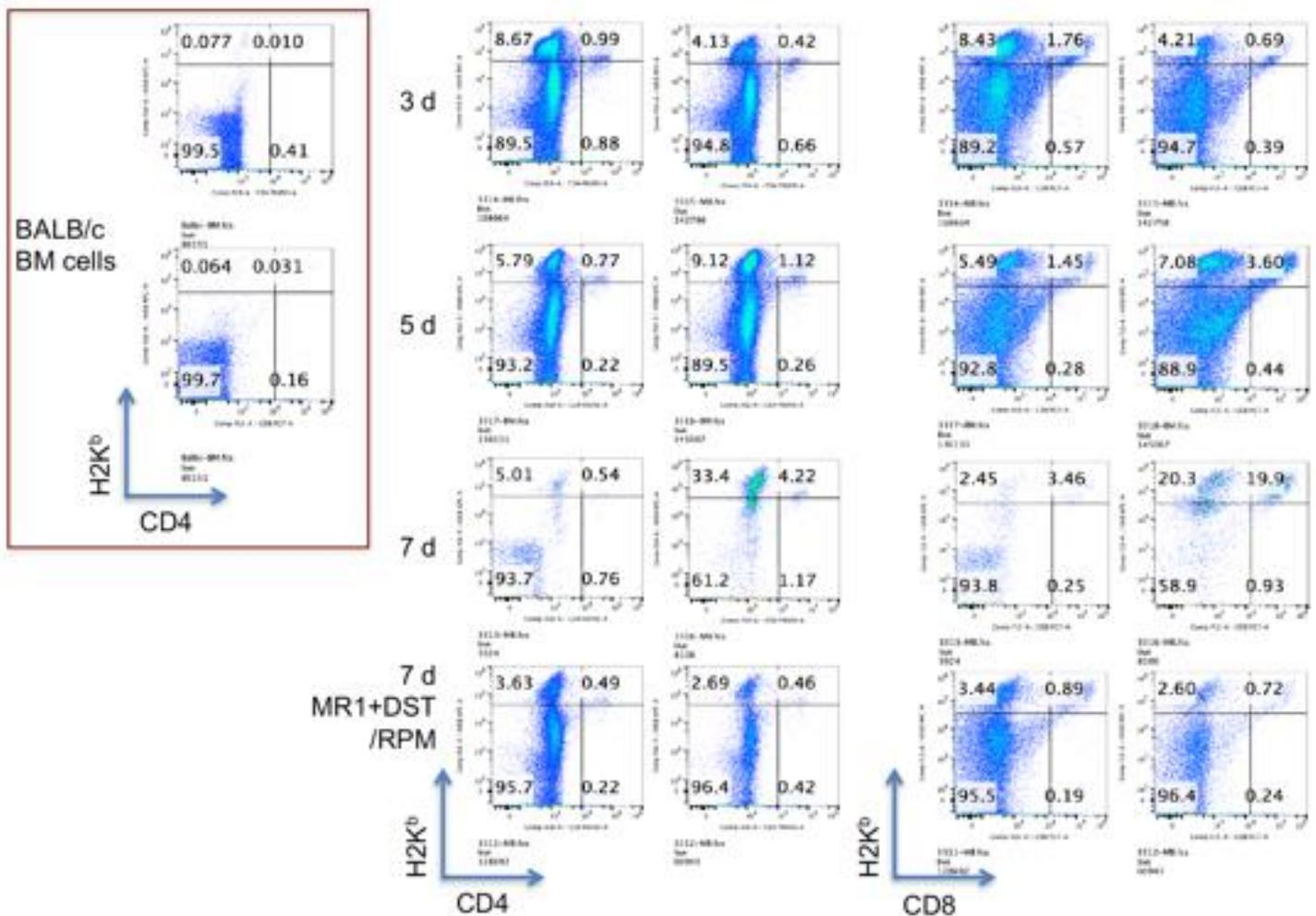


Fig. 3. Preservation of donor BM cells as assess by flow cytometry of BM cells isolated from hind-limb orthotopic hind-limb transplants in recipients treated with CD154/DST/RPM (day 7 samples). See text for summary of validation and experimental design.

3.4 Requirements for the efficacy of peri-transplant CTLA4Ig/RPM to induce long-term VCA survival.

We explored some of the requirements for CTLA4Ig/RPM-induced long-term VCA survival using dosing as shown in the Figure (Fig. 4).

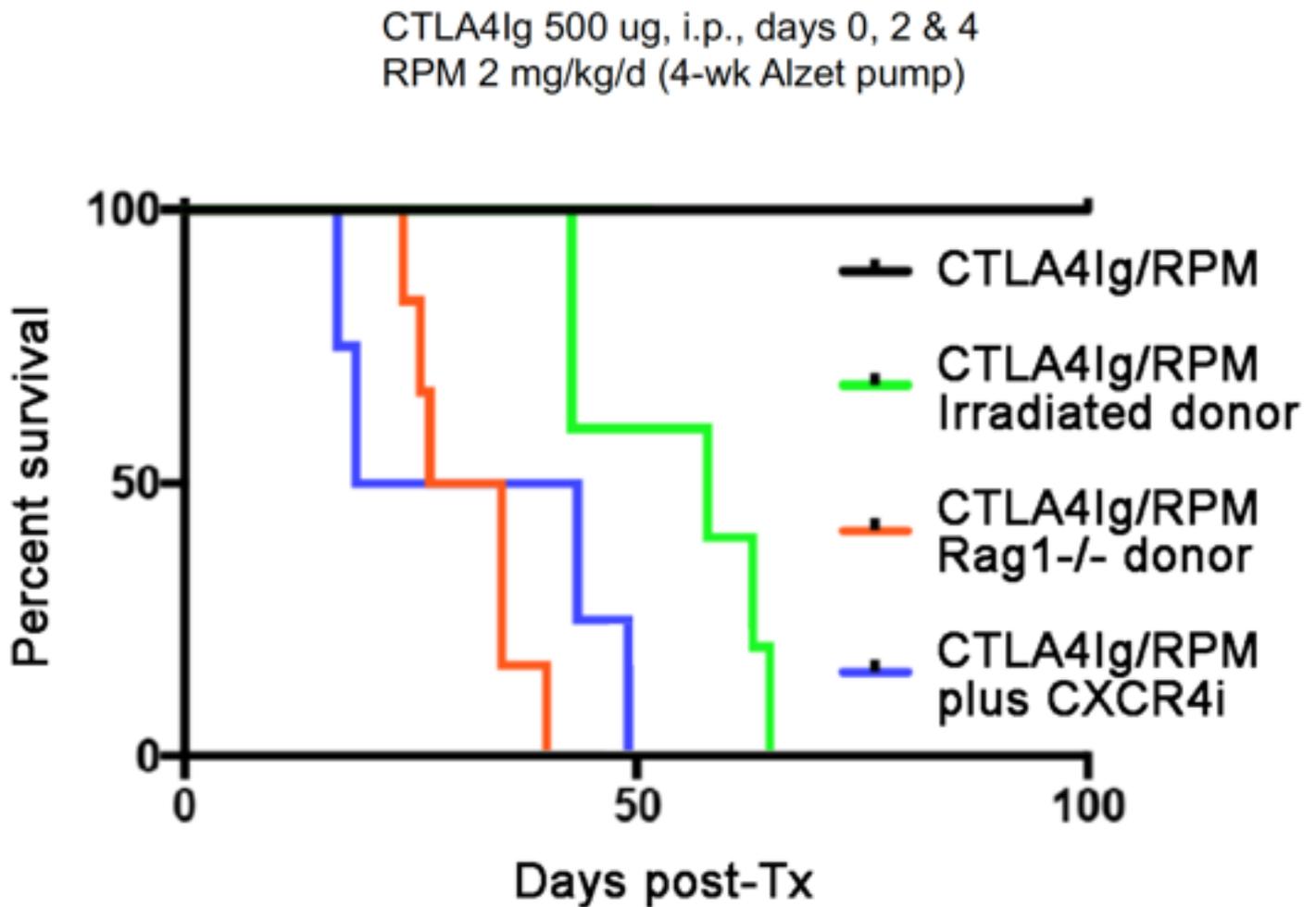


Fig. 4. The efficacy of CTLA4Ig/RPM to induce long-term VCA survival required the presence of a radiation-sensitive population, that is likely a T or B cell, and can be mobilized by therapy with a CXCR4 inhibitor ($p < 0.01$ for CTLA4Ig/RPM vs. all other groups).

These studies showed that CTLA4Ig/RPM was able to induce long-term hindlimb orthotopic VCA survival, but as with CD154/DST/RPM, efficacy was abrogated by irradiation, use of Rag1^{-/-} donors, or pre-transplant treatment of donors with a CXCR4 inhibitor (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 (1). 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 (1). 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 (2). Such considerations are of direct relevance to our findings, such that we have begun to undertake studies of Tregs present in donor limb BM samples pre-Tx.

3.5 Donor F_{oxp3}⁺ Treg depletion induced by DT in DERE_G 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 F_{oxp3}⁺CD4⁺ Treg cells (3). Administration of diphtheria toxin markedly reduced F_{oxp3}⁺ Tregs in donor BM pre-Tx (Fig. 5, left) and use of these treated donors blocked the efficacy of CTLA4Ig/RPM in promoting long-term survival of orthotopic hind-limb VCA (Fig. 5, right).

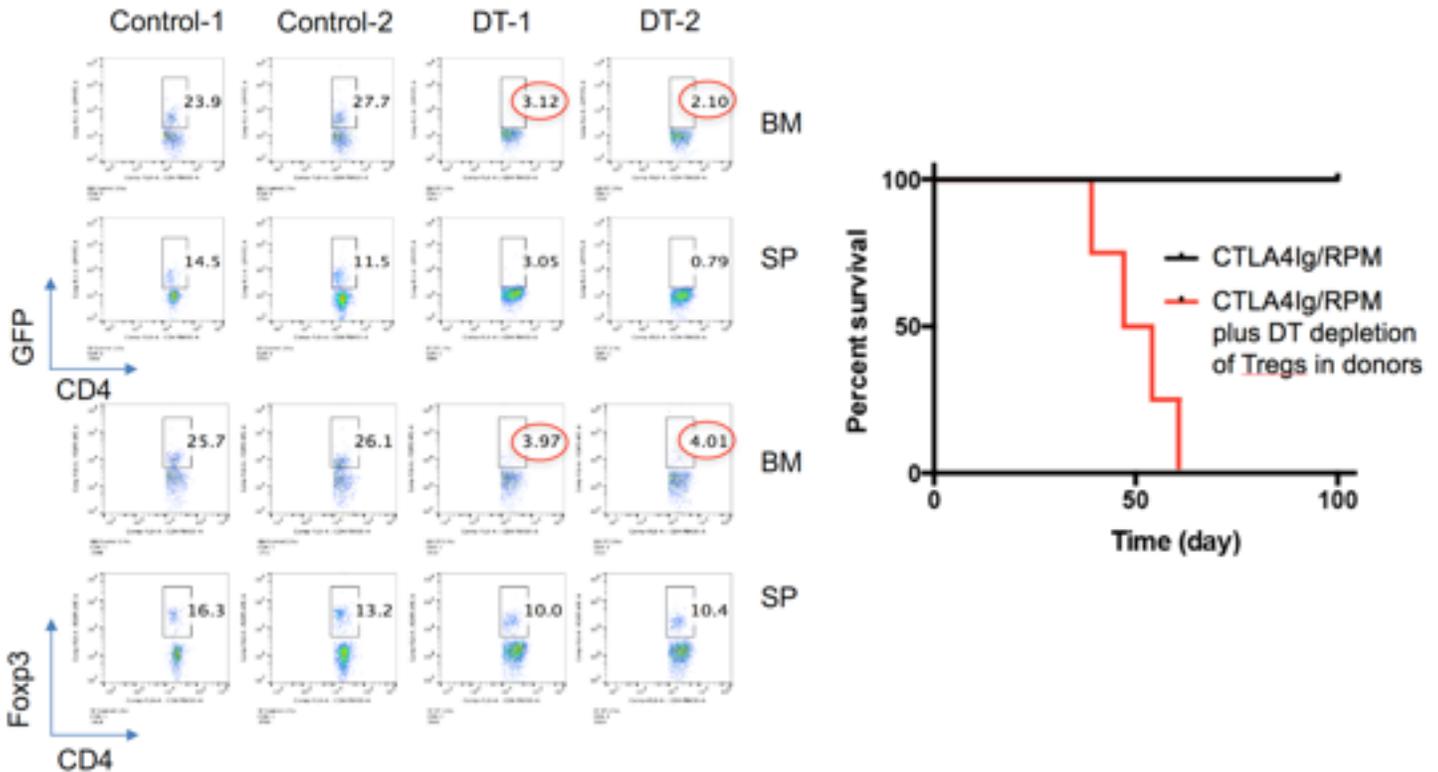


Fig. 5. Donor BM Tregs are essential to the efficacy of costimulation blockade-based therapy in VCA models of limb transplantation. Left, wild-type (control) or DERE_G mice were treated pre-Tx with diphtheria toxin (DT) and the levels of residual GFP⁺ and Fxp3⁺ Tregs assessed in donor BM and spleen (SP) samples. Right, DT-treated mice were then used as donors of hind-limbs in VCA procedures described above ($p < 0.01$ for survival in DT-treated vs. control VCA recipients).

These data are very provocative, but we are thinking should be complemented by use of one of more additional approaches that do not involve targeting with diphtheria toxin (!). Hence, as detailed under future plans, we plan to test the effects of CXCR4 deletion in donor BM Tregs, and also assess the efficacy of new generation antisense knockdown of Fxp3 in donor BM.

TASK 4: TREG-BASED THERAPIES IN VCA

4.1 Use of RPM and/or TGF- β to expand host Tregs.

We first explored options of using RPM and/or TGF- β therapeutically in a cardiac allograft survival, thinking these agents might each increase Treg numbers and thereby enhance VCA survival. Compared to use of either agent alone (**Fig. 6 and 7**), results in the cardiac allograft using both agents were excellent (**Fig. 8-11**).

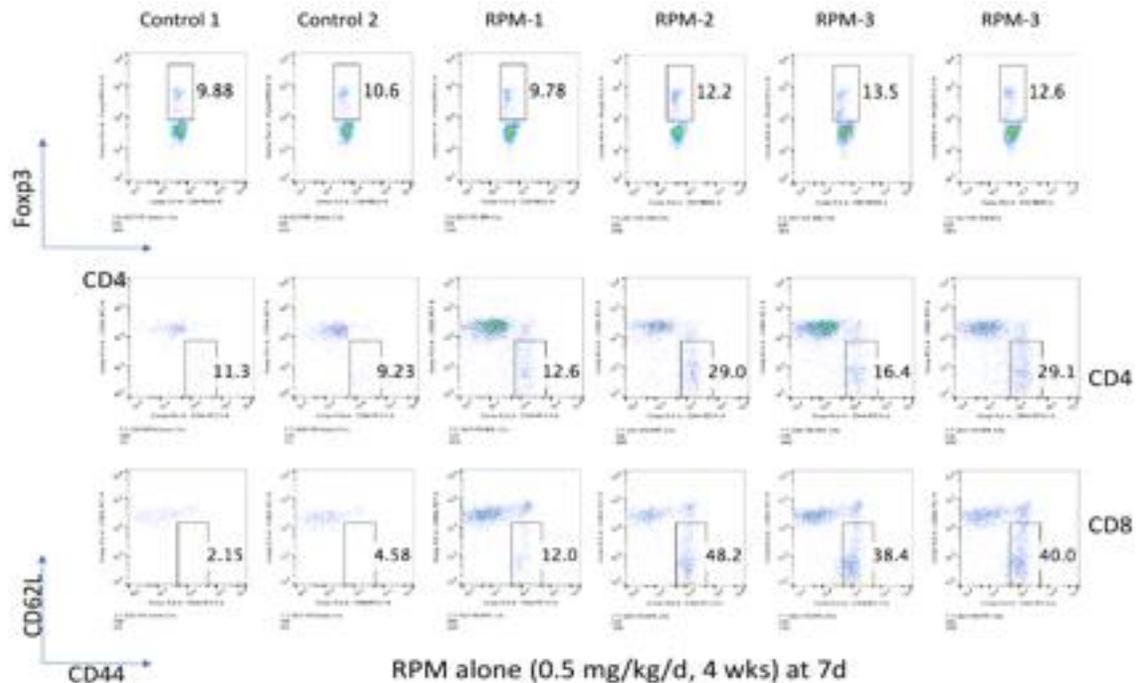


Fig. 6. At day 7 post-cardiac Tx, RPM alone caused only a slight expansion of blood Tregs and did not prevent Tx-induced host CD4 and CD8 T cell activation, i.e. increases in CD44^{hi}CD62L^{lo} cells.

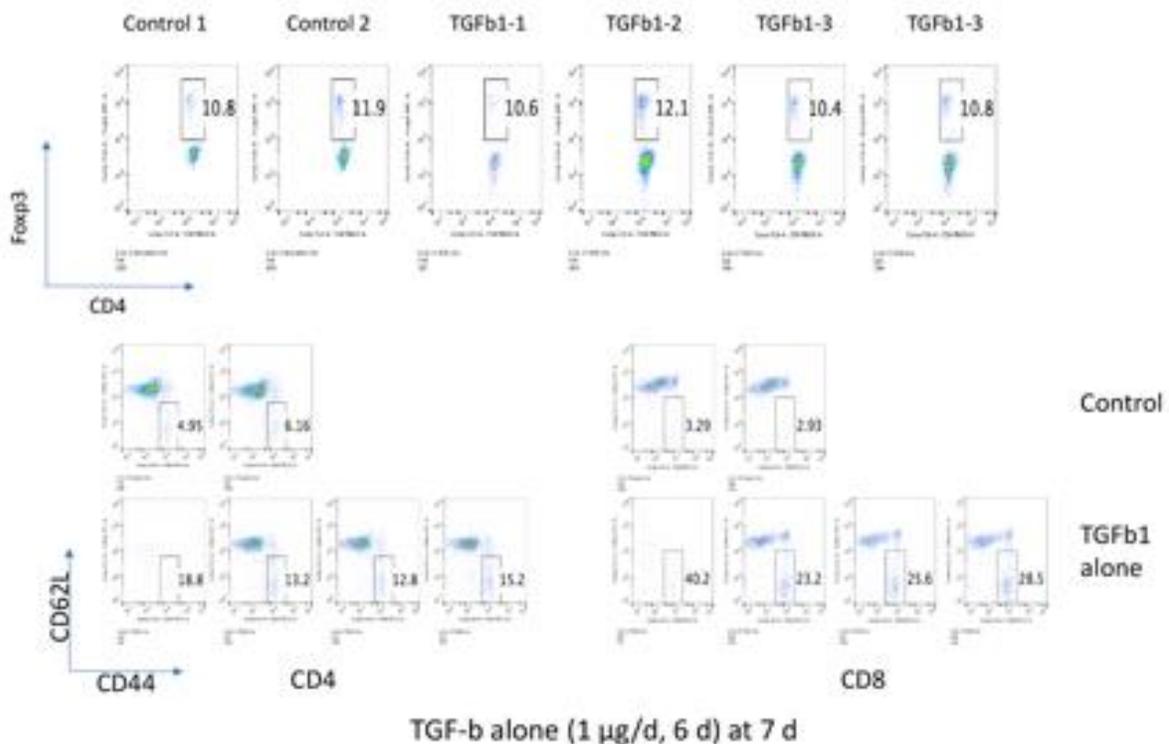


Fig. 7. At day 7 post-cardiac Tx, TGF- β alone had no obvious effect on blood Treg numbers and failed to prevent host CD4 and CD8 T cell activation, i.e. increases in CD44^{hi}CD62L^{lo} cells.

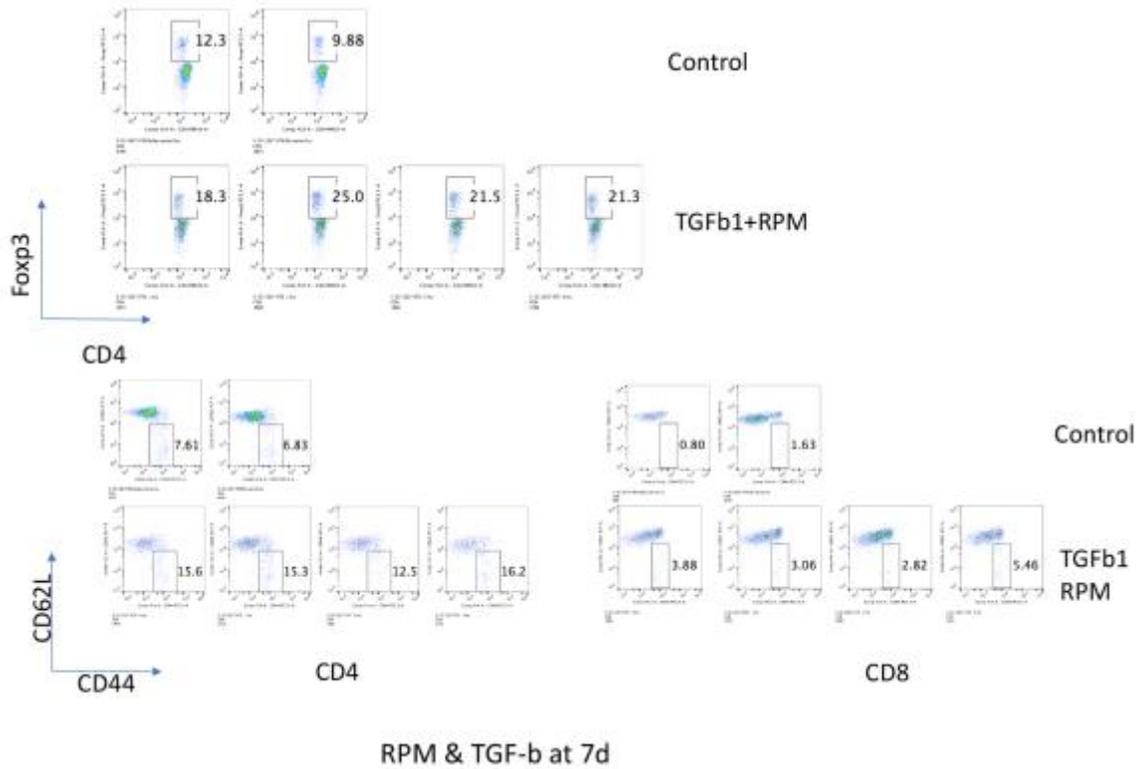


Fig. 8. In contrast to data using either agent alone, at day 7 post-cardiac Tx the combination of RPM and TGF- β roughly doubled blood Treg numbers and prevented host CD4 and CD8 T cell activation, i.e. increases in CD44^{hi}CD62L^{lo} cells.

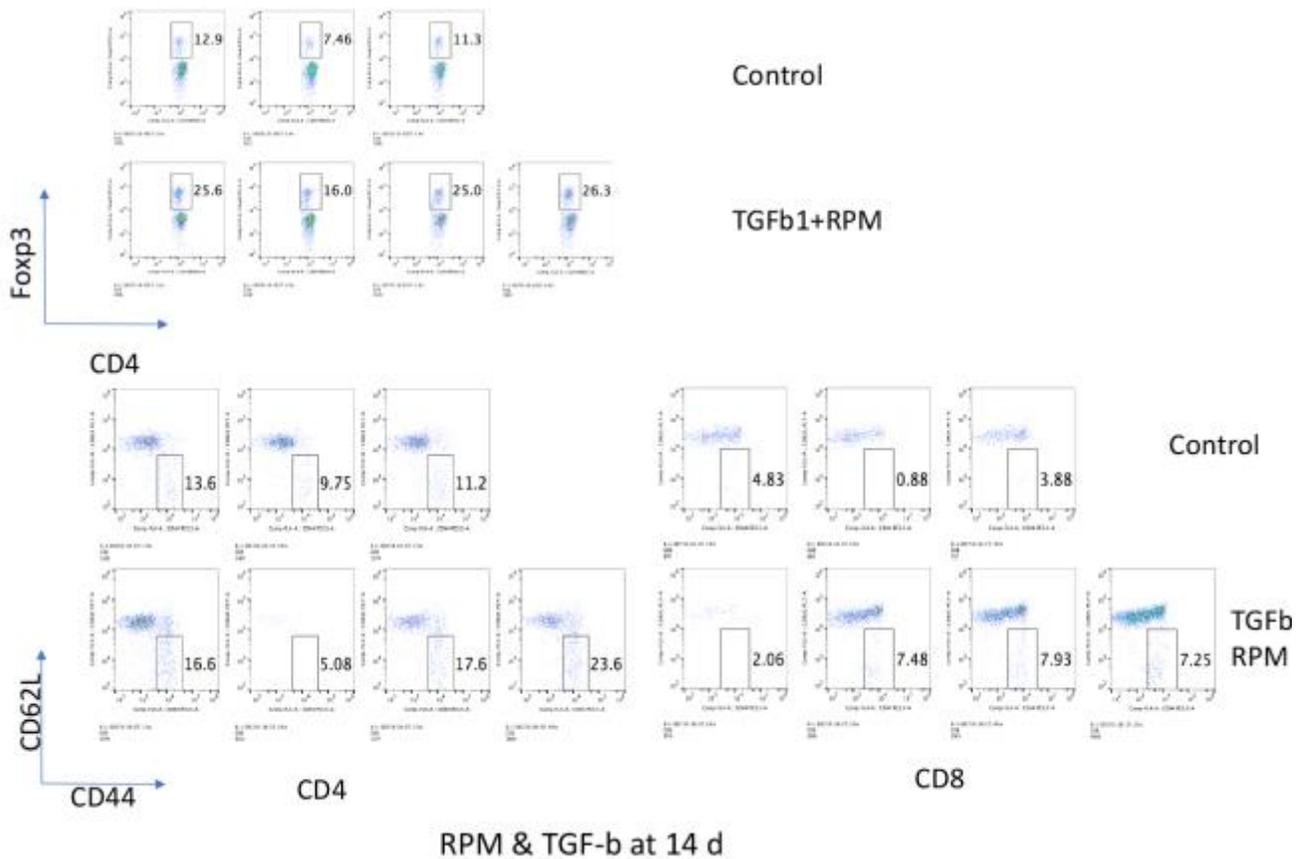


Fig. 9. Persistence of a doubling of Fc γ R3+ Tregs at day 14 post-Tx in mice treated with combined RPM and TGF- β , and control of CD44^{hi}CD62L^{lo} cells numbers.

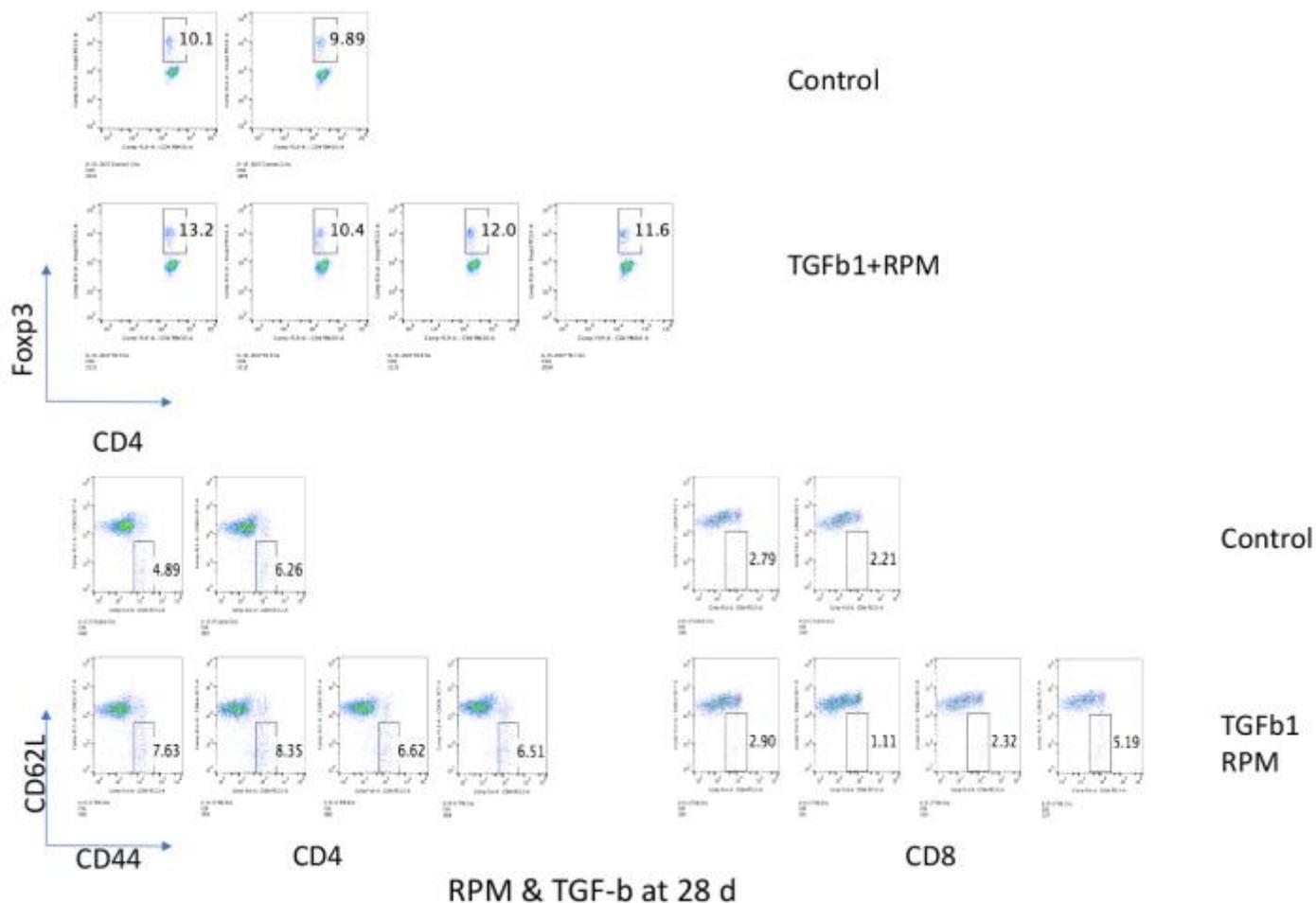


Fig. 10. Foxp3+ Tregs at day 28 post-Tx are back to normal in mice treated with RPM plus TGF- β , but control of CD44^{hi}CD62L^{lo} cell numbers persists.

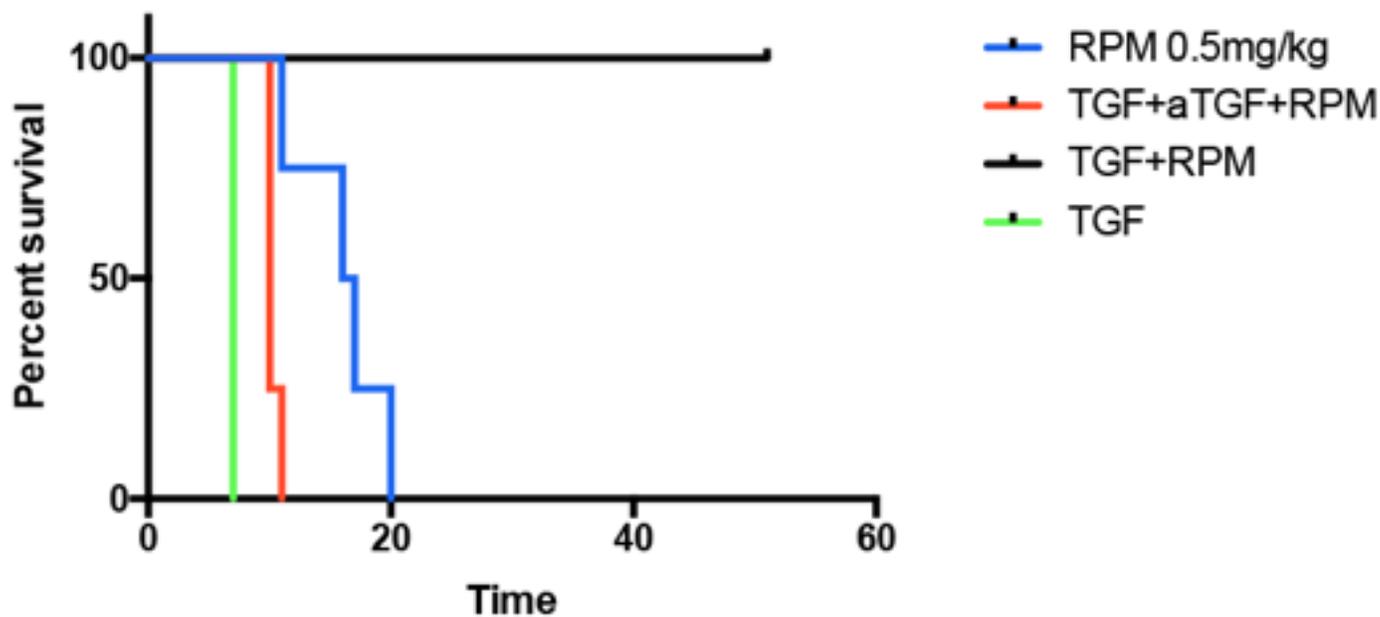


Fig. 11. Significant prolongation of cardiac allograft survival using the combined RPM/TGF- β therapy vs. RPM or TGF- β alone ($p < 0.01$), and abrogation of this beneficial effect by administration of a neutralizing mAb to TGF- β .

However, the combination of TGF- β and RPM did not prolong VCA survival (Fig. 12).

Survival proportions: Survival of TGF (1ug/mouse)+RPM (2mg/kg)

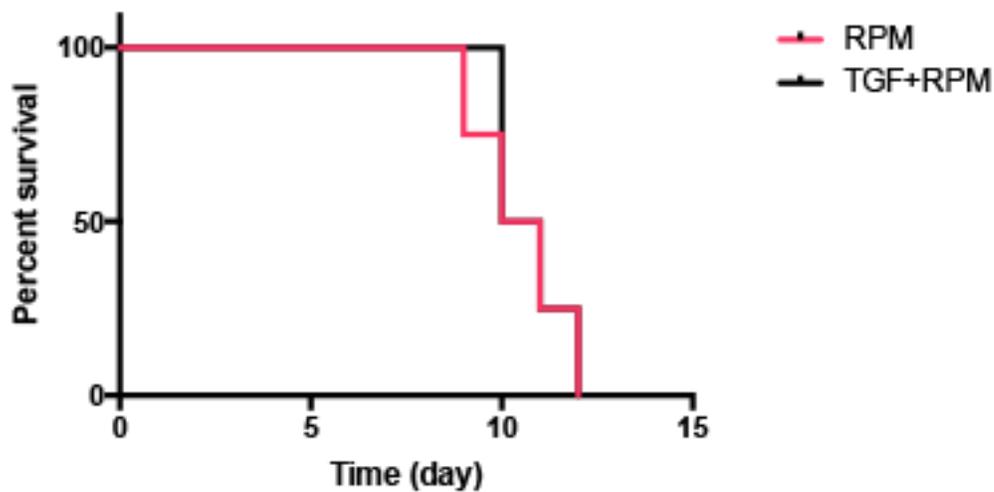


Fig. 12. Failure of combined RPM/TGF- β therapy to prolong murine orthotopic hind-limb VCA survival.

4.2 IL-2/anti-IL-2 monoclonal antibody complex (IL-2C) administration to expand the host Tregs

Given the failure of RPM/TGF- β to promote VCA survival, we set up 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 monoclonal antibody complex (IL-2C) administration as a means to expand the host Treg population and thereby attempt to promote Treg-dependent VCA survival.

IL-2C Therapy Increases the Number but Not Function of Foxp3 CD4+ Treg Cells

To test the effects of JES6-1 mAb-based IL-2C administration on Foxp3+ Treg cells in normal mice, wild-type (WT) C57BL/6 mice were injected with PBS or IL-2C for 3 consecutive days. Compared to PBS injections, by 2 days after the last injection, IL-2C increased by 4-5 fold the proportion ($p < 0.005$) and by 10-fold the total number ($P < 0.01$) of splenic Foxp3+CD4+ Treg cells (Figure 13A). The effects of IL-2C therapy were short-lived, as Foxp3+ Tregs levels peaked at day 5 and gradually declined thereafter (Figure 13B). IL-2C therapy did not appear to affect the properties of Foxp3 Treg cells, since their production of the cytokine, IL-10 (Figure 13C), their suppressive function (Figures 13D & 13E), and their levels of Foxp3 protein were unchanged (Figure 13F), compared to Tregs from PBS-treated control mice. Hence, IL-2C therapy appears to simply increase the total number of peripheral Foxp3+ Treg cells without modifying their function.

Pre- or Post-Tx IL-2C Therapy Alone or Plus RPM Prolongs VCA Survival

A mouse unilateral forelimb orthotopic transplantation model was established during this research, and used to test IL-2C-based therapy during the pre-Tx or post-Tx period. In initial studies (Figure 14A) 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 Figure 14B, and comparisons between groups were

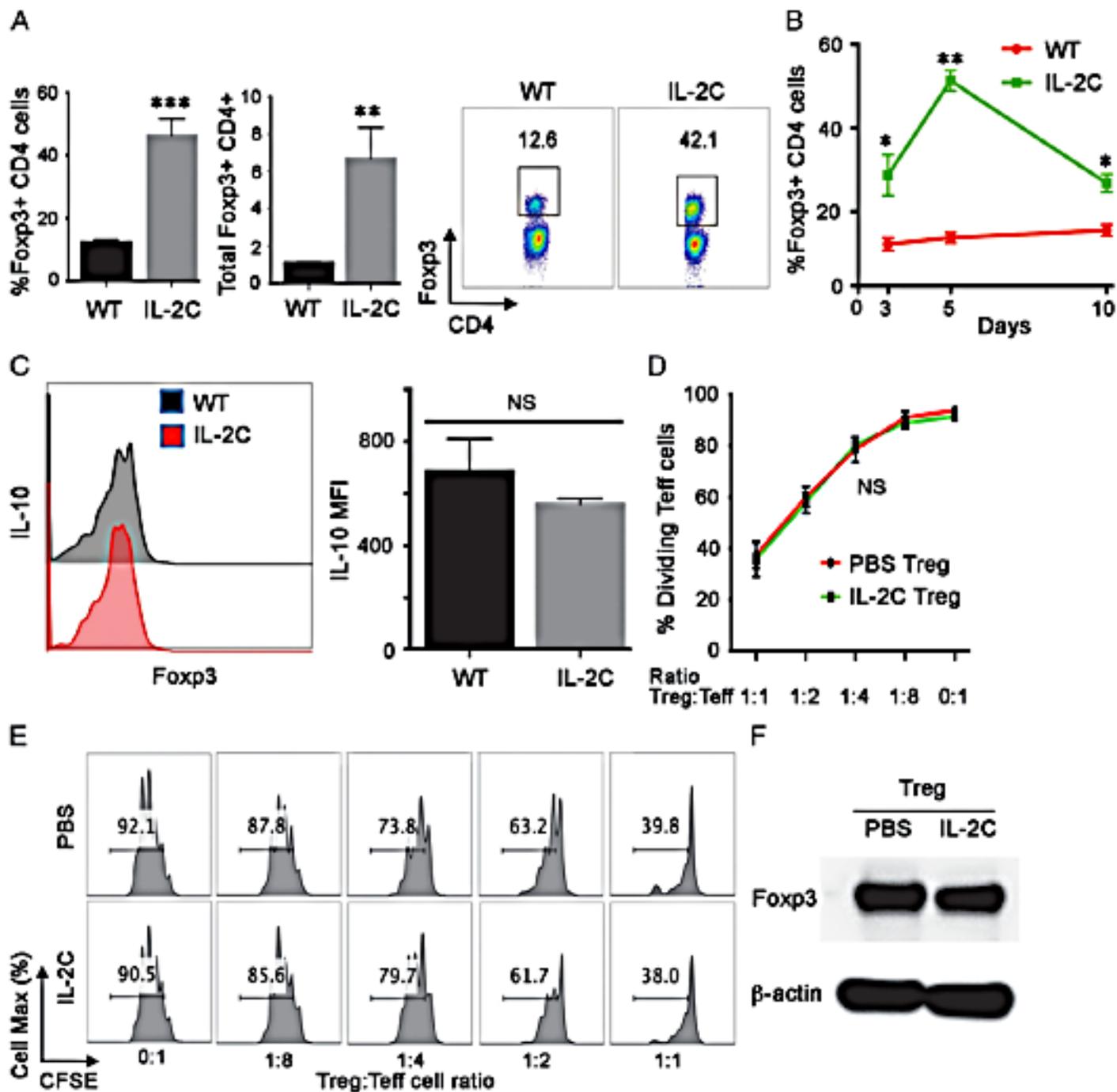


Fig. 13. IL-2C administration increases Treg cell numbers. A, IL-2C significantly increases the percentage of Fxp3+ Treg cells within the splenic CD4+ T fraction, and total Fxp3+ Treg cell numbers (106 cells/spleen); data (mean \pm SD) are from 4 animals/group/time-point, ** $P < 0.01$, *** $P < 0.005$. A representative flow plot is shown at right with the percentage of Fxp3 + CD4+ Treg cells indicated. B, IL-2C administration on days 0, 1 and 2 led to a peak in the percentage of Fxp3 + CD4+ Treg cells on day 5, with a decline thereafter towards baseline; data (mean \pm SD) are from 4 animals/group/time-point, * $P < 0.05$, ** $P < 0.01$. C, IL-2C administration did not affect expression of IL-10 by Treg cells harvested at 5 days after beginning IL-2C therapy; a representative flow plot is shown at left, and cumulative MFI data (mean \pm SD, $n = 4$ / group, NS, not significant) are shown at right. D, IL-2C administration did not affect Treg cell suppressive function as assessed by *in vitro* assays (mean \pm SD, $n = 4$ /group) using cells analyzed at day 5. E, An *in vitro* suppression assay, representative of 4 studies, with the proportion of dividing T effector cells shown in each panel. F, Western blots of Fxp3 protein expression in Treg cells isolated from mice treated with IL-2C or PBS (representative of 3 experiments). MFI, mean fluorescent intensity.

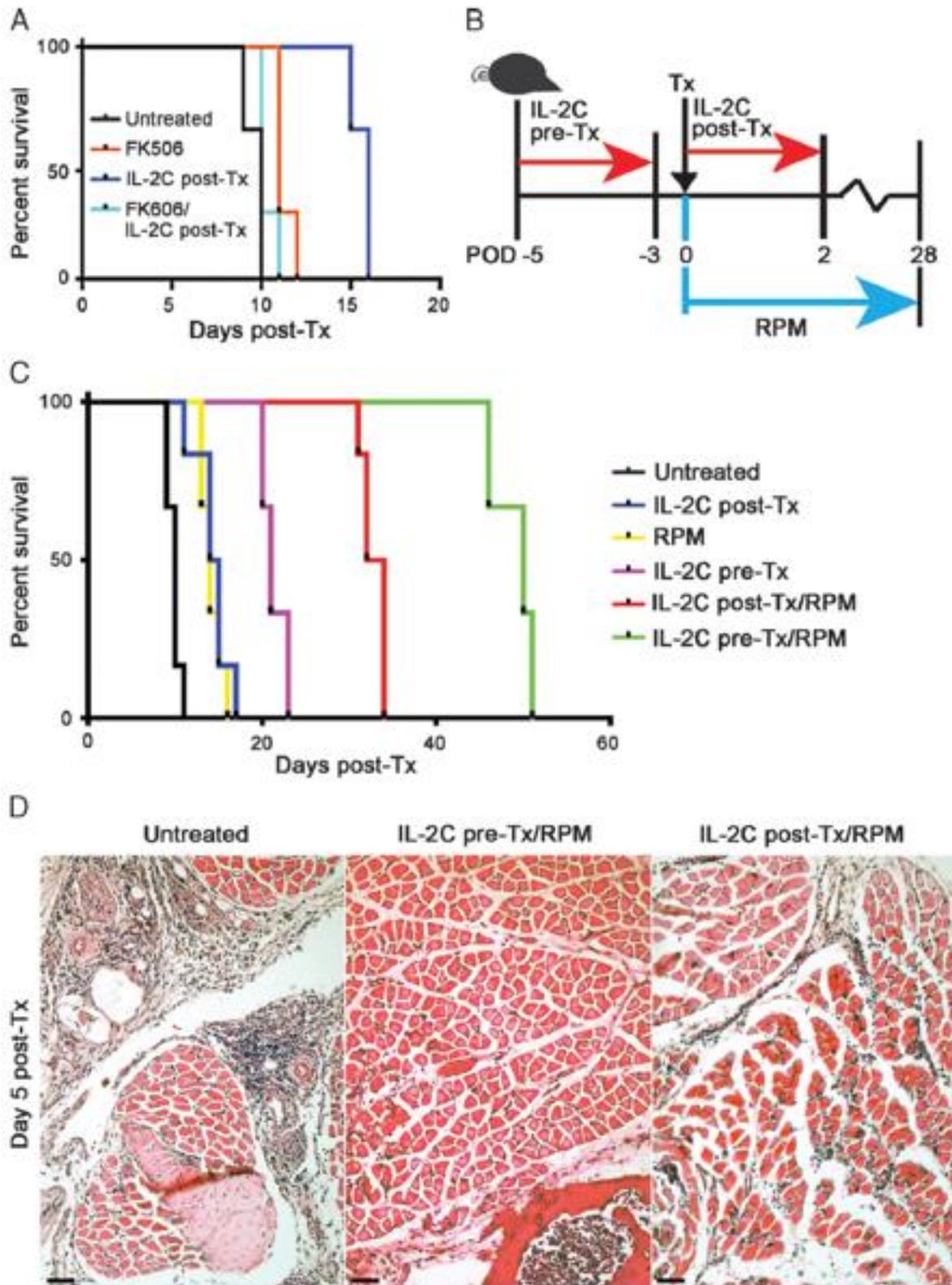


Fig. 14. Both pre- and post-Tx IL-2C treatment prolonged murine forelimb VCA survival. A, Post-Tx IL-2C therapy alone was significantly better ($P < 0.01$) at extending VCA survival than FK506 alone (1 mg/kg per day, i.p. for 14 days), or FK506 plus IL-2C therapy. B, Diagram summarizing use of pre-Tx 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.

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 (Figure 14C). 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 ($p < 0.05$, Figure 14C). Co-administration of IL-2C and post-Tx RPM had additional benefits, with pre-Tx IL-2C 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 (Figure 14). 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, Figure 14D). Infiltrates were absent in recipients receiving pre-Tx IL-2C plus post-Tx RPM (grade 0, Figure 14D), 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, Figure 14D). 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.

Differing effects of pre- or post-Tx IL-2C therapy on host Treg and Teff cells

At day 5 post-Tx, the proportions of Fcpx3+ CD4+ Treg cells in mice 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 (Figure 15A). Mice treated pre-Tx with IL-2C ($p < 0.05$)

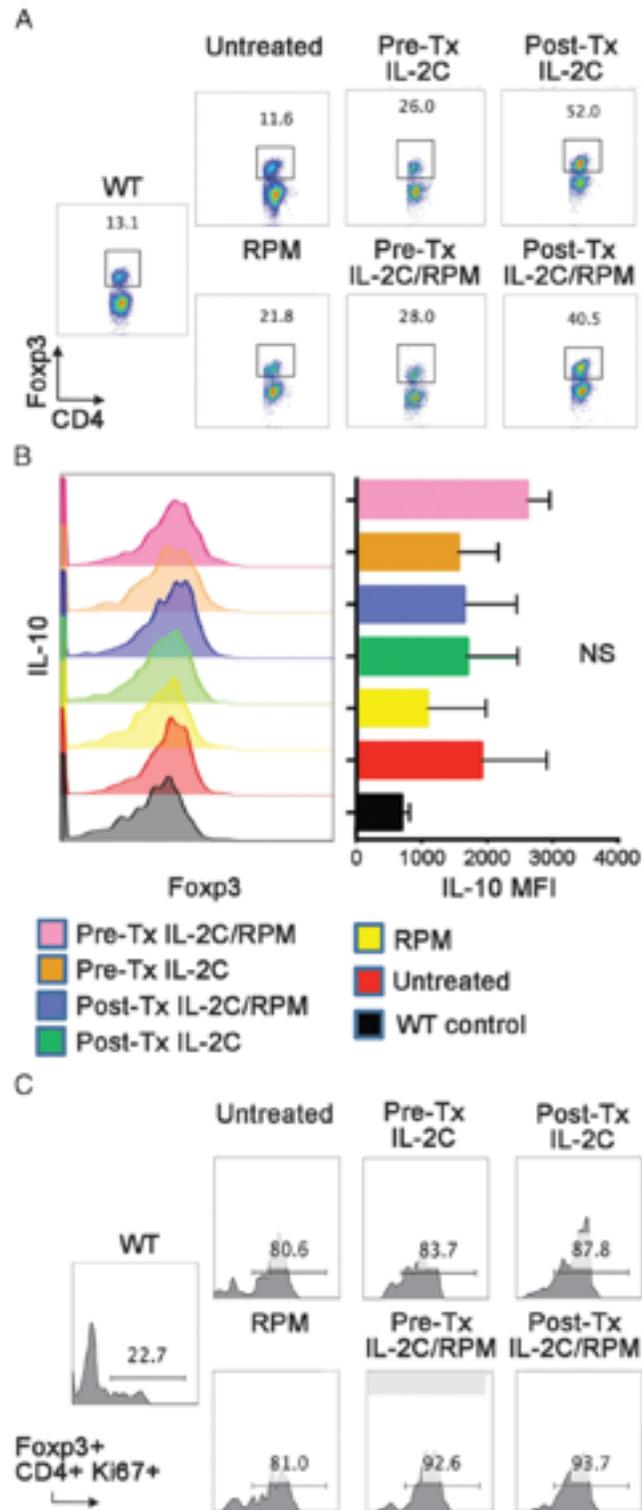


Fig. 15. Comparison of Treg cell populations at day 5 post-Tx (4 mice/group). A, Percentages of Fcpx3+ cells within the CD4+ T cells of Tx groups and WT mice are shown. B, Treg cell intracellular expression of IL-10, as determined by flow cytometry (MFI), did not significantly differ across groups. C, Percentages of proliferating (Ki67+) Fcpx3+ CD4+ Treg cells were comparable across groups but markedly increased compared with untransplanted WT control mice.

TABLE 1

Kaplan-Meier analysis of allograft survival in the various experimental groups^a

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	$P < 0.001$	$P < 0.001$	$P = 0.010$

^a Comparison of survival curves (log-rank test, P value) using six to eight allografts/group.

± post-Tx RPM ($p > 0.05$ vs. RPM alone) had lesser increases in Treg cells (Figure 15A). However, at day 5 post-Tx, Tregs isolated from all 6 groups of engrafted mice showed comparable levels of IL-10 (Figure 15B), GITR, ICOS and TGF- β , and comparable levels of cell proliferation (Ki67 expression) (Figure 15C).

Flow cytometric analysis of conventional CD4 and CD8 T cells at day 5 post-Tx showed comparable proportions of CD4 cells in untreated recipients and those receiving pre-Tx IL-2C ± RPM (Figure 16A). However, allograft recipients receiving post-Tx IL-2C ± RPM showed a 3-4 fold expansion of the CD8 population (Figure 16A). Analysis of Ki67 expression showed increased proliferation of CD4 (Fig. 16B) and especially CD8 T cells (Figure 16C) in all allograft groups compared to 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 (Figure 16C). Analysis of IFN- γ production by CD4 (Figure 16D) and CD8 T cells (Figure 16E) 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 (Figure 16E). Flow cytometric comparisons of the ratios of proliferating Tregs to CD4 or CD8 T cells at day 5 post-Tx (Figure 16F) 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 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.

Differing effects of pre- or post-Tx IL-2C therapy on intragraft gene expression

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 (Figure 17). Addition of RPM decreased expression of CD8, IFN- γ and granzyme B in the post-Tx IL-2C 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.

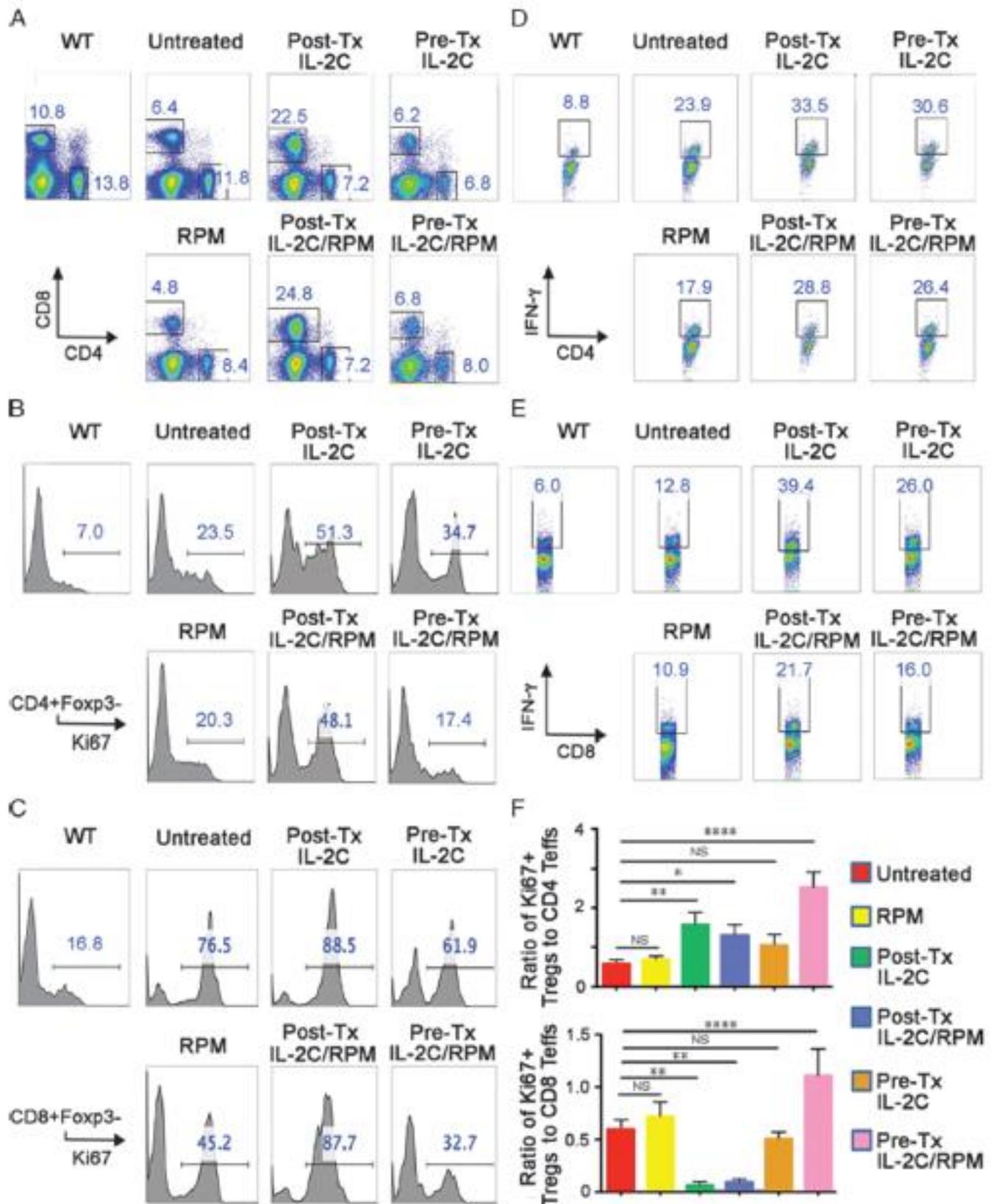


Fig. 16. Effects of IL-2C on non-Treg cell populations at day 5 post-Tx (4 mice/group). A, Increased percentage of CD8 T cells in allograft recipients treated with IL-2C post-Tx. B, Increased proliferation of CD4+ T cells in allograft recipients treated with IL-2C post-Tx. C, Increased proliferation of CD8+ T cells in allograft recipients treated with IL-2C post-Tx. D, Increased production of IFN- γ by CD4+ T cells in allograft recipients. E, Increased production of IFN- γ by CD8+ T cells in allograft recipients. F, Ratio of Ki67+ Treg cells to CD4 + Foxp3- or CD8 + Foxp3- T cells; higher ratios were associated with longer allograft survival.

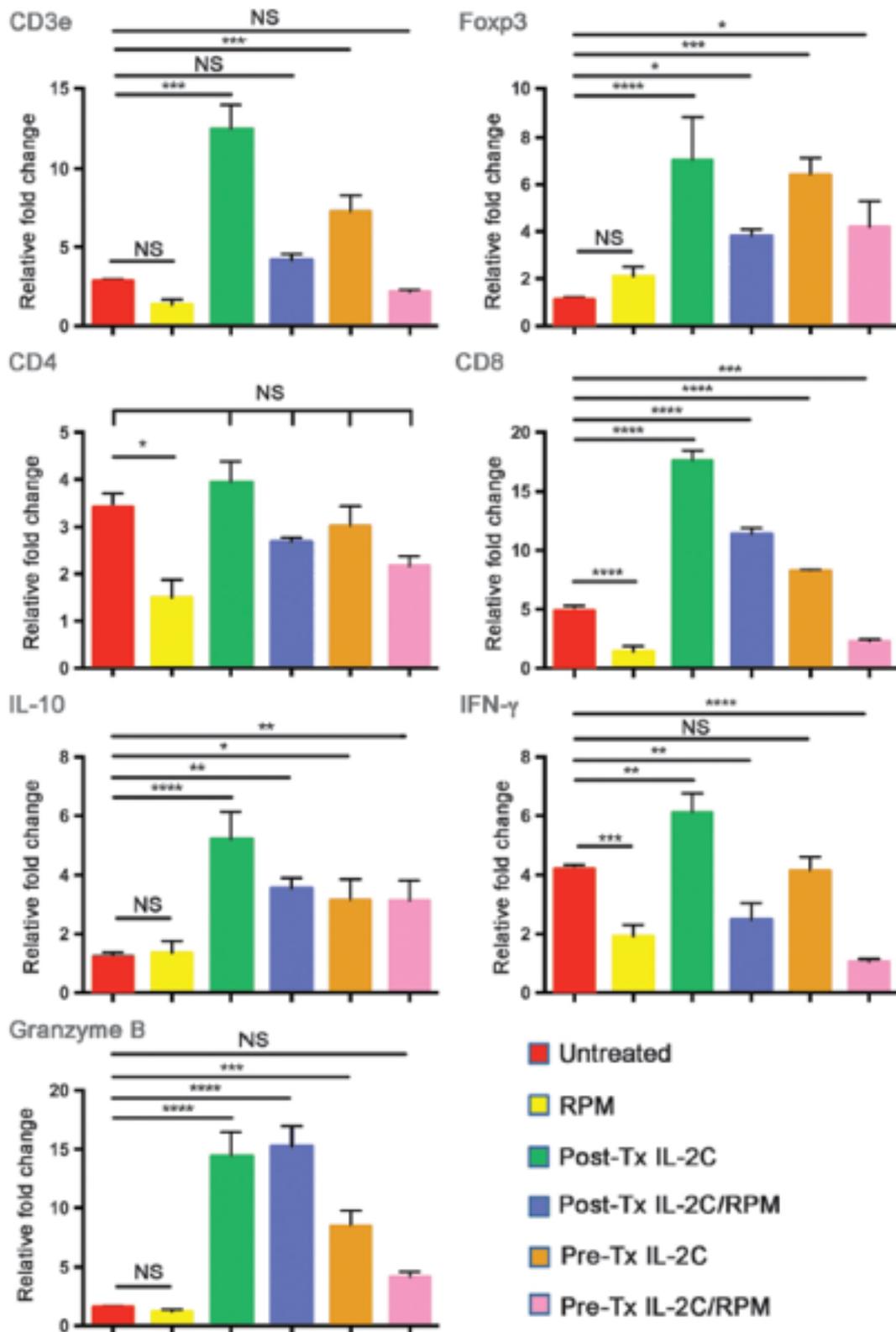


Fig. 17. Real-time qPCR analysis of gene expression within allografts at day 5 post-Tx (4 mice/group). IL-2C therapy pre-Tx was accompanied by increases in expression of Cd3e, Foxp3, Cd8, IL10, and Gzmb, but not Ifig, consistent with a Treg cell-dominant effect. The Cd8 increase was muted by concomitant RPM use. IL-2C therapy post-Tx was accompanied by increases in Cd3e, Foxp3, Cd8, IL10, Gzmb and Ifig, consistent with a prominent CD8 T-cell response occurring in addition to positive effects on Treg cells. The increases in Cd8 and Ifig were muted by concomitant RPM.

In summary, 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. These IL-2C studies are now “in press” (4).

TASK 5: OPTIMAL COMBINATION THERAPIES

As detailed with our costimulation blockade work, and Treg work, several therapeutic combinations are underway and being validated and the mechanisms dissected. This work is ongoing.

4. KEY RESEARCH ACCOMPLISHMENTS

- The efficacy of CD154/DST/RPM (COB/RPM) or CTLA4Ig/RPM is abrogated by irradiation of donor limbs, and depends on the presence of a cell type that absent from the BM of Rag1^{-/-} donor mice; i.e. efficacy depends upon a T or B cell population.
- Efficacy is also blocked by use of a CXCR4 inhibitor in the donor; i.e. there is a cell population that is mobilizable and whose residence in donor BM is dependent upon CXCR4.
- Efficacy is blocked by depletion of donor BM Foxp3⁺ Treg cells.
- An expanded Treg population can significantly prolong VCA survival.

We propose to continue this work into the nature of the BM cell responsible (including through use of conditional deletion of CXCR4 in donor Foxp3⁺ Treg cells, and by application of new generation FANA antisense approaches), and the effects of combination therapies involving costimulation blockade and Treg-based treatment

5. CONCLUSIONS

Our long-term goal is to improve the acceptability of limb and other forms of VCA as a therapeutic option in potential recipients. To that end, small animal studies using peri-transplant costimulation blockade/RPM therapy are proving very encouraging. Interesting biology is arising concerning donor/host cell interactions, especially with regard to donor bone marrow Foxp3⁺ Treg cells. Likewise, we have now shown that Treg-based therapies can prolong VCA survival. This area is still largely unexplored, but a model has been established that, with the usual small animal caveats, can be used to test the effects of various cell therapy-based strategies and/or pharmacologic approaches.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS

Publication

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 (In press)

Abstract

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 Society of Vascularized Composite Allotransplantation Salzburg, Austria, October 2017

Presentations

(by Dr. Hancock)

- 12/2016 "Novel Immunomodulatory Strategies for VCA"
Department of Defense
Fort Detrick, MD
- 7/2017 "An Update on Novel Immunomodulatory Therapies for VCA"
Department of Surgery, Duke University
Durham, NC

7. INVENTIONS, PATENTS AND LICENSES

None.

8. REPORTABLE OUTCOMES

None

9. OTHER ACHIEVEMENTS

None.

REFERENCES

1. Zou L, Barnett B, Safah H, Larussa VF, Evdemon-Hogan M, Mottram P, Wei S, David O, Curiel TJ, Zou W. Bone marrow is a reservoir for CD4+CD25+ regulatory T cells that traffic through CXCL12/CXCR4 signals. *Cancer Res.* 2004;64(22):8451-5.
2. Fujisaki J, Wu J, Carlson AL, Silberstein L, Putheti P, Larocca R, Gao W, Saito TI, Lo Celso C, Tsuyuzaki H, Sato T, Cote D, Sykes M, Strom TB, Scadden DT, Lin CP. In vivo imaging of Treg cells providing immune privilege to the haematopoietic stem-cell niche. *Nature.* 2011;474(7350):216-9.
3. Lahl K, Loddenkemper C, Drouin C, Freyer J, Arnason J, Eberl G, Hamann A, Wagner H, Huehn J, Sparwasser T. Selective depletion of Foxp3+ regulatory T cells induces a scurfy-like disease. *J Exp Med.* 2007;204(1):57-63.
4. Heng X, 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.* 2017:In Press.

11. APPENDICES

Revised Quad Chart.

Positioning Vascularized Composite Allotransplantation in the Spectrum of Transplantation

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



PI: Wayne W. Hancock

Org: Children's Hospital of Philadelphia & University of Pennsylvania

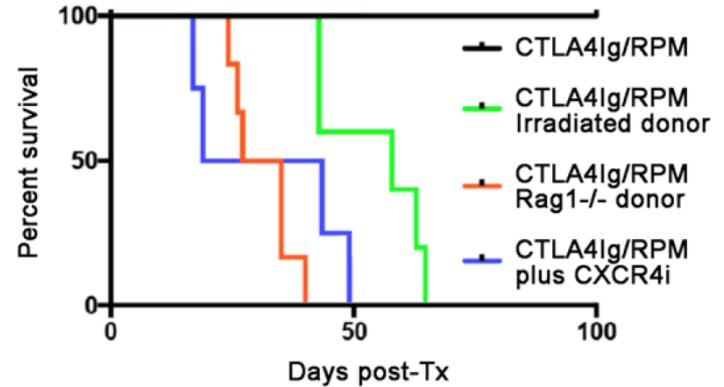
Award Amount: \$1,996,875

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 now know 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: October 14, 2017

Goals/Milestones

- ✓ **CY13 Goal** – We have established a VCA model and begun chemokine targeting (Task 1);
- ✓ **CY14 Goals** – We continuing chemokine/receptor targeting (Task 2), and using costimulation blockade, we have achieved considerable success (long-term engraftment with brief peri-Tx therapy);
- ✓ **CY15 Goal** - Complete costimulation blockade & Treg studies (Tasks 3 & 4)
- ✓ **CY16 Goal** - Undertake final studies using optimal combinations (Task 5); note there is an approved 1 year no-cost extension for further follow-up of this work.

Comments/Challenges/Issues/Concerns

- Ongoing studies and necessary follow-up have led to a 1 year no-cost extension of the project

Budget Expenditure to Date

Projected Expenditure: As budgeted
Actual Expenditure: As budgeted