

INVESTIGATING THE ROLE OF CYTOKINES AND REGULATORY T CELLS IN A  
NOVEL TREATMENT FOR GRAFT-VERSUS-HOST DISEASE USING EXOSOMES FROM  
ACTIVATED MESENCHYMAL STROMAL CELLS

by

Ella Martell

under the direction of

Christian Capitini, Department of Pediatrics  
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I have supervised this work, read this thesis and certify that it has my approval.

5/12/2025

Date

*Christian Capitini*

Thesis Advisor's Signature

## ABSTRACT

The development of graft-vs-host disease (GVHD) is a significant complication in the treatment of leukemia, wherein the graft of transplanted hematopoietic tissues from a donor (allo-HCT) attacks the recipient cells of the patient. The primary challenge in developing a treatment for GVHD is to establish a treatment that prevents GVHD while maintaining graft-vs-leukemia (GVL) activity. Previously, our lab has identified a T regulatory cell (Treg) population that is correlated with the prevention of GVHD. We have also developed a novel treatment involving exosomes collected from mesenchymal stromal cells stimulated with the lipid A analog CRX-527 to form immunosuppressive CRX-exosomes (CRX-exos) that limit GVHD without inhibiting GVL activity in an animal model. Here, I hypothesized that CRX-exos stimulate the expression of cytokine IL-6, which then induces Treg activation required for the prevention of xeno-GVHD. I tested this hypothesis by quantifying Tregs and their cytokine expression followed by blocking IL-6 receptors in both an in-vitro and in-vivo model. I found that blocking IL-6 receptors did not significantly reduce Tregs in both models, suggesting that IL-6 is not the main molecular mechanism by which CRX-exos induce Treg activation. Interestingly, blocking IL-6 receptors did increase CD4/CD8 double positive T cells (DPTs) and appeared to confer some protection from GVHD in the xenogeneic transplant model.

## INTRODUCTION

### **Graft-vs-Host Disease is a Major Concern in the allo-HCT Field**

Allogeneic hematopoietic cell transplantation (allo-HCT) is a procedure in which patients receive a replacement immune system from a healthy donor. It is a well-established treatment to control and cure many hematopoietic diseases and cancers such as leukemia, lymphoma, and myeloma<sup>1,3,5,9</sup>. Despite the success of allo-HCT as a treatment and its clinical expansion over the past few decades, the development of graft-vs-host disease (GVHD), an immune-mediated attack of the host's tissues, and the relapse of the patient's primary disease remain the major two causes of death following transplant<sup>3,5,6,9</sup>.

A major goal of the allo-HCT field is to develop a method to prevent GVHD while still maintaining an immune defense against the patient's primary disease, referred to as graft-vs-leukemia (GVL) activity. Prior research has found that donor-derived T cells have a GVL effect, but also mediate GVHD<sup>3</sup>. In our lab, we have looked further into this phenomenon

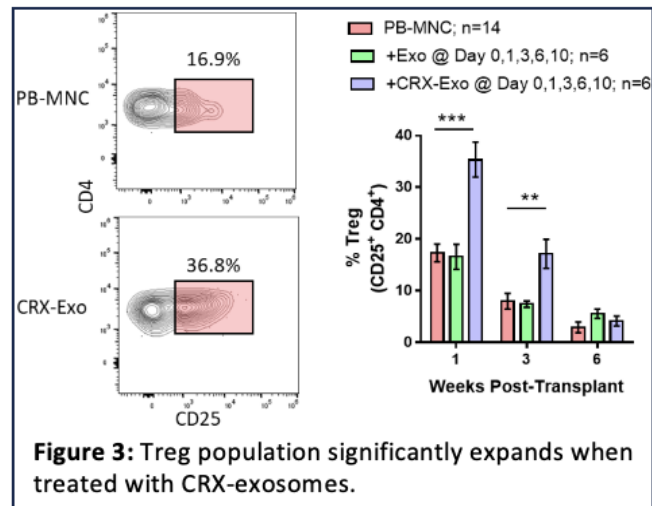
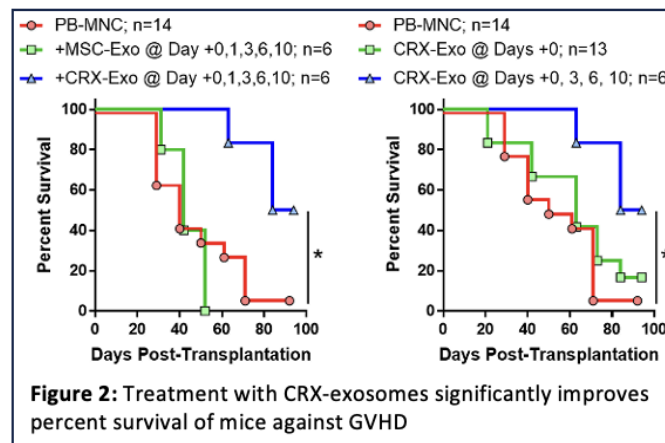
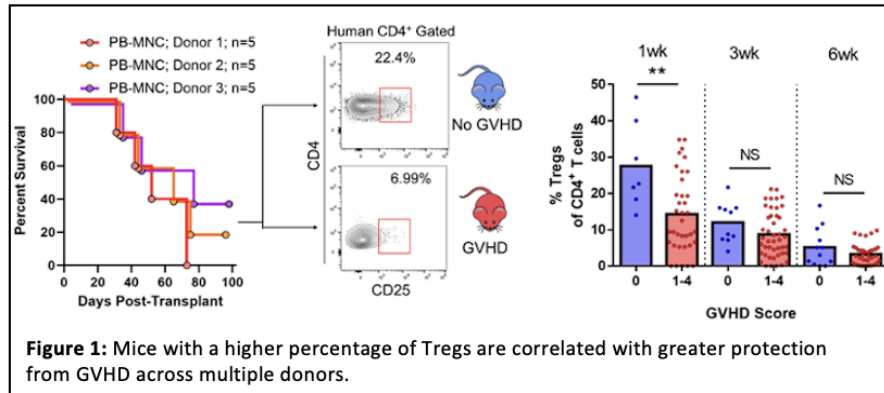
and have identified a regulatory T cell (Treg) population that can prevent GVHD while maintaining GVL activity.

### **The Role of Tregs in Preventing GVHD**

Thymus-derived CD4<sup>+</sup> regulatory T (tTreg) cells are essential in maintaining immune homeostasis<sup>1,9</sup>. Through our xenogeneic (xeno) transplant model, our lab has found that increased levels of Tregs were correlated with the prevention of lethal xeno-GVHD, while decreased Treg levels were predictive of a higher score of xeno-GVHD (Figure 1). Our findings are further supported by past literature that also found that administering tTregs did not limit GVL activity and increased suppression of GVHD in mice<sup>1,11</sup>.

### **CRX-Exos as a Novel Treatment for GVHD**

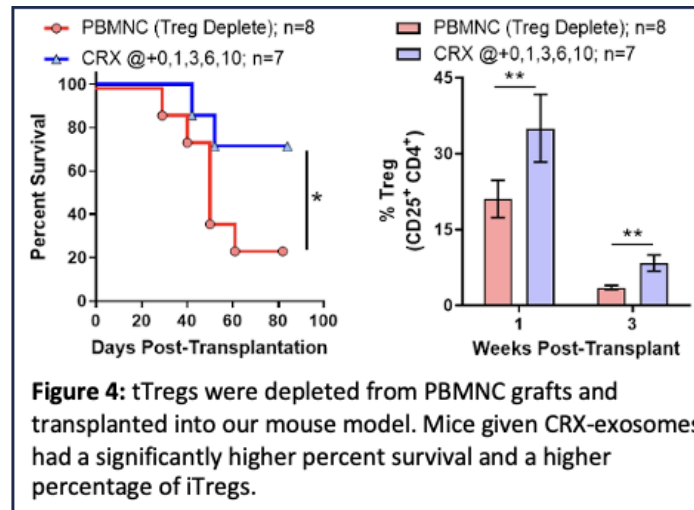
To develop a novel treatment for GVHD, our lab has been studying the effects of CRX-exosomes (CRX-exos) on Treg populations. Mesenchymal stromal cells (MSCs) are a subset of immunomodulatory cells that can differentiate into different cell types and migrate to various tissues<sup>2,6</sup>. MSCs exert their primary function through production of exosomes that transmit signals to recipient cells<sup>4,6,10,12</sup>. Our group has recently developed a novel mechanism to produce exosomes with increased potency for immune suppression by using a synthetic lipid A analog CRX-527 to stimulate the MSCs. We have found that multiple treatments of CRX-exos are able to prevent xeno-GVHD in our transplant model compared to unstimulated MSC-Exos (Figure 2), while preserving the GVL activity of human T cells. We also observed that CRX-exo treated mice exhibited a significant increase in Tregs (Figure 3).



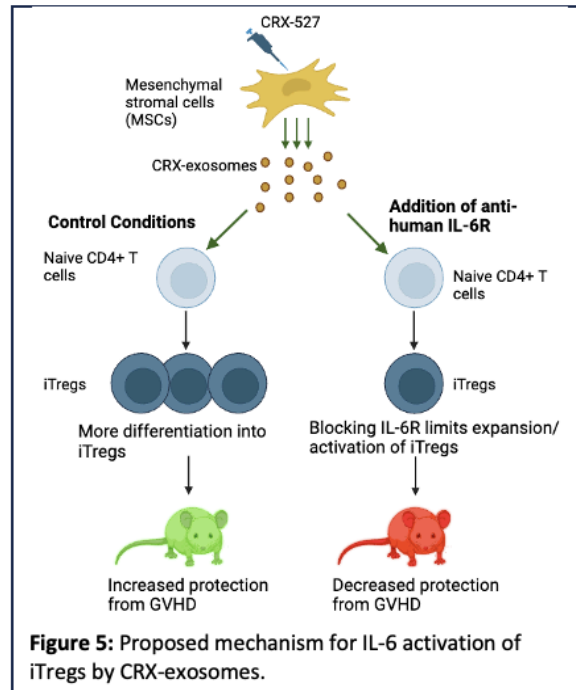
### Proposed Mechanism of CRX-exos Mediated Suppression of xeno-GVHD

To determine if CRX-exos are expanding an already existing tTreg population or transforming naive CD4<sup>+</sup> T cells into induced regulatory T cells (iTregs), we depleted the former

from the peripheral blood mononuclear cells (PB-MNCs) given to mice in our xeno-GVHD model. In past experiments done in our lab, we found that percent survival and percentage of Tregs were significantly higher after CRX-exo treatment, suggesting that iTreg induction is occurring (Figure 4). Prior literature also supports that iTregs have potent suppressive functions with the potential to inhibit GVHD<sup>1,11</sup>.



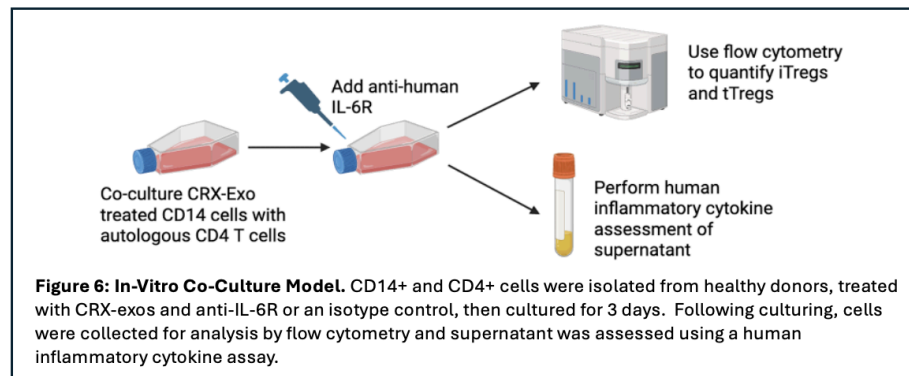
We believe that this induction of iTregs occurs through the secretion of cytokines from monocytes or macrophages educated by the CRX-exos. Our lab has found that after exosome education, IL-6 in these cells is by far the most upregulated gene<sup>6</sup>. Past research suggests that IL-6 signaling increases Treg activation and proliferation; however, there is a lack of research measuring the direct impact of IL-6 on human Tregs<sup>7,13</sup>. I hypothesized that Tregs, induced and activated by CRX-exo expression of IL-6, are required for the prevention of xeno-GVHD (Figure 5).



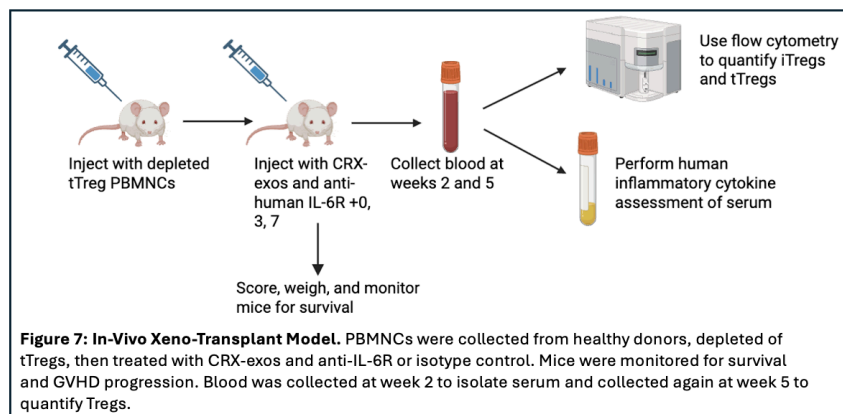
## METHODS

I used an in-vitro model to determine if CRX-exosome educated monocytes (CRX-EEMos) are inducing iTregs through the release of IL-6. First, I isolated and quantified CRX-exos by ultracentrifugation from MSCs stimulated with CRX-527. Next, I isolated fresh peripheral blood mononuclear cells (PBMCs) from healthy donors and further isolated monocytes (CD14<sup>+</sup> cells) and autologous T cells (CD4<sup>+</sup> cells) using MACS<sup>®</sup> Cell Separation. Then, I educated the monocytes with CRX-exos and co-cultured these CRX-EEMos with the autologous T cells and added either *InVivoSIM* anti-human IL-6R (Tocilizumab Biosimilar) or an isotype control antibody (RecombiMAB human IgG1 isotype control, anti-hen egg lysozyme) at a concentration of 200 µg/mL. I co-cultured these cells with ratios of 1:1 and 2:1 monocytes to T cells for three days, followed by the quantification of Tregs and monocytes by flow cytometry (Figure 6). Two sided T tests of equal variance were used to determine significant differences in Treg percentages at the 0.05 significance level. I also measured the cytokine expression profile

of the supernatant collected from these co-cultures using a human inflammatory cytokine kit that can detect multiple cytokines (Proinflammatory panel 1 assay – human from MSD®).



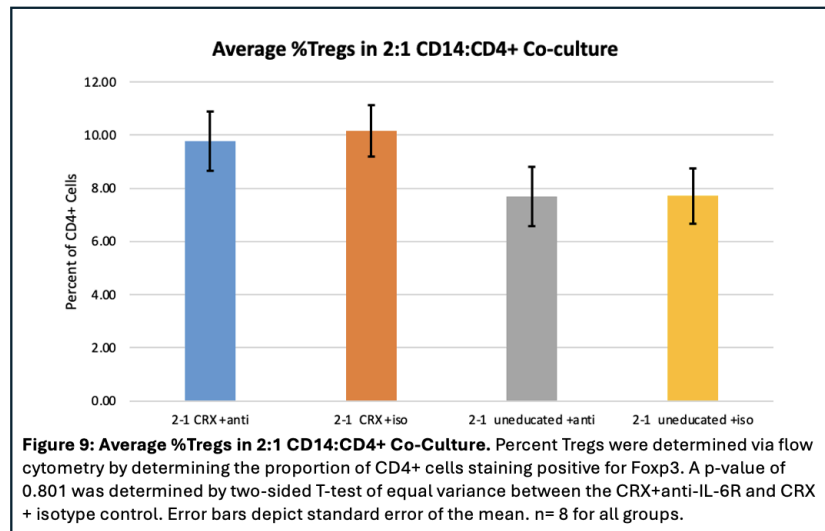
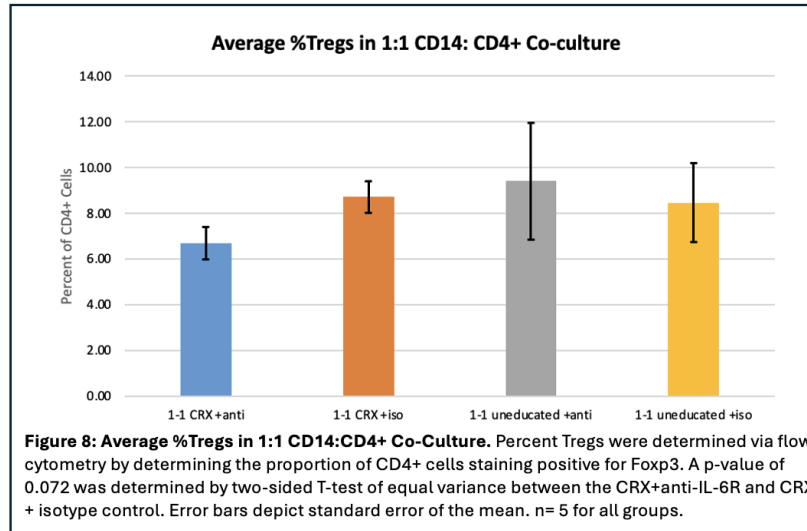
I then transitioned to an in-vivo xenogeneic transplant model<sup>8</sup> (Figure 7). Human PBMNCs were collected from healthy donors. Using a StemCell Technologies pan-CD25 depletion cocktail, I depleted CD25+ Tregs (tTregs) from PBMNC grafts prior to transplantation. The tTreg depleted grafts were transplanted into NSG mice at a dose of  $5 \times 10^6$  cells per mouse. Four experimental groups were prepared: one with just a PBMNC graft (3 mice), one with PBMNCs + CRX-exos (4 mice), one with PBMNCs + CRX-exos + isotype control antibody (3 mice), and one with PBMNCs + CRX-exos + anti-human IL-6R antibody (4 mice). The experimental mice were injected with CRX-exos ( $5 \times 10^7$  CRX-exos per mouse) and anti-human IL-6R or isotype control (250  $\mu$ g per mouse) at days +0, 3, and 7. The mice were monitored for survival and progression of GVHD by scoring the mice on a scale from 0-2 for severity of hair loss, presence of a hunch, and loss of activity. I also collected serum at week 2 to analyze cytokines using the multiplex human inflammatory cytokine kit from MSD®. Additionally, I collected blood at week 5 to quantify human Tregs and DPTs using flow cytometry. Two sided T tests of equal variance were used to determine significant differences in Treg and DPT percentages at the 0.05 significance level.



## RESULTS

### Co-Culture of Monocytes and T Cells

To determine if CRX-exo expression of IL-6 is inducing Treg activation and expansion, I measured the percentage of Foxp3<sup>+</sup> cells (from viable CD4<sup>+</sup> cells) from CD14<sup>+</sup> and CD4<sup>+</sup> co-cultures following treatment with CRX-exos and anti-IL-6R or an isotype control. Despite a lower average value of percent Tregs in the CRX-exos + anti-IL-6R group, there was no significant difference at the 0.05 level between the CRX-exos + anti-IL-6R treatment group and the CRX-exos + isotype control (p-value = 0.072, two-sided T test of equal variance) in the 1:1 co-culture (Figure 8). Furthermore, there was no significant difference between the CRX-exos + anti-IL-6R treatment group and the CRX-exos + isotype control (p-value = 0.801, two-sided T test of equal variance) in the 2:1 co-culture (Figure 9). No significant population of Helios<sup>+</sup> (iTregs) were detected in any samples.

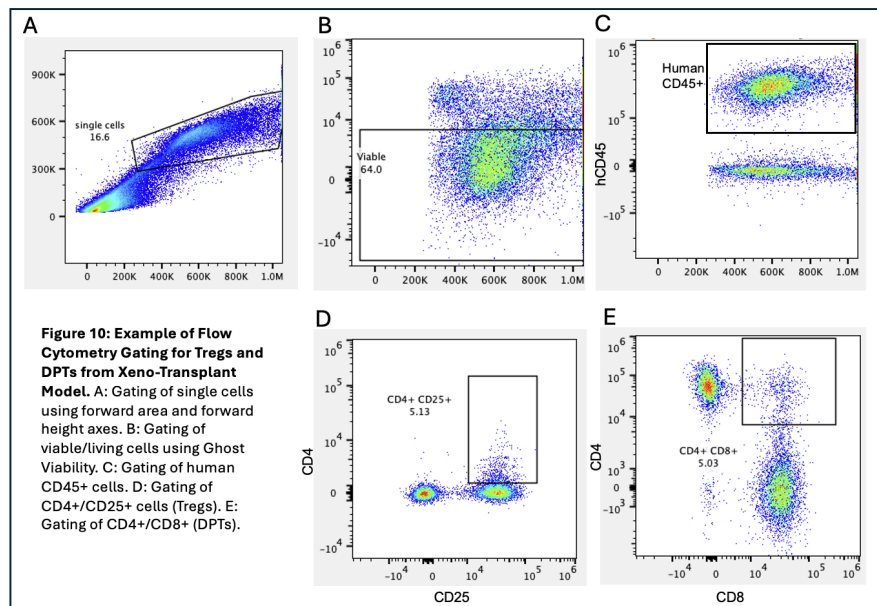


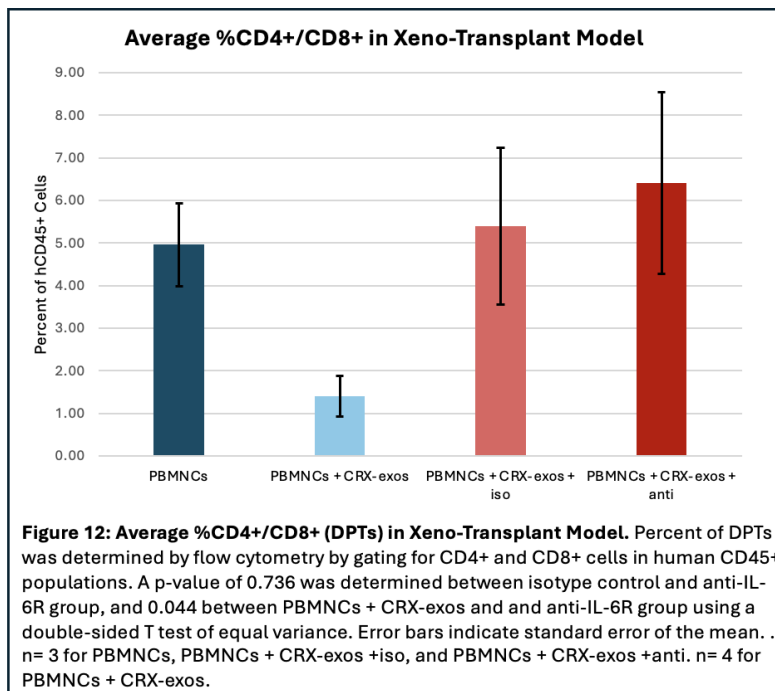
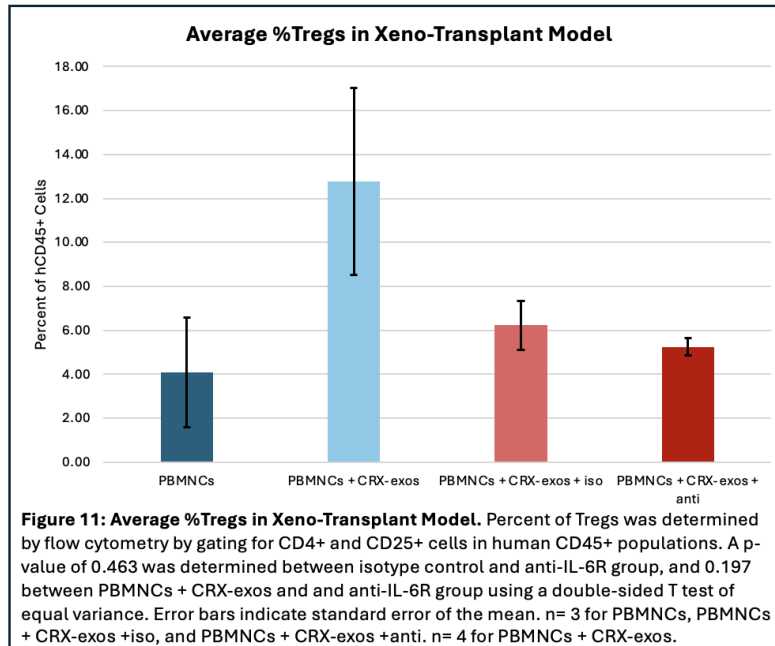
## Xenogeneic Transplant Model

Similar to the in vitro model, I expected to observe a higher percentage of Tregs in the CRX-exos and CRX-exos + isotype control group compared to both the PBMNC control group and the CRX-exos + anti-IL-6R group. Figure 10 depicts how flow cytometry was used to identify and quantify Tregs and DPTs from mouse blood samples. Although there was a higher average percentage of Tregs in the CRX-exos group, there was no significant difference in the percentage of Tregs between the CRX-exos and the CRX-exos + anti-IL-6R group (p-value = 0.197, two-sided T test of equal variance) (Figure 11). Furthermore, there was no significant

difference in the percentage of Tregs between the CRX-exos + anti-IL-6R group and the CRX-exos + isotype control group (p-value = 0.463, two-sided T test of equal variance).

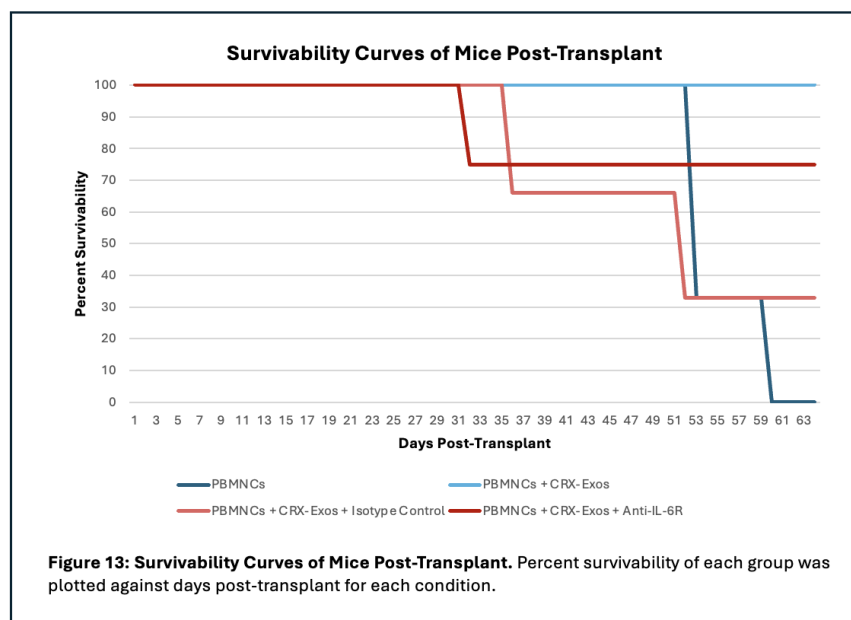
Figure 12 compares the average percentage of CD4/CD8 double positive T cells (DPTs) in each experimental group. Interestingly, there was a significant difference in the percentage of DPTs between the CRX-exos and the CRX-exos + anti-IL-6R groups (p-value = 0.044, two-sided T test of equal variance). However, there was no significant difference between the CRX-exos + isotype control and the CRX-exos + anti-IL-6R groups (p-value = 0.736, two-sided T test of equal variance).





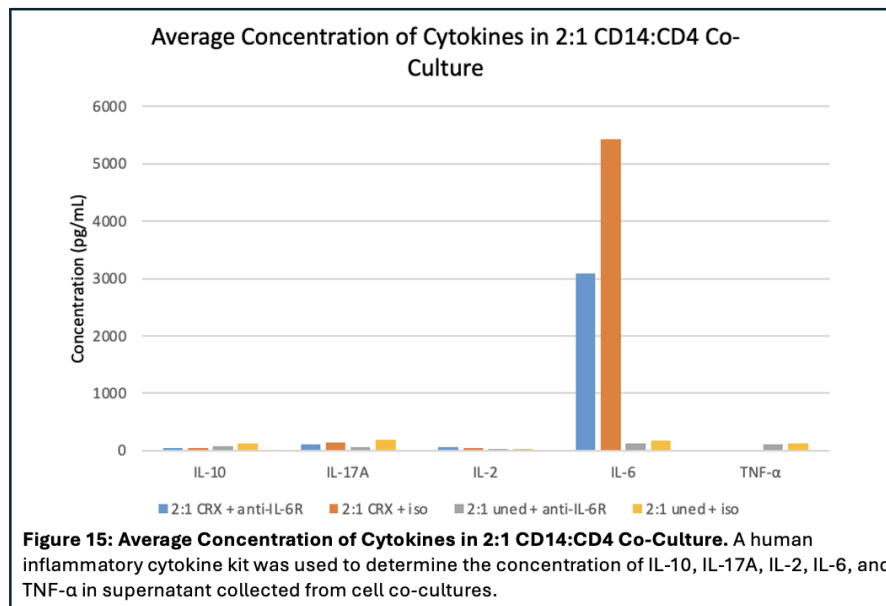
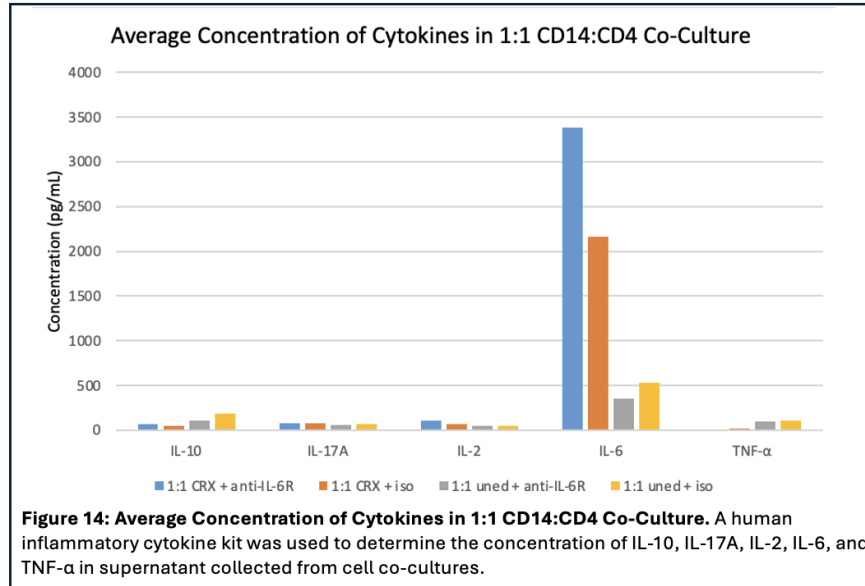
In looking at the overall survivability of the mice, the CRX-exos group fared best with percent survivability remaining at 100% through the entirety of the study (Figure 13). The CRX-exos + anti-IL-6R group exhibited the next best outcome with percent survivability remaining at 75% from day 32 until the end of the study. The CRX-exos + isotype control group

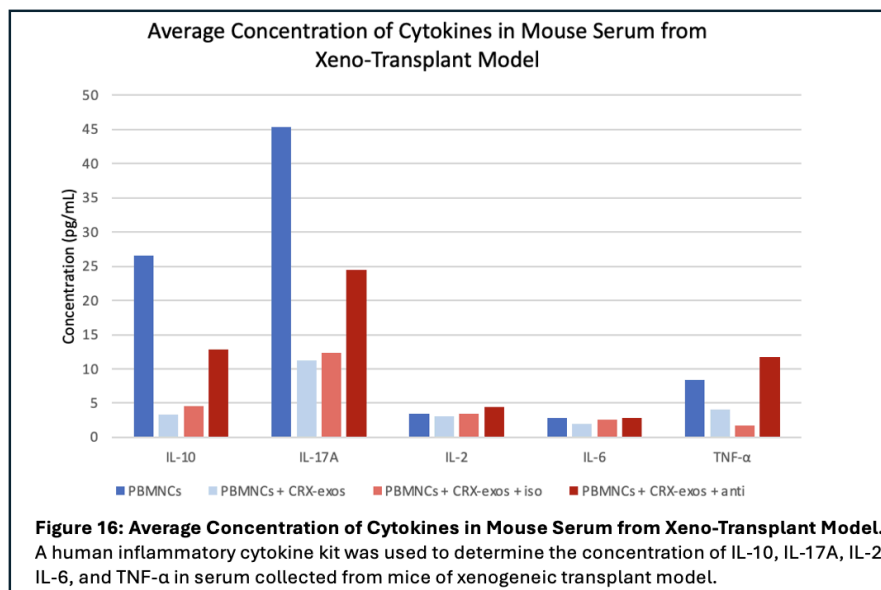
had the next death at day 36, with final percent survivability of 33%. The PBMNC group exhibited the worst outcome, with a sharp dropoff on day 53 and 0% survivability by day 59.



## Human Inflammatory Cytokine Assay

A human inflammatory cytokine assay was performed to obtain a better picture of the molecular profile of the CRX-exo treatment response and identify other possible targets for inhibition in future studies. In both the 1:1 and 2:1 co-cultures, IL-6 was by far the most concentrated cytokine in both CRX-exo treated groups (Figures 14 and 15). There were relatively similar levels of IL-17A, IL-2, and IL-10 between all experimental groups, and a higher concentration of TNF- $\alpha$  in the uneducated conditions. In the cytokine assay of mouse serum, the PBMNC and + anti-IL-6R groups had the highest concentrations of IL-10, IL-17A, and TNF- $\alpha$ . Concentrations of IL-2 and IL-6 were similar between all groups (Figure 16).





## DISCUSSION

### IL-6 Likely Not Main Driver of Treg Activation Following CRX-exo Treatment

If CRX-exos are inducing Treg activation through expression of IL-6 as hypothesized, I expected to observe a decrease in Tregs following treatment with anti-IL-6R antibody compared to the isotype control in both the in-vitro and in-vivo models. As seen in Figures 8 and 9, there was no significant decrease in Treg expression quantified by flow cytometry in either the 1:1 or 2:1 co-culture conditions. Since blocking IL-6R is still allowing for Treg activation, these results suggest that IL-6 is not the main molecular driving force for Treg activation by CRX-exos.

However, I also expected to see a significant increase in Tregs with CRX-exos + isotype control compared to the uneducated conditions. As this increase was not observed, it is possible that this in-vitro model is simply not able to capture and reflect the biological complexity of GVHD. If there are other types of cells and cytokines involved in the cellular response to CRX-exos, a co-culture of only monocytes and T cells may not be sufficient to observe changes in Treg activation. Therefore, I moved to an in-vivo xenogeneic transplant model.

As seen in Figure 11, there was no significant difference in Tregs between the +anti-IL-6R group and the +isotype control group in vivo, suggesting that blocking IL-6R does not affect Treg activation. This conclusion is further supported by there being no significant difference in Tregs between the +anti-IL-6R group and the +CRX-exos group. One interesting observation is that the data collected from the +CRX-exos group and the +isotype control group looks very different. As the isotype control antibody was a human IgG1 isotype control, it should not react with human cells, and the results collected from this group should have looked very similar to the +CRX-exos group. It is possible that maybe another component in the isotype control is affecting the mice, or that there was a health issue with the mice in this condition; it was observed that these mice did have more inflamed mouth and nasal regions compared to the other groups. Due to these possible confounding variables with the isotype control group, it is difficult to draw any definitive conclusions on the role of IL-6 in Treg activation in our transplant model.

### **Possible Role of IL-6 in DPT Suppression**

Interestingly, there was a significant difference in the % DPTs between the +anti-IL-6R group and the +CRX-exos group (Figure 12). Although the aim of this study focused on Tregs, not DPTs, prior research done by the Capitini lab has shown that a human CD4/CD8 double positive T cell (DPT) population develops in mice with lethal xenogeneic GVHD (xeno-GVHD)<sup>5</sup>. We have also shown that isolated DPTs are sufficient to mediate lethal xeno-GVHD, the depletion of DPTs in vivo can rescue mice, and the DPT population has minimal GVL activity against a human B-ALL cell line. Furthermore, we have identified DPTs in primary human allo-HCT recipients and correlated their presence with clinical GVHD development. In summary, DPTs have been found to be upregulated in GVHD and suppressed

upon treatment with CRX-exos. Although these results may suggest a possible role for IL-6 in DPT suppression, because the isotype control also exhibited an increase in DPTs no definitive conclusions can be drawn. Future studies specifically targeting IL-6 suppression of DPTs are needed to investigate this phenomenon further.

### **Improved Survivability with anti-IL-6R**

Based on previous research, I expected to observe increased survivability upon treatment with CRX-exos. My results supported these previous findings, as the CRX-exo treated group exhibited the highest percent survivability (Figure 13). However, I hypothesized that blocking IL-6R would decrease survivability assuming that Treg proliferation would be inhibited. As the +anti-IL-6R in vivo group exhibited the second best survivability, these results further support the flow cytometry data that Treg proliferation was not reduced. Additionally, IL-6 is known to have proinflammatory effects<sup>14</sup>. Blocking IL-6 receptors may have helped to reduce inflammation and presentation of GVHD in the CRX-exo+anti-IL-6R group in vivo.

### **Human Inflammatory Cytokine Assay**

As our lab previously identified IL-6 as the most upregulated gene in an in-vitro model<sup>6</sup>, observing IL-6 to be the highest concentration in CRX-exo treated co-cultures in both the 1:1 and 2:1 conditions aligns with previous research. Conflictingly, the concentration of IL-6 was relatively the same between all treatment groups in the in vivo model. It is possible that the concentration of anti-IL-6R (250 µg per mouse) was not sufficient to significantly block IL-6 receptors and induce decreased expression of IL-6.

It is also interesting to observe that the concentration of TNF- $\alpha$  was higher in the uneducated groups; it is possible that CRX-exos are reducing the expression or production of TNF- $\alpha$  in the in vitro model. There was also a reduced expression of TNF- $\alpha$  in the PBMNC

group compared to the CRX-exo and CRX-exo + isotype control treated groups in vivo; however, the concentration of TNF- $\alpha$  was also high in the CRX-exo + anti-IL-6R group. It also appears that treatment with CRX-exos in vivo decreased expression of IL-10 and IL-17A. For future studies, it may be interesting to try blocking these cytokines to determine their role in the molecular mechanism of GVHD protection by CRX-exos.

## CONCLUSION

With the expansion of allo-HCT as a therapy for patients, developing a treatment for GVHD that will not inhibit GVL activity is a huge unmet medical need and the primary goal for improving patient outcomes. Due to the ease and “off the shelf” application of CRX-exos, CRX-exos are an exciting new treatment with high potential for clinical use. Identifying the specific mechanisms induced by CRX-exos to promote Treg development and activation will provide additional rigor to their mechanism of action and is an important step in moving their application from the benchtop to to the patient. Although this study suggests that IL-6 is not the primary mechanism by which CRX-exos induce Treg activation, this study suggests other cytokine targets for future studies and offers potential for the role of IL-6 in DPT development.

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