Human CD4⁺CD25⁺CD127^{lo}Treg In Vitro Activation With Alloantigen and rIL-4 Induces Receptor for Interleukin 5 (IL-5rα)

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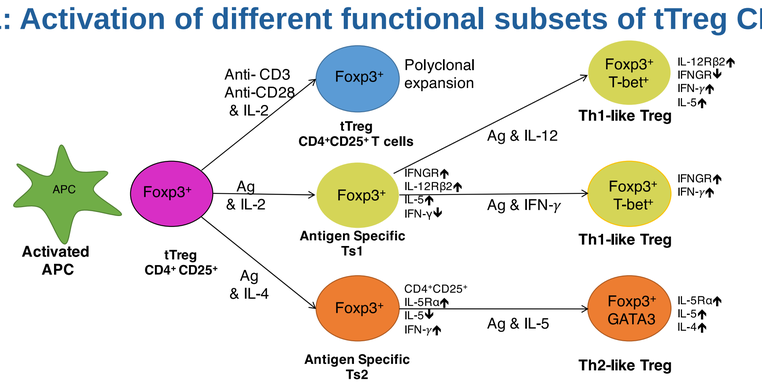
INTRODUCTION

CD4⁺CD25⁺FoxP3⁺Treg (tTreg) have been extensively studied over the last 25 years. Transplant tolerance induction requires tTreg and therefore expanding tTreg for therapy is an attractive option. However, tTreg population comprises of Treg with various antigen specificity and even after expansion are required in large numbers (in ratios of 1:1 or higher to effector cells) to suppress immune responses. We first described alloantigen-specific tolerance mediating CD4⁺CD25⁺CD45RC⁺MHC ClassII⁺T cells in rats (Hall et al, 1990), which can control donor specific cardiac allograft rejection in much lower ratios. These activated antigen-specific Treg rapidly die ex-vivo as they need continuous antigen stimulation and cytokines. The precise cytokine combination to support their ex-vivo survival and growth are not identified, however, IL-2 can expand them non-specifically. In our rat model, we found that tTreg proliferate with alloantigen and rIL-4. Further, IL-4 with alloantigen, in the absence of IL-2, can induce changes in resting tTreg from naïve animals that have TCR for stimulating donor antigen, inducing the receptor for IL-5 (IL-5Rα) (Verma et al, 2009) (Figure 1). These activated tTreg are further expanded by IL-5 to increase their capacity to suppress allograft rejection. We have also demonstrated antigen-specific Treg in autoimmunity are dependent on IL-5 (Tran et al, 2012). Human Treg are identified as CD4⁺CD25⁺CD127^{lo}FoxP3⁺ and are heterogenous comprising of 3 populations based on expression of CD25/Foxp3 and CD45RA. Population I is resting Treg as CD25⁺/Foxp3⁺CD45RA⁺, population, Population II is activated Treg as CD25^{hi}/Foxp3^{hi}CD45RA⁻ and Population III is cytokine secreting as CD25⁺/Foxp3⁺CD45RA⁻ and may include activated effector CD4⁺ T cells.

Here, we examined if human Treg from a healthy donor could be (a) activated by IL-4 and alloantigen to become activated Treg that express IL-5Rα, (b) if these cells express chemokine receptors, c. if renal transplant patients with long surviving stable graft (>10 years) will exhibit changes in Treg populations compared to healthy volunteers.

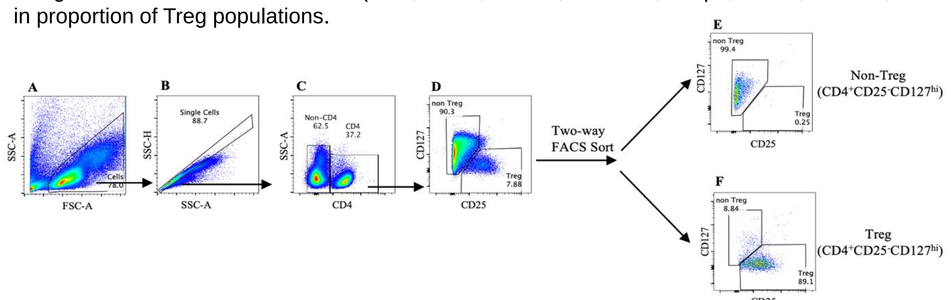
Figure 1: Activation of different functional subsets of tTreg CD4⁺CD25⁺ tTreg



Suppression	**In vitro**	**1:32- 64**	**1:1000**
	In vivo	**1:10**	**1:100**

METHODS

Treg were isolated from healthy human blood using Human CD4⁺CD127^{lo}CD25⁺Treg isolation Kit or CD4⁺CD25⁺ Treg isolation kit for some experiments. Treg were subjected to in vitro culture with IL-4 and alloantigen for 4 days with allogeneic stimulator cells for assessment of proliferation, Real-time PCR for expression of IL-5Rα and multicolour flow cytometry using mouse anti-human antibodies (CD4, CD25, CD127, CD45RA, Foxp3, CCR4, CXCR3, CCR6) for markers for changes in proportion of Treg populations.



Purity of Sorts

	Treg	Non-Treg
Percent (%)	97	98.4
purity of four	89.5	99.6
sorts	91.7	97.5
	92.5	92.5
Mean	92.68	97
Standard Deviation	3.15	3.12

4-5 Day Culture

Treg Alone
Treg + Allo-Ag
Treg + IL-4
Treg + Allo-Ag + IL-4

Figure 2: *PBMC isolated from peripheral blood were stained with antibodies against CD4, CD25, CD127 and CD45RA and subjected to flow cytometry (A). Doublets were excluded (B) before gating on CD4⁺ cells (C), which were further gated based on CD25 and CD127 (D). Tregs (CD4⁺CD25⁺CD127^{lo}) were FACS sorted as shown in Treg (F) and Non-Treg (E). Allogeneic PBMC were X-irradiated at 30 gray to be used in culture as allo-stimulators (Allo-Ag). Sort purity was 89-97% (92.68 ± 3.1%) for Treg and 92.5-99.6% (97 ± 3.1%) for non-Treg.*

Treg proliferate in response to antigen and IL-4

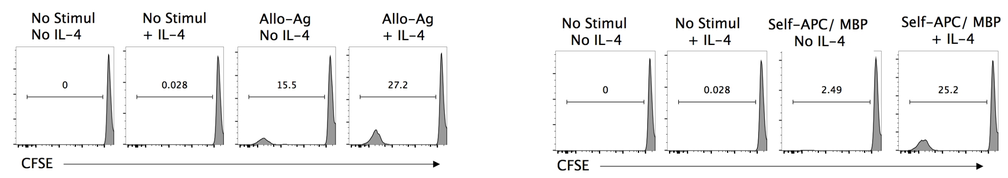


Figure 3: *Tregs were cultured with allo-Ag (Left panel) or Auto-APC primed with auto-antigen (right Panel) with or without IL-4. Proliferation was enhanced when both antigen and IL-4 was present in culture*

RESULTS

Gating Strategy for Treg populations and chemokine expression

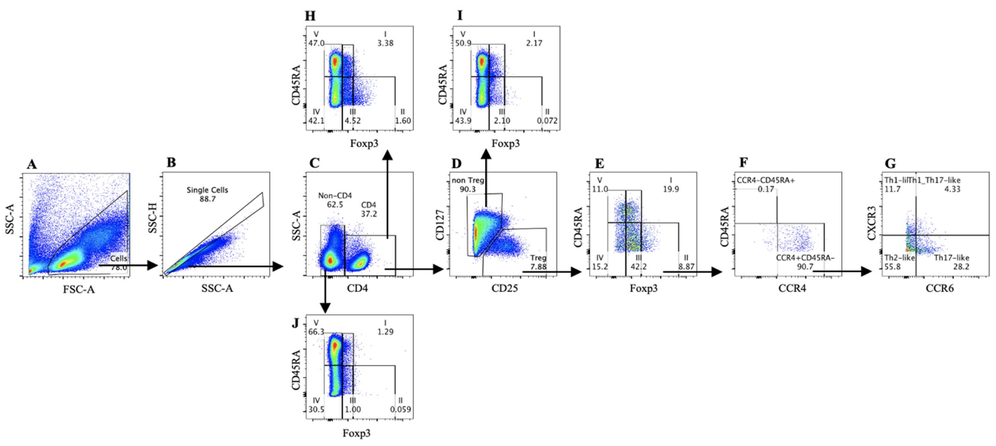


Figure 4: *Cells were subjected to exclude doublets and were gated to identify CD4⁺ cells and subsequently as CD25⁺CD127^{lo} Treg and CD25⁻CD127^{hi} as non-Treg. CD4⁺, non-Treg and Treg were gated on Foxp3 and CD45RA to examine proportions of Foxp3⁺CD45RA⁺ (Pop I), Foxp3^{hi}CD45RA⁻ (Pop II), and Foxp3⁺CD45RA⁻ cells (Pop III).*

Effect of different rIL-4 types and concentrations on Treg Pop

rIL-4 (CHO expressed)

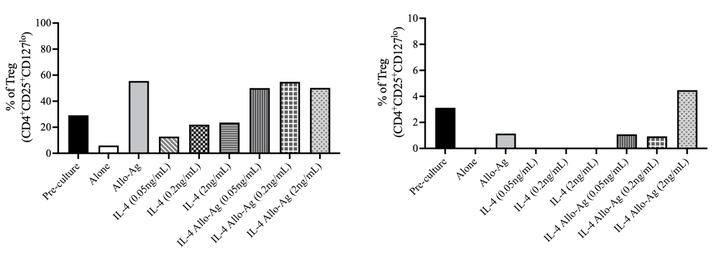


Figure 5: *Enriched Treg were cultured with rIL-4 expressed from various sources (E.coli, CHO or HEK293 cells) and irradiated allogeneic PBMS (Allo-Ag) for 4 days. Results for CHO-IL-4 are shown. Cells were harvested and subjected to flow cytometry to examine proportion of Treg Pop I and II within whole Treg were examined in each culture conditions. CHO rIL-4 maintained pop I .*

Effect of rIL-4 on Treg in culture

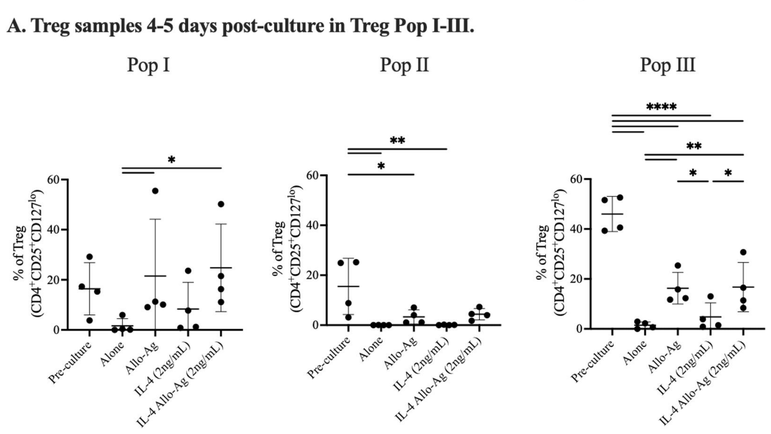


Figure 6: Pooled results for effect of rIL-4 on Treg. *Enriched Treg were cultured with rIL-4 expressed from CHO cells and irradiated allogeneic PBMS (Allo-Ag) for 4 days (n=4). Cells were harvested and subjected to flow cytometry to examine proportion of Treg Pop I, II and III. Of the few cells surviving when cultured alone CD4⁺CD25⁺CD127^{lo} cells were 65.7±18.5% but had less than 2% Foxp3⁺ cells and none Foxp3^{hi} cells. Culture with rIL-4 alone had reduced Pop I (7.2 ± 8.7%) and almost no cells as Pop II (0.27 ±0.48). With allo-Ag and rIL-4, Pop I was preserved (25.2 ±15.2) and about 1/4th cells in Pop II survived (4.2 ± 1.9%).*

Proportions of CCR4⁺CXCR3⁻CCR6⁻ cells in Treg populations 4 days post-culture

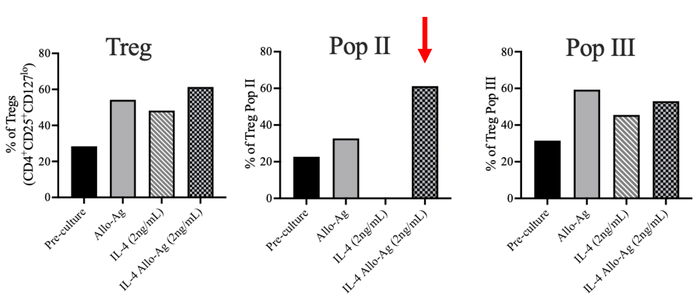


Figure 7: *Enriched Treg were cultured with rIL-4 expressed from CHO cells and irradiated allogeneic PBMS (Allo-Ag) for 4 days. Cells were harvested and subjected to flow cytometry to examine proportion of CCR4, CXCR3 and CCR6 in whole Treg and Treg Pop II and III. CXCR3, CCR6 proportions were similar prior to and after culture, however, CCR4⁺CXCR3⁻CCR6⁻ (Th2-like cells) were increased in Pop II in cultures with allo-Ag and rIL-4.*

IL-5Rα expression in Treg populations 4 days post-culture

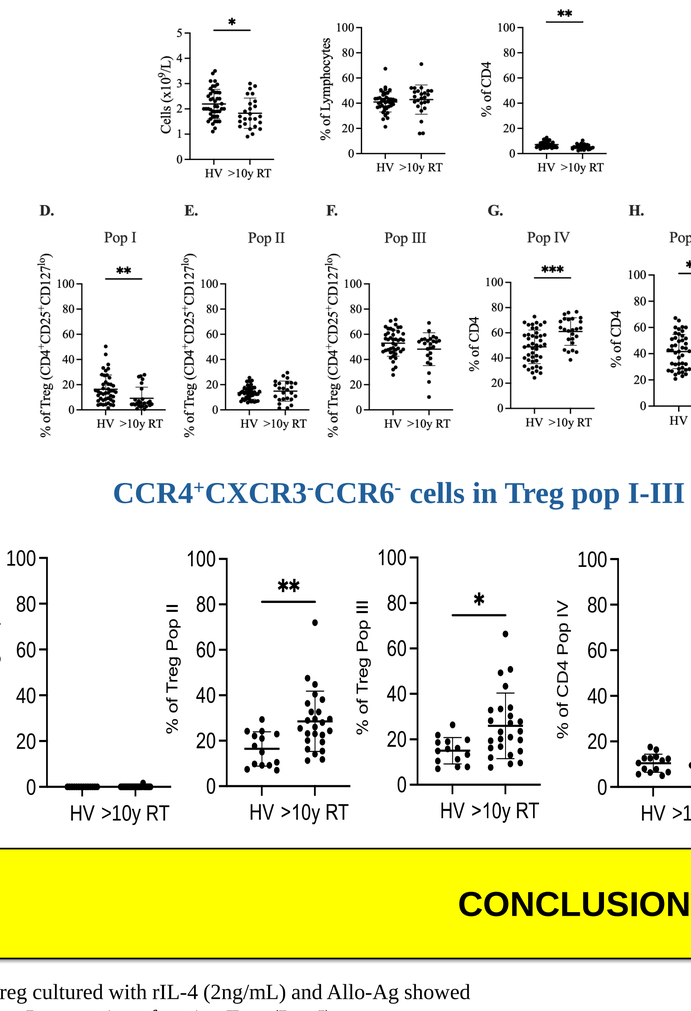


Figure 8: *Enriched Treg were cultured with rIL-4 expressed from CHO cells and irradiated allogeneic PBMS (Allo-Ag) for 4 days. Cells were harvested and subjected to flowcytometry to examine if proportion of IL5Rα⁺ cells changed after culture in whole Treg and Treg Pop I, II and III. IL-5Rα increased in Pop I cultured with allo-Ag alone or rIL-4/allo-Ag, also in Pop II after culture with allo-Ag/rIL-4 (26%) compared to fresh tTreg (0.24), allo-Ag (11.8%) or rIL-4 alone (0%). Pop III expressed IL-5R α with rIL-4, higher than with allo-Ag/rIL-4 (15.2 vs 4.41%).*

Lymphocyte populations in renal transplant (RT) patients

Figure 9: *Lymphocyte subsets in healthy volunteers a (HV) and transplant patients with kidney grafts surviving over 10 years. Lymphocyte subsets were examined in peripheral blood of HV and RT using flow cytometry. RT had lower lymphocyte counts with lower proportions of total Treg and Treg pop I (resting Treg). Within CD4⁺ cells, proportions of activated T cells (Pop IV) was higher whereas pop V, which includes resting T cells and TEMRA was lower.*

CCR4⁺CXCR3⁻CCR6⁻ cells in Treg pop I-III and CD4⁺ pop IV and V

Figure 10: *Th2-like Treg (CCR4⁺CXCR3⁻CCR6⁻) in renal transplant patients. Proportion of Th2-like Treg was higher within activated Treg (pop II), which indicated potential involvement of this subset in graft acceptance.*

CONCLUSIONS

Treg cultured with rIL-4 (2ng/mL) and Allo-Ag showed

- Preservation of resting Treg (Pop I).
- Induction of Ts2 Treg with IL-5Rα expression within activated Treg.
- Induction of Th2-like Treg (CCR4⁺CXCR3⁻CCR6⁻) within Treg Pop II.

RT patients with graft surviving over 10 years had

- Significantly higher Treg Pop II expressing CCR4 but not CXCR3 or CCR6 compared to HV suggesting presence of Th2-like Treg (CCR4⁺CXCR3⁻CCR6⁻) in Treg Pop II.

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Authors have no conflict of interest for this study