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Isolation and Expansion of Clinical Grade T Regulatory Cells for GvHD Prophylaxis

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Abstract: In hematopoietic stem cell transplantation (HSCT), donor-derived allogeneic T cells contribute to the graft-versus-tumor (GVT) effect but can also trigger graft-versus-host disease (GvHD), a major source of post-transplant morbidity and mortality. Regulatory T cells (Tregs), particularly FoxP3$^+$ Tregs, play a pivotal role in promoting immune tolerance and preventing GvHD, as established in murine models. Translating these findings to human therapy requires the isolation and expansion of pure, stable Treg populations suitable for clinical infusion. This study investigates whether clinical-grade CD45RA$^+$ naïve Tregs—known for their stem-like properties and enhanced persistence—can be isolated and expanded while retaining their phenotype and immunosuppressive function. Peripheral blood mononuclear cells (PBMCs) were used to isolate naïve Tregs defined as CD45RA$^+$CD4$^+$CD25$^+$CD127$^-$. Artificial antigen-presenting cells (aAPCs) derived from genetically engineered K562 cells expressing CD64, CD86, and CD137L were loaded with OKT3 monoclonal antibody to stimulate Treg cell expansion. Tregs were re-stimulated on days 0 and 10 and assessed on day 21 using flow cytometry, CFSE suppression assays, and TCR repertoire analysis. Results showed that CD45RA$^+$ Tregs expanded approximately 500-fold, compared to 200-fold for CD45RA$^-$ Tregs, with >80% maintaining FoxP3 expression. Cytokine production remained low, with <5% IL-2 and <2% IFNγ and TNFα. TCR analysis revealed a maintained broad polyclonal repertoire, supporting the diversity and functional stability of expanded Tregs. This study demonstrates a feasible and scalable method for generating clinical-grade, stable, and suppressive CD45RA$^+$ Tregs suitable for immunotherapy. These findings support further clinical trials evaluating their efficacy in preventing or treating GvHD in HSCT recipients.

Keywords: Clinical grade Treg cells, Ex vivo expansion of Tregs, GvHD

1. Introduction

Allogeneic stem cell transplantation (allo-SCT) represents a potentially curative strategy for a range of hematologic malignancies and certain non-malignant conditions. Despite its promise, the presence of alloreactive T cells within the graft initiate an immune response against recipient cells, leading to graft versus host disease (GvHD) [1,2]. GvHD remains a major complication of allogeneic hematopoietic stem cell transplantation (HSCT), contributing to significant morbidity and mortality in these patients. The incidence of GvHD varies between 25–

60% in matched related donor transplants and 40–70% in matched unrelated donor recipients despite the use of pharmacologic immunosuppression for prophylaxis [3,4]. Clearly, the immunosuppressive agents currently employed—such as methotrexate, steroids, mTOR inhibitors and calcineurin inhibitors are inadequate, underscoring the need for more effective strategies to mitigate GvHD, which continues to represent the leading cause of mortality in this patient population [5,6].

Historically, the acute manifestations of GvHD have been attributed primarily to T cells present in the donor graft. Consequently, several clinical trials investigated T cell-depleted grafts as a means to reduce GvHD. However, this approach has been accompanied by an increased incidence of tumor relapse and a higher risk of opportunistic infections and secondary lymphoproliferative disorders [7–9]. These findings highlight the necessity for innovative approaches to effectively manage GvHD in the context of allogeneic HSCT.

In a series of landmark studies, Sakaguchi et al. identified a subset of $CD4^+$ T cells characterized by high expression of CD25 (the IL-2 receptor α-chain) and its association with GvHD. Depletion of these $CD4^+CD25^{+hi}$ cells from T cell suspensions of wild-type BALB/c mice, followed by inoculation into BALB/c nu/nu mice, resulted in the development of inflammatory lesions and GvHD-like wasting disease. Conversely, co-infusion of $CD4^+CD25^{+hi}$ cells prevented the onset of GvHD [10]. Further investigation revealed that IPEX syndrome—a condition involving immune dysregulation, polyendocrinopathy, and enteropathy—was linked to a loss-of-function mutation in the FoxP3 gene. FoxP3 emerged as a transcriptional regulator that suppresses T cell activation [11,12], and the $CD4^+CD25^{+hi}$ cells identified by Sakaguchi et al. and those deficient in IPEX syndrome were not mutually exclusive [13].

These $CD4^+CD25^{+hi}$ FoxP3-expressing cells, further characterized as natural regulatory T cells (nTregs), have shown great potential for down-regulating inflammatory responses by inhibiting T cell activation. In murine models, nTregs effectively induce self-tolerance in alloantigen and infection settings [13–16]. Notably, elegant experiments by Negrin et al. demonstrated that the adoptive transfer of nTregs could prevent GvHD in mouse models [17]. Tregs have also been shown to promote donor bone marrow graft acceptance while facilitating immune recovery in GvHD-prone animals [18,19]. In further studies, Blazar et al. revealed that the infusion of ex vivo cultured Tregs from mice could significantly lessen the severity of lethal GvHD [20]. Cohen et al. found that Tregs infused with recipient-specific allo-antigens mitigated the intensity of acute GvHD while preserving the graft versus leukemia (GVL) effect [21,22]. Consequently, Tregs present an enticing therapeutic avenue for preventing and managing GvHD in allogeneic transplant patients.

A number of clinical trials have investigated the safety and efficacy of infusing ex vivo expanded Tregs in the context of HSCT. For instance, the NCT00602693 trial involved isolating Tregs from umbilical cord blood and expanding them ex vivo with anti-CD28/CD3 dynabeads. Results indicated a reduced incidence of grade II to IV acute GvHD among patients receiving these expanded Tregs compared to a historical control group, notably without an increased risk of infections, relapses, or early mortality [23].

Despite these promising results, various challenges must be addressed before Tregs can effectively transition to clinical application. One significant barrier is the relative scarcity of nTregs, which constitute only 1–2% of total peripheral blood mononuclear cells. Moreover, the intrinsic anergic phenotype of Tregs complicates their expansion in ex vivo culture systems. It has been shown that appropriate co-stimulation using anti-CD86/CD3 can overcome this anergy and enhance expansion [24,25]. However, during this process, $CD4^+CD25^{+hi}$ cells can lose FoxP3 expression and differentiate into Th17 cells secreting IL-17 and IFN-γ raising concerns of potential phenotype plasticity. This plasticity can lead to contamination by non-Tregs, such as $CD25^+$ T-effector or T-memory cells, which could adversely affect the efficacy of Tregs [26]. Additionally, ensuring the preservation of Treg CD3 receptor repertoire diversity post-expansion remains crucial.

Recently, several laboratories have explored various strategies for Treg expansion. For example, Bluestone et al. employed anti-CD86/CD3 beads along with IL-2 and rapamycin, achieving 2 to 3 logs of expansion over 14 days while maintaining around 80% FoxP3 expression and about 60% suppression in a 1:5 ratio of Tregs to responder cells [27,28]. Meanwhile, the Dr. Blazar's group successfully expanded $CD4^+CD25^{+hi}$ Tregs from umbilical cord blood using OKT3-loaded artificial antigen presenting cells (aAPC) expressing CD64 and CD86. Their subsequent clinical trial (NCT00602693) reported a statistically significant reduction in the incidence of grade II to IV GvHD among recipients of these Tregs compared to historical controls. The Blazar team's work additionally demonstrated that $CD4^+CD25^{+hi}$ Tregs isolated from peripheral blood could be expanded up to 5×10^6-fold after multiple stimulations [23,29]. Moreover, researchers in Europe, including Andrew Bushnell's group, have investigated the application of cilostamide, a phosphodiesterase 3 inhibitor, to enhance the number and functional capabilities of FoxP3+ Tregs in culture [30].

In this study, we describe our methodology for isolating $CD4^+CD25^{+hi}$ $CD45RA^+$ Tregs and expanding them using OKT3-loaded aAPCs derived from K562 cells engineered to express CD64, CD86, membrane-bound IL-15,

and 41BB ligand to generate quality clinically significant number of bonafide nTreg cells. Our results demonstrate that the expanded Treg populations retain FoxP3 expression and exhibit significant suppressive functions against CTLs, comparable to the findings reported by others [23,29]. Notably, our aAPC platform is a cost-effective alternative to dynabead technology, is cGMP-certified, and is approved for clinical use [31–33]. These aAPC are available as a master cell bank and offer an off-the-shelf solution for Treg expansion, making this an appealing strategy for cellular therapy to treat GvHD.

2. Materials and Methods

2.1. Isolation and Characterization of Treg Cells

Leukocyte preparations were performed from buffy coats obtained from deidentified donors from the Gulf Coast Blood Bank, Houston, TX. Buffy coats were prepared using Ficoll Hypaque density gradient centrifugation. Regulatory T cell (Treg) isolation was performed based on the expression of surface markers: CD4+ CD25+ $CD127^{-ve}$, utilizing the BD Aria II sorter at the MD Anderson Flow Cytometry Core Laboratory, in collaboration with BD Biosciences, which provided GMP-grade antibodies for Treg cell isolation. To differentiate naïve Tregs from memory Tregs, CD4+ CD25+ $CD127^{-ve}$ cells were further stained with CD45RA, sorting $CD45RA^{+}$(naïve Tregs) and $CD45RA^{-}$ (memory Tregs) cells via fluorescence-activated cell sorting (FACS), as illustrated in Figure 1.

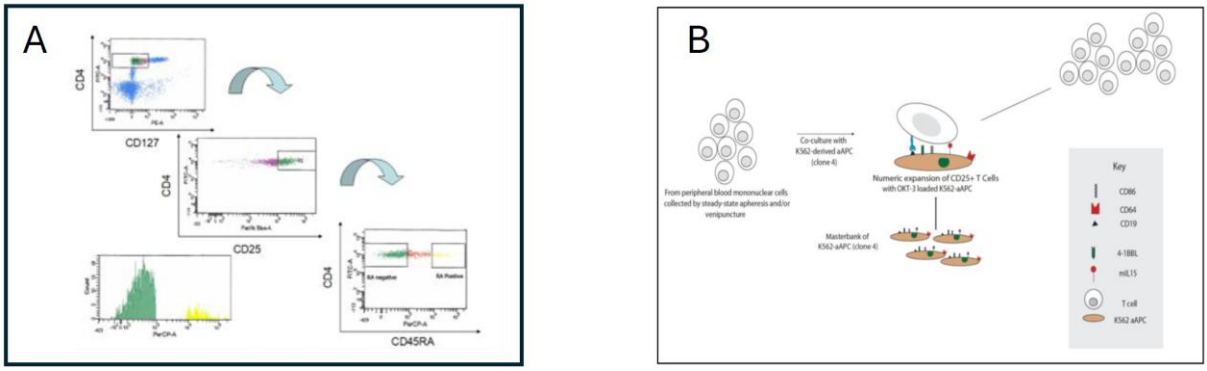

Figure 1. (**A**). Isolation strategy for naïve and memory T regulatory cells using flow cytometry. After magnetic bead based $CD25^{+}$ selection using AutoMACS the $CD25^{+}$ fraction was labeled with CD4FITC CD25Brilliant violet CD127PE CD 45RA PE Cy5.5. From the $CD127^{neg}$ fraction in P4 the CD25hi cell were selected in P5. From this P5 fraction CD45RA $^{+}$ and CD45RA- cells were isolated as different fractions. (**B**). Expansion of $CD4^{+}$ CD25hi $CD127^{neg}$ cells on K562 aAPCs that express CD86 CD64 CD19 4-1BB ligand and membrane bound IL15.

2.2. OKT3 Loaded Clone 4 aAPCs

To expand Treg cells a master cell bank (MCB) of clinical-grade artificial antigen presenting cells (aAPC, kind gift from Dr. Carl June, University of Pennsylvania, Philadelphia) was used. It was developed by genetically engineering K562 cells to express CD64 (Fc receptor) and the T-cell co-stimulatory molecules CD86 and CD137L. The aAPCs were loaded with clinical-grade monoclonal antibody (mAb) OKT3 that imparts a T-cell proliferative signal upon cross-linking CD3 on T cells. $CD25^{+}$ cells were positively enriched from peripheral blood mononuclear cells using anti-CD25 antibody coated magnetic beads, and then $CD4^{+}$ $CD25^{+}$ $CD127^{-}$ Tregs were isolated using fluorescent activated cell sorting (FACS). $CD4^{+}$ $CD25^{-}$ $CD127^{+}$ cells were also isolated and used in place of responder cells in a suppression assay.

2.3. Expansion of Sorted Treg Cells

Expansion of Treg cells was conducted using designer artificial antigen-presenting cells (aAPCs). K562 cells modified to express CD64, alongside co-stimulatory molecules CD137L and CD86, and membrane-bound IL-15, were utilized as APCs. These aAPCs were GMP-certified and stored as a master cell bank. Isolated Treg cells were co-cultured with irradiated aAPCs at a 1:1 ratio in the presence of IL-2 at a final concentration of 200 IU/mL on Day 0 and Day 10. Additional IL-2 and Rapamycin (100 nM/mL) were administered on Days 0, 3, 5, 8, 12, 15, 18, and 20. The cultured Tregs were evaluated for expression of CD4, CD25, CD127, and FoxP3 using flow cytometry with a BD FACS Calibur on Days 5, 8, 12, 18, and 21.

2.4. Suppression Assay

The immunosuppressive capacity of expanded Treg cells was assessed through a suppression assay, where their ability to inhibit the proliferation of conventional T cells in the presence of anti-CD3 antibody (OKT3) was evaluated. CD4+ T cells were isolated from a buffy coat sample followed by Treg isolation via flow cytometry based on $CD25^+$ $CD127^-$ Tregs selection. The $CD25^-$ $CD127^+$ fraction was used as T effector cells. $HLA\text{-}DR^+$ cells were further selected from the $CD4^-$ fraction using magnetic beads on an AutoMACS. These $HLA\text{-}DR^+$ cells were cultured with OKT3 at a concentration of 0.25 μg/mL. T effector cells were labeled with CFSE and subsequently added to the APCs with serial dilutions of Tregs at ratios of 1:2, 1:4, and 1:8. After 5 days, suppression was measured based on CFSE dilution in T effector cells.

2.5. Cytokine Secretion

To assess the secretion of immunosuppressive cytokines like IL-10 by Treg cells, intracellular cytokine staining was performed following PMA/ionomycin stimulation. We anticipated that Treg cells would exhibit an anergic state, exhibiting no secretion of IFN-γ or IL-17 upon stimulation. Fresh Treg cells were cultured in supplemented RPMI medium for 4 h with or without PMA (2 ng/mL) and ionomycin (1 μg/mL). Following stimulation, cells were harvested and stained for CD4, CD25, FoxP3, and cytokines (IL-2, IL-4, IL-17, and IFN-γ) or granzyme B using a standard FoxP3 intracellular staining kit.

2.6. TCR Repertoire Abundance and Diversity

Detailed digital TCR profiling using nCounter analysis (NanoString Technologies, Seattle, USA) was previously described [34].This method was employed to quantify mRNA transcripts for 45 TCR α alleles, 46 TCR β alleles, 13 TCR γ alleles, and 5 TCR δ alleles from RNA samples obtained from Day 0 and Day 20 Treg cells expanded on OKT3 loaded with clone #4. For donors K and L, both Day 0 and expanded Day 20 Tregs were sorted into $CD45RA^+$ and $CD45RA^-$ populations. Total RNA was extracted from the cell pellets using a commercial DNA/RNA preparation kit (Qiagen) and stored at −80 °C in nuclease-free water until further analysis. Custom-designed TCR gene code sets were hybridized with 100 ng of total RNA samples, combined with hybridization buffer (10 μL), reporter probes (10 μL), and capture probes (5 μL). The reaction mixture was then incubated at 65 °C for 12–18 h in a thermal cycler with a heated lid (Pelletier, BIO-RAD DNA Engine). Post-hybridization, samples were processed using an nCounter PrepStation and counted using an nCounter Digital Analyzer (NanoString Technologies) [35]. TCR expression data were normalized relative to both spiked positive control RNA and housekeeping genes (ACTB, G6PD, OAZ1, POLR1B, POLR2A, RPL27, RPS13, and TBP). The spiked positive control normalization factor was calculated as the average of sums for all samples divided by the sum of counts for each individual sample, and similar methodology was applied for the normalization of geometric means. Normalized counts were reported accordingly.

3. Results

3.1. Isolation of T Regulatory Cells by FACS

We employed a FACS-based approach to obtain a highly purified population of Tregs. The sorting process focused on $CD4^+CD25^+CD127^-$ cells, which were subsequently expanded in culture as outlined in the methods. To distinguish between naïve and memory phenotypes, we further sorted the $CD4^+CD25^+CD127^-$ Tregs into $CD45RA^+$ and $CD45RA^-$ populations. Post-sorting analysis confirmed greater than 90% purity based on the relevant surface markers. The yield of sorted cells ranged from 0.5×10^5 to 2×10^6 on average. See Figure 1A for the sorting strategy and post-sort analysis details. The genetically modified K562 aAPCs used in our experiments expressed CD86 and CD64, allowing for the attachment of the Fc region of the CD3 antibody to the CD64 receptor. Additionally, membrane-bound IL15 (mIL15) provided robust stimulation and improved Treg survival as illustrated in Figure 1B.

3.2. Expansion Kinetics of Treg Cells

Normal donor's peripheral blood mononuclear cells (PBMC) were isolated from their buffy coats of 4 normal donors after receiving signed consensus form at Gulf Coast Blood Bank. To numerically expand Treg cells to clinically sufficient numbers, a clinical-grade artificial antigen presenting cells aAPC was used to stim on Day 1 and on Day10. Also, included rapamycin (100 ng/mL) in the culture media to selectively expanded $CD4^+CD25^+FoxP3^+$ Tregs while inhibiting T effector cell proliferation. FoxP3 expression was measured to

evaluate the purity of Treg population in the FACS sorted population and expanded Treg population. Initially, on day 0, CD45RA$^+$ and CD45RA$^-$ Tregs exhibited 40–85% FoxP3 positivity, averaging around 70% (Figure 2A). Remarkably, these cells maintained 80–90% FoxP3 expression at the end of the 21-day culture (Figure 2B).

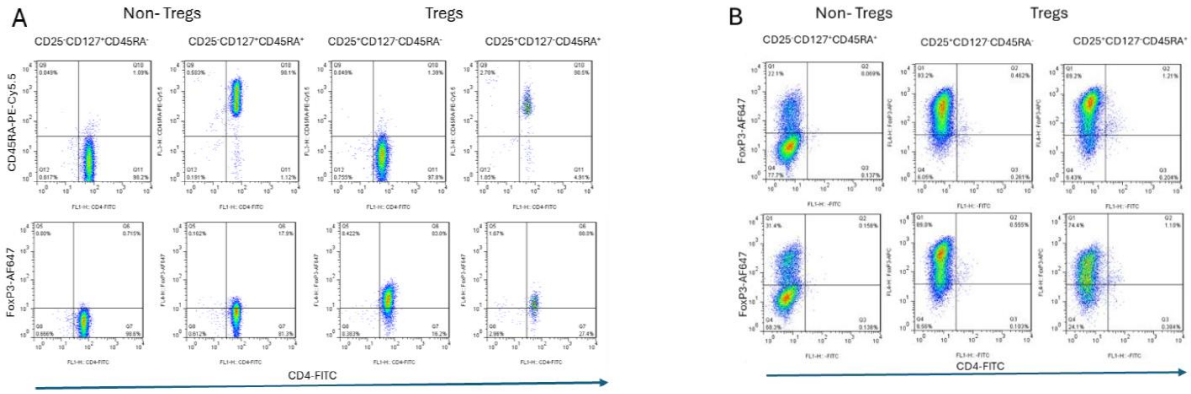

Figure 2. Phenotypical characterization studies: **(A).** Post sort analysis of a representative donor showing FoxP3 purity after sorting based on CD4$^+$CD25$^+$CD127$^-$ CD45RA$^+$ and CD45RA$^-$ sorting (upper panel). FoxP3 expression of corresponding samples are shown in lower panel. (**B**). FoxP3 expression of 2 representative donors after 21 days in culture. The first column shows foxP3 expression in the control population of CD4$^+$CD25$^-$CD127$^-$ cells. The 2nd column shows CD45RA$^+$ naïve Tregs from two donors, third column shows FoxP3 expression in CD45RA memory Tregs. These cells were stimulated 48hrs prior to flow analysis.

3.3. Suppressive Function of T Regulatory Cells

After 21 days of culture, we observed approximately 500-fold expansion of CD45RA$^+$ Tregs and about 200-fold expansion of CD45RA$^-$ Tregs (Figure 3A–D) on average from 4 donors. To confirm that expanded Tregs retained their suppressive function, we co-cultured CFSE-labeled CD25neg responder cells with HLA-DR+ APCs and evaluated proliferation in the presence or absence of allogeneic Tregs. After 4 to 5 days, flow cytometry analysis revealed that Tregs expanded on aAPCs exhibited approximately 60% suppression of responder cell proliferation (Figure 3E–H).

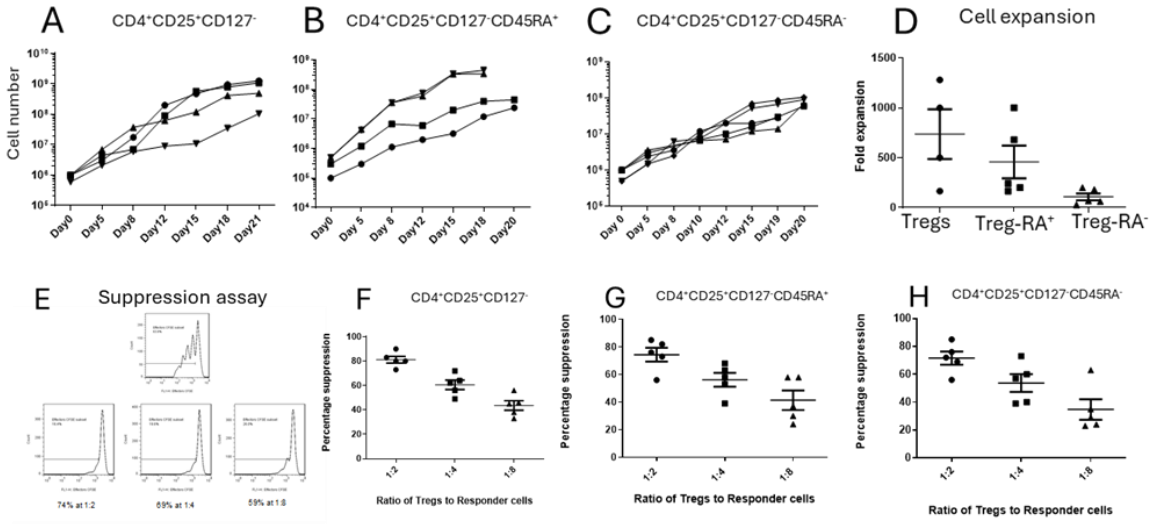

Figure 3. Expansion kinetics. **(A)** Sort purified Treg cells based on the CD4$^+$CD25$^+$CD127$^-$ selection were propagated on OKT3 loaded clone 4 in the presence of IL2 and rapamycin. (**Upper panel**) shows cell expansion kinetics of 4 representative donors of CD4$^+$CD25$^+$CD127$^-$ Tregs; (**B**) shows expansion of CD4$^+$CD25$^+$CD127$^-$ CD45RA$^+$ naïve Tregs and (**C**) shows expansion of CD4$^+$CD25$^+$CD127$^-$ CD45RA-memory Tregs. (**D**) compares the cell expansion kinetics in fold with various Treg fractions. In lower panel, (**E**). shows the suppression at 1:2 1:4 and 1:8 dilution of expanded Tregs to CD4$^+$CD25$^+$CD127^{neg} responder cells. (**F**). shows CD4$^+$CD25$^+$CD127$^-$Tregs (**G**). CD45RA$^+$ Tregs and (**H**). CD45RA$^-$ Tregs.

3.4. Cytokine Profile

The issue of Treg plasticity remains controversial. Previous studies, such as those by the Bluestone group, indicated that cells lose their FoxP3 phenotype and suppressive function after bead-based stimulation. In our assessment of the cytokine profile post 21 days in culture, we induced Tregs with PMA-ionomycin for intracellular staining of IL10, IL17, and IFNγ. Our findings revealed that less than 2% of the expanded cells secreted IL17, while 3–5% secreted IL2, and again less than 2% produced IFNγ. Collectively, these results indicate that the Tregs retained their lineage and suppressive capabilities after 21 days in culture (Figure 4A).

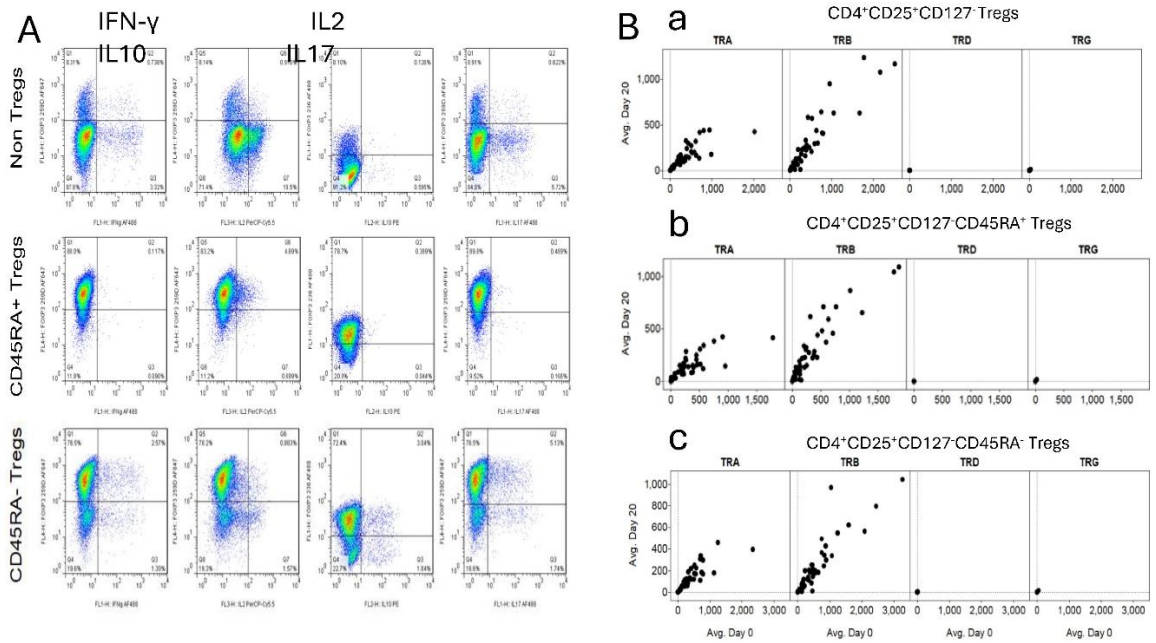

Figure 4. (**A**). Cytokine profile of one representative donor. CD45RA$^+$ Tregs and B CD45RA-Tregs Compared to non Tregs CD4$^+$CD25$^-$CD127$^+$ cells propagated in similar fashion. Cells were stimulated with PMA ionomycin in the presence of golgi-plug and tested for presence of IFNγ, IL2, IL10 and IL17. (**B**). TCR repertoire of TCR-α, TCR-β, TCR-δ and TCR-γ shown in (**a**). CD4$^+$CD25$^+$CD127$^-$ population, (**b**). CD4+CD25+CD45RA+ population and (**c**). CD4+CD25+CD45RA- population.

3.5. Maintenance of a Polyclonal TCR Repertoire

To evaluate the TCR repertoire of expanded Tregs, we analyzed the expression of TCR Vα, TCR Vβ, TCR Vγ, and TCR Vδ chains. A direct TCR expression assay (DTEA) assessed the diversity of TCR chains across time in culture. With the capacity to detect 45 TCR Vα alleles, 46 TCR Vβ alleles, 13 TCR Vγ alleles, and 5 TCR Vδ alleles, we identified the availability of diverse TCR repertoires pre- and post-expansion [35]. Analyses indicated that the aAPC design, alongside high concentrations of IL2, supported the numeric expansion of Treg populations while preserving a broad TCR repertoire. Spearman's non-parametric correlation confirmed that the TCR repertoires showed a strong correlation (coefficient ≥ 0.8, $p \geq 0.8$) within the 95% confidence interval, supporting the polyclonal nature of the Treg repertoire expanded under these conditions ((Figure 4B).

4. Discussion

The therapeutic potential of regulatory T cells (Tregs) in preventing GvHD and various autoimmune disorders has been extensively demonstrated in murine models by multiple research groups [18,21]. However, translating these findings into effective therapies for human diseases poses significant challenges, most notably in generating enough Tregs and ensuring their safety characterized by stable FoxP3 expression. Critical here is maintaining Treg integrity without shifting towards a Th17 phenotype in vivo.

Current clinical approaches have largely focused on Tregs isolated from umbilical cord blood cells, which presents limitations due to variability in cell numbers and availability. For example, in the NCT0602693 trial, up to 30×10^5 Tregs per kg body weight were infused shortly after transplantation. Some patients received a second infusion two weeks post-transplant [23]. In contrast, the use of peripheral blood allows for the isolation of approximately 0.5 to 1 million CD4$^+$CD25$^+$CD127$^-$ cells from a typical buffy coat, and an apheresis pack yields

roughly ten-fold this amount. Through our protocol utilizing activated artificial antigen-presenting cells (aAPCs) irradiated at 100Gy, we achieve Treg expansion resulting in an estimated 10×10^9 cells—adequate for clinical application. Notably, while our aAPCs, originating from a chronic myeloid leukemia cell line, lose viability after 7 to 10 days in culture, the GMP-approved Clone 4 aAPCs facilitate robust Treg expansion.

In our findings, we consistently observe a 200 to 250-fold increase in Treg populations following two stimulation rounds with aAPCs and IL-2. Starting from 10 to 20 million Tregs isolated from a leukopheresis pack, this process allows us to generate a minimum of 2×10^9 Tregs for infusion, with the option of repeat donations from donors if additional cells are needed. Thus, peripheral blood emerges as a superior source for Treg harvesting compared to umbilical cord blood.

Ensuring the generation of pure and stable Tregs is vital for their therapeutic use. Extended ex vivo stimulation can inadvertently result in the loss of Treg suppressive phenotype and contamination with T cells producing IL-17 and IFN-γ, leading to adverse effects. Addition of rapamycin in our expansion protocol has proven instrumental; we observed over 80% FoxP3 positivity after 21 days of culture, alongside preserved Treg suppressive function. In addition, flow cytometry analysis indicated minimal presence (~0.48%) of IL-17 or IFN-γ expressing cells, in $CD45RA^+$ ve population compared to ~5% seen in CD45RA-ve Treg population confirming that the Treg phenotype of the expanded cells. Furthermore, assessment of the TCR Vβ repertoire revealed it remained unchanged pre- and post-expansion, reinforcing the notion that our protocol is efficacious in generating a polyclonal Treg population suited for the treatment of GvHD, autoimmunity, and other conditions.

Future Directions

Research conducted by the Bluestone group and others suggests that CD45RA+ naïve Tregs maintain stable FoxP3 expression post-expansion [23,27,28]. We hypothesize that while CD45RA+ naïve Tregs may have stable FoxP3 expression, their restricted TCR repertoire might attenuate their ability to suppress GvHD compared to the $CD45RA^-$ fractions. Testing this hypothesis through in vitro assays presents challenges; thus, we plan to implement a humanized GvHD mouse model to verify our hypothesis. Current experiments focus on assessing the impact of infusing $CD4^+CD25^+CD127^-$ cells on mouse weight, fur loss, and overall survival following the administration of allogeneic PBMCs, with and without Tregs.

In summary, our Treg expansion methodology utilizing aAPCs presents a promising avenue for developing an "off-the-shelf" cellular therapy using Tregs derived from HLA-matched donors boasting established efficacy and safety profiles. This advancement could significantly impact the field of cell therapy, providing scalable and reliable treatment options for GvHD and other immune-mediated conditions.

Author Contributions

PK.: conceptualization, methodology, data collection, analyzing, writing-original draft, reviewing; SM.: data collection, PJH: Data analysis, JR: Data analysis, HS.: visualization, data analysis; CD.: Methodology, data collection, writing—reviewing and editing. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement

Not applicable.

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Conflicts of Interest

The authors declare no conflict of interest.

Use of AI and AI-Assisted Technologies

No AI tools were utilized for this paper.

References

1. Blazar, B.R.; Hill, G.R.; Murphy, W.J. Dissecting the biology of allogeneic HSCT to enhance the GvT effect whilst minimizing GvHD. *Nat. Rev. Clin. Oncol.* **2020**, *17*, 475–492. https://doi.org/10.1038/s41571-020-0356-4.
2. Welniak, L.A.; Blazar, B.R.; Murphy, W.J. Immunobiology of allogeneic hematopoietic stem cell transplantation. *Annu. Rev. Immunol.* **2007**, *25*, 139–170. https://doi.org/10.1146/annurev.immunol.25.022106.141606.
3. Fonseca-Santos, M.; Bailen, R.; Lopez-Godino, O.; et al. Characterization of Chronic Graft-versus-host Disease After Haploidentical Stem Cell Transplantation with Posttransplant Cyclophosphamide: A Study on behalf of GETH-TC. *Transplantation* **2024**, *108*, 2134–2143. https://doi.org/10.1097/TP.0000000000005034.
4. Goerner, M.; Gooley, T.; Flowers, M.E.; et al. Morbidity and mortality of chronic GVHD after hematopoietic stem cell transplantation from HLA-identical siblings for patients with aplastic or refractory anemias. *Biol. Blood Marrow Transplant.* **2002**, *8*, 47–56. https://doi.org/10.1053/bbmt.2002.v8.pm11858190.
5. Lum, S.H.; Eikema, D.J.; Piepenbroek, B.; et al. Outcomes of hematopoietic stem cell transplantation in 813 pediatric patients with Fanconi anemia. *Blood* **2024**, *144*, 1329–1342. https://doi.org/10.1182/blood.2023022751.
6. Nagler, A.; Ngoya, M.; Galimard, J.E.; et al. Comparable relapse incidence after unrelated allogeneic stem cell transplantation with post-transplant cyclophosphamide versus conventional anti-graft versus host disease prophylaxis in patients with acute myeloid leukemia: A study on behalf of the Acute Leukemia Working Party of the European Society for Blood and Marrow Transplantation. *Am. J. Hematol.* **2024**, *99*, 1732–1745. https://doi.org/10.1002/ajh.27383.
7. Halahleh, K.A.; Ma'koseh, M.; Sultan, I.; et al. High Incidences of Acute and Chronic Graft-Versus-Host Disease after Hematopoietic Cell Transplants for Acute Myeloid Leukemia Using Thiotepa, Busulfan, and Fludarabine Pretransplant Conditioning. *Acta Haematol.* **2023**, *146*, 88–91. https://doi.org/10.1159/000528306.
8. Satty, A.M.; Klein, E.; Mauguen, A.; et al. T-cell depleted allogeneic hematopoietic stem cell transplant for the treatment of Fanconi anemia and MDS/AML. *Bone Marrow Transplant.* **2024**, *59*, 23–33. https://doi.org/10.1038/s41409-023-02113-1.
9. Wagner, J.E.; Thompson, J.S.; Carter, S.L.; et al. Effect of graft-versus-host disease prophylaxis on 3-year disease-free survival in recipients of unrelated donor bone marrow (T-cell Depletion Trial): A multi-centre, randomised phase II-III trial. *Lancet* **2005**, *366*, 733–741. https://doi.org/10.1016/S0140-6736(05)66996-6.
10. Sakaguchi, S.; Sakaguchi, N.; Asano, M.; et al. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J. Immunol.* **1995**, *155*, 1151–1164.
11. Bennett, C.L.; Christie, J.; Ramsdell, F.; et al. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat. Genet.* **2001**, *27*, 20–21. https://doi.org/10.1038/83713.
12. Zheng, Y.; Rudensky, A.Y. Foxp3 in control of the regulatory T cell lineage. *Nat. Immunol.* **2007**, *8*, 457–462. https://doi.org/10.1038/ni1455.
13. Sakaguchi, S.; Sakaguchi, N.; Shimizu, J.; et al. Immunologic tolerance maintained by $CD25^+$ $CD4^+$ regulatory T cells: Their common role in controlling autoimmunity, tumor immunity, and transplantation tolerance. *Immunol. Rev.* **2001**, *182*, 18–32. https://doi.org/10.1034/j.1600-065x.2001.1820102.x.
14. Belkaid, Y.; Piccirillo, C.A.; Mendez, S.; et al. $CD4^+CD25^+$ regulatory T cells control Leishmania major persistence and immunity. *Nature* **2002**, *420*, 502–507. https://doi.org/10.1038/nature01152.
15. Kursar, M.; Bonhagen, K.; Fensterle, J.; et al. Regulatory $CD4^+CD25^+$ T cells restrict memory CD8+ T cell responses. *J. Exp. Med.* **2002**, *196*, 1585–1592. https://doi.org/10.1084/jem.20011347.
16. Taylor, P.A.; Noelle, R.J.; Blazar, B.R. $CD4^+CD25^+$ immune regulatory cells are required for induction of tolerance to alloantigen via costimulatory blockade. *J. Exp. Med.* **2001**, *193*, 1311–1318. https://doi.org/10.1084/jem.193.11.1311.
17. Nguyen, V.H.; Shashidhar, S.; Chang, D.S.; et al. The impact of regulatory T cells on T-cell immunity following hematopoietic cell transplantation. *Blood* **2008**, *111*, 945–953. https://doi.org/10.1182/blood-2007-07-103895.
18. Edinger, M.; Hoffmann, P.; Ermann, J.; et al. $CD4^+CD25^+$ regulatory T cells preserve graft-versus-tumor activity while inhibiting graft-versus-host disease after bone marrow transplantation. *Nat. Med.* **2003**, *9*, 1144–1150. https://doi.org/10.1038/nm915.
19. Ermann, J.; Hoffmann, P.; Edinger, M.; et al. Only the CD62L+ subpopulation of $CD4^+CD25^+$ regulatory T cells protects from lethal acute GVHD. *Blood* **2005**, *105*, 2220–2226. https://doi.org/10.1182/blood-2004-05-2044.
20. Taylor, P.A.; Lees, C.J.; Blazar, B.R. The infusion of ex vivo activated and expanded $CD4^+CD25^+$ immune regulatory cells inhibits graft-versus-host disease lethality. *Blood* **2002**, *99*, 3493–3499. https://doi.org/10.1182/blood.v99.10.3493.
21. Cohen, J.L.; Trenado, A.; Vasey, D.; et al. $CD4^+CD25^+$ immunoregulatory T Cells: New therapeutics for graft-versus-host disease. *J. Exp. Med.* **2002**, *196*, 401–406. https://doi.org/10.1084/jem.20020090.

22. Trenado, A.; Charlotte, F.; Fisson, S.; et al. Recipient-type specific $CD4^+CD25^+$ regulatory T cells favor immune reconstitution and control graft-versus-host disease while maintaining graft-versus-leukemia. *J. Clin. Investig.* **2003**, *112*, 1688–1696. https://doi.org/10.1172/JCI17702.
23. Brunstein, C.G.; Miller, J.S.; Cao, Q.; et al. Infusion of ex vivo expanded T regulatory cells in adults transplanted with umbilical cord blood: Safety profile and detection kinetics. *Blood* **2011**, *117*, 1061–1070. https://doi.org/10.1182/blood-2010-07-293795.
24. June, C.H.; Blazar, B.R. Clinical application of expanded CD4+25+ cells. *Semin. Immunol.* **2006**, *18*, 78–88. https://doi.org/10.1016/j.smim.2006.01.006.
25. Li, L.; Godfrey, W.R.; Porter, S.B.; et al. $CD4^+CD25^+$ regulatory T-cell lines from human cord blood have functional and molecular properties of T-cell anergy. *Blood* **2005**, *106*, 3068–3073. https://doi.org/10.1182/blood-2005-04-1531.
26. Beyer, M.; Schultze, J.L. Plasticity of T(reg) cells: Is reprogramming of T(reg) cells possible in the presence of FOXP3? *Int. Immunopharmacol.* **2011**, *11*, 555–560. https://doi.org/10.1016/j.intimp.2010.11.024.
27. Battaglia, M.; Stabilini, A.; Migliavacca, B.; et al. Rapamycin promotes expansion of functional $CD4^+CD25^+FOXP3^+$ regulatory T cells of both healthy subjects and type 1 diabetic patients. *J. Immunol.* **2006**, *177*, 8338–8347. https://doi.org/10.4049/jimmunol.177.12.8338.
28. Putnam, A.L.; Brusko, T.M.; Lee, M.R.; et al. Expansion of human regulatory T-cells from patients with type 1 diabetes. *Diabetes* **2009**, *58*, 652–662. https://doi.org/10.2337/db08-1168.
29. Hippen, K.L.; Merkel, S.C.; Schirm, D.K.; et al. Massive ex vivo expansion of human natural regulatory T cells (T(regs)) with minimal loss of in vivo functional activity. *Sci. Transl. Med.* **2011**, *3*, 83ra41. https://doi.org/10.1126/scitranslmed.3001809.
30. Feng, G.; Nadig, S.N.; Bäckdahl, L.; et al. Functional Regulatory T Cells Produced by Inhibiting Cyclic Nucleotide Phosphodiesterase Type 3 Prevent Allograft Rejection. *Sci. Transl. Med.* **2011**, *3*, 83ra40. https://doi.org/10.1126/scitranslmed.3002099.
31. Kebriaei, P.; Singh, H.; Huls, M.H.; et al. Phase I trials using Sleeping Beauty to generate CD19-specific CAR T cells. *J. Clin. Investig.* **2016**, *126*, 3363–3376. https://doi.org/10.1172/JCI86721.
32. Singh, H.; Srour, S.A.; Milton, D.R.; et al. Sleeping beauty generated CD19 CAR T-Cell therapy for advanced B-Cell hematological malignancies. *Front. Immunol.* **2022**, *13*, 1032397. https://doi.org/10.3389/fimmu.2022.1032397.
33. Srour, S.A.; Singh, H.; McCarty, J.; et al. Long-term outcomes of Sleeping Beauty-generated CD19-specific CAR T-cell therapy for relapsed-refractory B-cell lymphomas. *Blood* **2020**, *135*, 862–865. https://doi.org/10.1182/blood.2019002920.
34. Beard, R.E.; Abate-Daga, D.; Rosati, S.F.; et al. Gene expression profiling using nanostring digital RNA counting to identify potential target antigens for melanoma immunotherapy. *Clin. Cancer Res.* **2013**, *19*, 4941–4950. https://doi.org/10.1158/1078-0432.CCR-13-1253 From NLM Medline.
35. Caruso, H.G.; Torikai, H.; Zhang, L.; et al. Redirecting T-Cell Specificity to EGFR Using mRNA to Self-limit Expression of Chimeric Antigen Receptor. *J. Immunother.* **2016**, *39*, 205–217.