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FOOD AND DRUG ADMINISTRATION
Center for Biologics Evaluation and Research
Office of Therapeutic Products
Office of Pharmacology/Toxicology

BLA NUMBER: STN #125868.000.011.018.023

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DATE REVIEW COMPLETED: June 12, 2026

PRODUCT²: TREGZI

APPLICANT: Orca Biosystems Inc.

PROPOSED INDICATION³: Use in matched donor hematopoietic stem cell (HSC) transplantation with myeloablative preparative regimen, for hematopoietic and immunologic reconstitution and to improve survival free of chronic graft-versus-host disease (cGVHD) in the treatment of adults with hematologic malignancies.

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EXECUTIVE SUMMARY⁴:

TREGZI (Orca-T) is an allogeneic hematopoietic stem and progenitor cell (HSPC), and T-cell immunotherapy designed to improve survival free of cGVHD in adult patients with hematologic malignancies undergoing a myeloablative preparative regimen. TREGZI consists of four drug products (DPs): three cellular DPs and one Tcon Diluent DP. The three cellular DPs contain distinct cell types derived from human leukocyte antigen (HLA)-matched donor peripheral blood apheresis: HSPCs, regulatory T cells (Tregs), and conventional T cells (Tcons). Following an appropriate myeloablative conditioning (MAC) regimen, HSPC DP (dose level: $\geq 1.0 \times 10^6$ viable HSPCs/kg per patient) followed by Treg DP (dose level range: $(b)(4) \times 10^6$ to 3.5×10^6 viable Tregs/kg per patient) are intravenously (IV) administered on day 0, and Tcon DP (dose level range: $(b)(4) \times 10^6$ to 6.9×10^6 viable Tcons/kg per patient) admixed with Tcon Diluent DP is IV administered on day +2 to day +3 after HSPC DP and Treg DP administrations.

While no formal nonclinical pharmacology, distribution, or toxicology studies were conducted with TREGZI, the applicant provided the scientific rationale for this application with published data from nonclinical studies evaluating the mechanisms of action (MOAs) of hematopoietic stem cells (HSCs), Tregs, Tcons, and their combinations regarding hematopoietic and immunologic reconstitution and GVHD. Data from these published studies demonstrated key mechanistic findings that contributed to understanding T cell biology and functions in GVHD development and suppression. Additionally, the applicant provided a study report summarizing multiple proof-of-concept (POC) studies evaluating the ability of human Tcons to induce xenogeneic GVHD and the ability of Tregs co-administered with Tcons to mitigate Tcon-induced xenogeneic GVHD in sub-lethally irradiated immunodeficient mice.

Published data show that HSCs could be successfully isolated from murine and human bone marrow (BM) using specific cell surface markers: Thy-1^{lo}Lin⁻Sca-1⁺ cells in mice (0.05% of BM cells) and Thy-1⁺ Lin⁻CD34⁺ cells in humans (0.05-0.1% of fetal BM cells). These purified HSCs were shown to be essential for hematopoietic engraftment and immune reconstitution, with as few as 30 murine HSCs sufficient to proliferate, differentiate into most blood lineages (myelomonocytic, B, and T cells), and rescue lethally irradiated mice upon transplantation.

Published nonclinical studies also show that Tregs suppressed Tcon activation and proliferation through direct cell contact, inhibiting interleukin (IL)-2 production and promoting recipient dendritic cell death. Foxp3 expression was essential for Treg development and function, and highly purified, functionally suppressive Tregs could be successfully isolated using specific cell surface markers: CD4⁺CD25⁺CD127^{low/-}.

Studies using murine GVHD models demonstrate that mature donor T cells mediated GVHD following allogeneic BM transplantation, while T cell-depleted BM (TCD-BM) prevented GVHD and preserved engraftment and immune reconstitution. A 1:1 donor Treg:Tcon ratio prevented GVHD when administered on the same day as TCD-BM. Early Treg administration, two days prior to Tcon administration, maximized GVHD suppression at lower Treg doses (1:10 Treg:Tcon ratio) while preserving graft-versus-leukemia (GVL) effects. Data from this study provided the scientific rationale for administering Tregs two days earlier than Tcons clinically.

In the applicant's POC studies using xenogeneic models, co-administration of human donor Tregs with Tcons demonstrated dose-dependent GVHD inhibition in sub-lethally irradiated immunodeficient mice. Coadministration of a 2:1 Treg:Tcon ratio provided significantly longer median survival (78.8 days) compared to 1:1 (60.6 days) or Tcons alone (22.5 days).

No genotoxicity, carcinogenicity, and developmental and reproductive toxicity (DART) studies were conducted for TREGZI. These studies are not warranted based on the product characteristics, biological activity, and safety profile.

PHARMACOLOGY/TOXICOLOGY RECOMMENDATION⁵:

No nonclinical deficiencies have been identified in this submission. There are no outstanding requests for additional nonclinical studies to evaluate TREGZI. The nonclinical information in this BLA submission supports approval of the licensure application.

Formulation and Chemistry⁶:

A single dose of TREGZI contains four single-dose infusion bags: HSPC DP, Treg DP, and Tcon DP (approximate (b) (4) and Tcon diluent DP (approximate (b) (4)). For each patient, three distinct cellular products, HSPCs, Tregs, and Tcons, are manufactured from granulocyte colony-stimulating factor (G-CSF)-mobilized peripheral blood apheresis product collected from an 8/8 HLA-matched related or unrelated donor (HLA-A, -B, -C, and -DRB1). HSPC DP, immediately followed by Treg DP, are administered IV through a central venous catheter on day 0, and Tcon DP admixed with Tcon diluent DP is administered IV on day +2 to day +3 (48 to 72 hours after HSPC DP and Treg DP administrations).

HSPC DP

HSPC DP is formulated in approximately 100 mL of Multiple Electrolytes Injection (MEI; Type 1, (b) (4) and 2% weight per volume (w/v) human serum albumin (HSA) and shipped fresh. Viable HSPCs are (b) (4) CD34⁺ and viability-positive (b) (4). The dose level is $\geq 1.0 \times 10^6$ viable HSPCs/kg per patient. The primary container is a sterile, (b) (4) bag (150 mL), and the secondary container is a sterile Ziplock bag (b) (4).

Treg DP

Treg DP is formulated in the identical final volume and excipients as HSPC DP and shipped fresh. Viable Tregs are both (b) (4) CD4⁺CD25⁺(b) (4) CD127^{low/-} and viability-positive. The dose level range is (b) (4) $\times 10^6$ to 3.5×10^6 viable Tregs/kg per patient. Treg DP uses the same primary and secondary containers as HSPC DP.

Tcon DP

Tcon DP is formulated in approximately 15 mL of MEI (b) (4) (b) (4) HSA (3% to 4% [w/v]), dimethyl sulfoxide (7.5% [v/v]), sodium chloride injection (b) (4), and Hetastarch (1.8% [w/v]) and cryopreserved. Viable Tcons are CD3⁺(b) (4) and viability positive. The dose level range is (b) (4) $\times 10^6$ to 6.9×10^6 viable Tcons/kg per patient. The primary container is an (b) (4) (b) (4) (b) (4) and the secondary container is a sterile Ziplock (b) (4) bag (b) (4) Tcon DP and Tcon diluent DP are shipped together in a vapor dry shipper box.

Tcon Diluent DP

Tcon diluent DP formulation consists of 30 mL of (b) (4) MEI and 2% (w/v) HSA. The primary container is a 50 mL end-ported biocontainer (bag) made of (b) (4) -based (b) (4) film, and the secondary container is a sterile clear self-sealing plastic bag. Prior to administration, Tcon DP is thawed and diluted with 30 mL of Tcon diluent DP.

Reviewer's Comment:

- TREGZI contains impurities. Product-related impurities include (b) (4)

(b) (4). These impurities have been communicated to the CMC reviewer (Karin Knudson). Our P/T discipline defers to Dr. Knudson to use the CMC data to address any potential safety issues regarding the presence of these impurities in the final drug products.

Abbreviations

BM	Bone marrow
BMT	BM transplantation
CFU-S	Splenic colony-forming unit
CFU-T	Thymic colony-forming unit
DART	Developmental and reproductive toxicity
DCs	Dendritic cells
DP	Drug product
ELISPOT	Enzyme-linked immunosorbent spot
FACS	Fluorescence-activated cell sorting
Foxp3	Forkhead box protein P3
G-CSF	Granulocyte colony-stimulating factor
GVHD	Graft-versus-host disease
GVL	Graft versus leukemia
HCT	Hematopoietic cell transplantation
HLA	Human leukocyte antigen
HSCs	Hematopoietic stem cells
HSPCs	Hematopoietic stem and progenitor cells
IL-2	Interleukin 2
IFN- γ	Interferon-gamma
IV	Intravenously
Lin ⁻	Absence of lineage markers
mAb	Monoclonal antibody
MAC-allot	Myeloablative conditioning-allogeneic HCT
MCMV	Murine cytomegalovirus
MEI	Multiple Electrolytes Injection
MHC	Major histocompatibility complex
MLRs	Mixed lymphocyte reactions
MOA	Mechanism of action
(b) (4)	
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PFU	Plaque-forming units
qPCR	Quantitative polymerase chain reaction
Rh-123	Rhodamine 123
Sca-1	Stem cell antigen-1
SCID	Severe combined immunodeficiency
TCD-BM	T cell-depleted BM
TCR	T cell receptor

TGF- β	Transforming growth factor β
Tcons	Conventional T cells
Tregs	Regulatory T cells
USP	United States Pharmacopeia
w/v	Weight per volume

Related File:

IND #18873: Allogeneic Hematopoietic Stem and Progenitor Cells, Tregs, Conventional T Cells (Orca-T [Formally Treg Graft]); Prevention of Moderate to Severe Chronic GVHD and/or Survival in Adult Patients with Acute Leukemia and Myelodysplastic Syndrome; Orca Biosystems Inc.; Active.

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INTRODUCTION

Hematologic malignancies are cancers originating in blood-forming tissues, including the BM, lymphatic system, and blood cells^{1,2}. These malignancies result from uncontrolled proliferation of abnormal hematopoietic or lymphoid cells, disrupting normal blood cell production and immune function. They primarily localize in the BM, peripheral blood, and secondary lymphoid organs and are broadly categorized into three main groups: leukemias, lymphomas, and multiple myelomas. Each category represents various heterogeneous disease groups comprising numerous distinct biological entities^{3,4}. In the United States (US), an estimated 192,070 combined cases of leukemia, lymphoma, or multiple myeloma are expected to be diagnosed in 2025, with 56,110 deaths anticipated⁵.

¹ Pulte D, Jansen L, Brenner H, et al. Changes in long term survival after diagnosis with common hematologic malignancies in the early 21st century. *Blood Cancer J* 2020; 10:56

² Wesson W, Galate VL, Sborov DW, et al. Characteristics of clinical trials for haematological malignancies from 2015 to 2020: a systematic review. *Eur J Cancer* 2022; 167:152-160

³ Arber DA, Orazi A, Hasserjian R, et al. The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. *Blood* 2016; 127:2391-2405

⁴ Swerdlow SH, Campo E, Pileri SA, et al. The 2016 revision of the World Health Organization classification of lymphoid neoplasms. *Blood* 2016; 127:2375-2390

⁵ Siegel RL, Kratzer TL, Kratzer TB, et al. Cancer statistics, 2025. *CA Cancer J Clin* 2025; 75:10-45

MAC-allogeneic hematopoietic cell transplantation (MAC-alloHCT) is a standard treatment option for various high-risk hematologic malignancies in the US⁶. However, its therapeutic utility remains limited by potentially lethal complications. MAC-alloHCT is curative in only some patients, with 5-year survival rates ranging from 20% to 60%, depending on donor type, disease type, and disease stage. Leading causes of death following MAC-alloHCT from HLA-matched donors include relapse (29% to 57%), opportunistic infection (11% to 19%), GVHD (8% to 10%), and organ failure (8% to 19%)⁷. Despite advances in transplantation techniques and supportive care, severe GVHD and treatment-related mortality after standard MAC-alloHCT approaches remain an unmet medical need⁸.

TREGZI consists of allogeneic HSPCs, Tregs, and Tcons derived from G-CSF-mobilized peripheral blood apheresis collected from an 8/8 HLA-matched donor (related or unrelated) for each patient. Based on cited nonclinical studies and a phase 1/2 clinical study with a similar clinical product consisting of HLA matched CD34-selected HSPCs, Tregs, and Tcons⁹, it is hypothesized that TREGZI will reduce GVHD while preserving GVL effects and immune reconstitution in adult patients with hematologic malignancies¹⁰.

Each patient will receive a MAC regimen according to institutional practice prior to IV administration of TREGZI¹¹. The MAC regimen is utilized in alloHCT to maximize disease control, provide adequate immunosuppression to prevent graft rejection, and create marrow “space” for a new healthy blood system to replace cancer cells. TREGZI is IV administered following the MAC regimen. The HSPC DP (dose level: $\geq 1.0 \times 10^6$ viable HSPCs/kg per patient), immediately followed by Treg DP (dose level range: $(b)(4) \times 10^6$ to 3.5×10^6 viable Tregs/kg per patient) are IV administered on day 0, followed by Tcon DP admixed with Tcon diluent DP administration (dose level range: $(b)(4) \times 10^6$ to 6.9×10^6 viable Tcons/kg per patient) on day +2 to day +3 (48 to 72 hours after HSPC DP and Treg DP administrations).

⁶ Bejanyan N, Zhang N, Bo-Subait K, et al. Myeloablative conditioning for allogeneic transplantation results in superior disease-free survival for acute myeloid leukemia and myelodysplastic syndromes with low/intermediate, but not high disease risk index: A CIBMTR Study. *Transplant Cell Ther* 2021; 27:68. e1-68. e9

⁷ D'Souza A, Fretham C. Current Uses and Outcomes of Hematopoietic Cell Transplantation (HCT): CIBMTR Summary Slides, 2018. Available at <https://www.cibmtr.org>. Accessed 19 October 2019

⁸ Styczynski J, Tridello G, Koster L, et al. Death after hematopoietic stem cell transplantation: changes over calendar year time, infections and associated factors. *Bone Marrow Transplant* 2020; 5:126-136

⁹ Meyer EH, Laport G, Xie BJ, et al. Transplantation of donor grafts with defined ratio of conventional and regulatory T cells in HLA-matched recipients. *JCI Insight* 2019; 4: e127244

¹⁰ Meyer EH, Hoeg R, Moroz A, et al. Orca-T, a precision Treg-engineered donor product, prevents acute GVHD with less immunosuppression in an early multicenter experience with myeloablative HLA-matched transplants. *Blood* 2020; 36(Supplement 1):47-48.

¹¹ Hoeg RT, Moroz A, Gandhi A, et al. Orca-T results in high GVHD-free and relapse-free survival following myeloablative conditioning for hematologic malignancies: results of a single center Phase 2 and a multicenter Phase 1b study. *Blood* 2021; 138 (Supplement 1):98

NONCLINICAL STUDIES**PHARMACOLOGY STUDIES****Summary List of Pharmacology Studies⁷**

No formal nonclinical pharmacology, pharmacokinetic (distribution), or toxicology studies were conducted for TREGZI. Instead, in Module 4 of the BLA, the applicant submitted 45 publications delineating the MOAs of HSCs (mouse and human), Tregs (mouse), Tcons (mouse), and their combinations (mouse). Additionally, the applicant submitted one study report (#OBS-24-001) that included a summary of 18 independent nonclinical studies evaluating the onset and severity of GVHD and survival of sub-lethally irradiated immunodeficient mice following IV administration of human Tcons alone or human Tcons with human Tregs.

In Vitro and In Vivo MOA Studies

Study Number	Publication Citation
1	Spangrude GJ, Heimfeld S, Weissman IL. Purification and characterization of mouse hematopoietic stem cells. Science 1988; 241:58-62
2	Baum CM, Weissman IL and Tsukamoto AS, et al. Isolation of a candidate human hematopoietic stem-cell population. Proc. Natl. Acad. Sci. USA 1992; 89:2804-2808
3	Korngold R and Sprent J. Lethal graft-versus-host disease after bone marrow transplantation across minor histocompatibility barriers in mice. Prevention by removing mature T cells from marrow. J Exp Med 1978; 148:1687-1698
4	Thornton AM and Shevach EM. CD4 ⁺ CD25 ⁺ Immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. J Exp Med 1998; 188:287-296
5	Fontenot JD, Gavin MA and Rudensky AY. Foxp3 programs the development and function of CD4 ⁺ CD25 ⁺ regulatory T cells. Nat. Immunol 2003; 4:330–336
6	Liu W, Amy L Putnam AL, Zhou Xu-Yu, et al. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4 ⁺ T reg cells. J Exp Med 2006; 203:1701-1711
7	Hoffmann P, Ermann J, Edinger M, et al. Donor-type CD4 ⁺ CD25 ⁺ regulatory T cells suppress lethal acute graft-versus-host disease after allogeneic bone marrow transplantation. J Exp Med 2002; 196:389-399
8	Edinger M, Hoffmann P, Joerg 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
9	Nguyen VH, Zeiser R, DaSilva DL, et al. In vivo dynamics of regulatory T-cell trafficking and survival predict effective strategies to control graft-versus-host disease following allogeneic transplantation. Blood 2007; 109:2649-2656

Study Number	Publication Citation
10	Nguyen VH, Shashidhar S, Chang DS, et al. The impact of regulatory T cells on T-cell immunity following hematopoietic cell transplantation. Blood 2008; 111:945-953
11	Lin KL, Fulton LM, Berginski M, et al. Intravital imaging of donor allogeneic effector and regulatory T cells with host dendritic cells during GVHD. Blood 2014; 123:1604-1614

In Vivo MOA Studies

Study Number	Study Title	Report Number
12	Activity of Orca-T in a Xenogeneic Murine Model of GVHD	OBS-24-001

Reviewer's Notes⁸:

- The 11 key publications were selected for inclusion in this memo because the reported studies delineated the MOAs of HSCs (mouse and human), Tregs (mouse), Tcons (mouse), and their combinations (mouse). The study report #OBS-24-001 contains multiple POC studies evaluating the ability of human Tcons to induce xenogeneic GVHD and the ability of Tregs co-administered with Tcons to mitigate Tcon-induced xenogeneic GVHD in sub-lethally irradiated immunodeficient mice. These are detailed in the “Overview of Nonclinical Pharmacology Studies” section below.
- The terms and phenotypes for HSPCs, Tregs, and Tcons vary across the cited publications and the study report #OBS-24-001 compared to those used for TREGZI. Therefore, this review memo retains the specific terms and phenotypes as used in each original source.

Overview of Nonclinical Pharmacology Studies

Study #1

Spangrude GJ, Heimfeld S and Weissman IL. Purification and characterization of mouse hematopoietic stem cells. Science 1988; 241:58-62

Objective: To isolate mouse HSCs from mouse BM using cell-surface differentiation antigens in vitro, and to evaluate their pluripotent differentiation capacity in vivo.

Methods

- BM cells were harvested from femurs of C57BL/Ka-Thy-1.1 mice and purified using immunomagnetic bead enrichment and fluorescence-activated cell sorting (FACS) for the following cell surface markers: low expression of Thy-1 (Thy-1^{lo}), absence of lineage markers (Lin⁻) including B220, Mac-1, Gr-1, CD4, and CD8, and positive expression of stem cell antigen-1 (Sca-1⁺).

- BM and purified stem cells were IV administered to irradiated mice to evaluate pluripotent proliferation and differentiation capacity using the following functional assays:
 - Spleen Colony Formation Unit (CFU-S) Activity Assays: BM or purified Thy-1^{lo}Lin⁻Sca-1⁺ and Thy-1^{lo}Lin⁻Sca-1⁻ cells were administered to lethally irradiated mice (900 rads). Spleen colonies were counted for their numbers at 12 days or microscopically assessed for myeloerythroid differentiation potential at 8, 9 and 12 days following a single IV administration.
 - Thymic Colony Formation Unit (CFU-T) Activity Assays: HSCs were administered to sub-lethally irradiated congenic mice (700 rads). Thymic colonies were assessed for T-cell lineage potential using two color FACS analysis four weeks after intra-thymic transfer.
 - Multiple Hematolymphoid Lineage Reconstitutions: Forty Thy-1^{lo}Lin⁻Sca-1⁺ cells (C57BL/6-Ly-5.2) were administered into lethally irradiated (900 rads) Ly-5 congenic mice (C57BL/Ka, Ly-5.1) along with 200 host-derived stem cells. Phenotype of donor peripheral blood cells from congenic mice were determined by two-color FACS analysis at six weeks following a single IV administration.
 - Radioprotection Assays: Groups of 10 to 20 mice were lethally irradiated (900 rads) and reconstituted IV with specified numbers of Thy-1^{lo}Lin⁻Sca-1⁺ cells. Percent survival was determined to assess the ability of these cells to rescue irradiated mice.

Results

- CFU-S were enriched 1000-fold in Thy-1^{lo}Lin⁻Sca-1⁺ cells compared to one CFU-S per ten whole BM control cells at Day 12 following administration.
- Thy-1^{lo}Lin⁻Sca-1⁺ cells contained more primitive precursors, as the ratio of day 12 to day 8 CFU-S was 10. Thy-1^{lo}Lin⁻Sca-1⁻ cells contained primarily differentiated (or committed) progenitors, as the ratio of day 12 to day 8 CFU-S is 0.7.

Reviewer's Notes¹²:

- Day-8 CFU-S mostly represents committed progenitors, while Day-12 CFU-S represents primitive progenitors. Day-12 CFU-S best correlates with true pluripotent HSC activity.
- Committed progenitors are late-stage and lineage-restricted hematopoietic cells with the following characteristics:
 - Limited or no self-renewal capacity
 - Restricted differentiation potential (uni- or oligopotential)
 - Short-term reconstitution ability only
 - Actively cycling

¹² Molineux G, Schofield R, Testa NG. Development of spleen CFU-S colonies from day 8 to day 11: relationship to self-renewal capacity. *Exp. Hematol* 1986. 14, 710-713

- Express lineage-specific markers
- Primitive progenitors are early-stage hematopoietic cells with multipotent or pluripotent differentiation capacity. Their characteristics include:
 - High self-renewal capacity
 - Multipotent or pluripotent differentiation potential
 - Long-term reconstitution ability (>16 weeks in mice)
 - Quiescent or slow cycling (only ~3.9% in S/G2/M phases)
 - Express stem cell markers
- Approximately one in five Thy-1^{lo}Lin⁻Sca-1⁺ cells administered could establish CFU-T, in contrast, Thy-1^{lo}Lin⁻Sca-1⁻ cells failed to establish CFU-T despite containing CFU-S activity.
- The Thy-1^{lo}Lin⁻Sca-1⁺ population, but not the Thy-1^{lo}Lin⁻Sca-1⁻ population, is capable of self-renewal and differentiation into all major blood cell lineages with near-unit efficiency.
- Forty Thy-1^{lo}Lin⁻Sca-1⁺ cells were able to reconstitute all major hematolymphoid lineages, including 60% of T cells, 50% of B cells, and 50% of neutrophils. Thirty Thy-1^{lo}Lin⁻Sca-1⁺ cells rescued 50% of lethally irradiated mice. In contrast, administration of 900 Thy-1^{lo}Lin⁻Sca-1⁻ cells resulted in death of mice due to lack of long-term reconstitution of HSCs.

Conclusions

The purification method based on Thy-1^{lo}Lin⁻Sca-1⁺ cell phenotype resulted in the isolation of a near-pure population of multipotent HSCs that show functional equivalence to pluripotent HSCs. These studies provided a novel approach for isolating and evaluating the biological function, development and MOA of HSCs that was included into the rationale for TREGZI development.

Reviewer's Comments:

- The publication noted the Thy-1^{lo}Lin⁻Sca-1⁻ population includes a significant number of cells capable of forming CFU-S. The observed CFU-S activity in the Thy-1^{lo}Lin⁻Sca-1⁻ population was related to either stem cell activity or primitive myeloid erythroid progenitors because the markers used to select the Lin⁻ population included only a subset of the myelomonocytic class (Mac-1 and Gr-1), and excluded markers of the erythroid, megakaryocytic, NK, mast cells, or eosinophil lineages that are derived from HSCs.
- While the study provides functional evidence for self-renewal through long-term reconstitution, it lacks direct serial transplantation data, which is an accepted standard for definitive evidence of HSC self-renewal capacity¹³.

Study #2

¹³Wilkinson AC, Igarashi KJ and Nakauchi H. Hematopoietic stem cell self-renewal in vivo and ex vivo. Nature Reviews Genetics 2020; 21:541-554.

Baum CM, Weissman IL and Tsukamoto AS, et al. Isolation of a candidate human hematopoietic stem-cell population. *Proc. Natl. Acad. Sci. USA* 1992; 89:2804-2808

Objective: To identify and isolate a human HSC population with multipotent differentiation capacity from heterogeneous CD34⁺ populations.

Methods

- Human fetal BM cells were subjected to FACS-based sorting of different cell subpopulations based on the expression of CD34, Thy-1, lineage markers, and rhodamine 123 (Rh-123) dye retention to identify subpopulations with HSC activity using specific mAbs.
- Sorted CD34⁺ stem cell subpopulations were evaluated for: 1) the ability to establish long-term cultures (>100 progeny) and multilineage (e.g., myeloid and B-lymphoid) hematopoiesis *in vitro* using mouse stromal cell-dependent coculture assays; and 2) the ability to engraft and differentiate within human fetal bone cylindrical sections (18-24 weeks) and human fetal thymus fragments (20 weeks of gestation) transplanted in severe combined immunodeficiency (SCID)-hu mouse model (SCID-Hu mice).

Reviewer's Notes:

- Rh-123 is a fluorescent mitochondrial dye used to identify HSCs in sorted cell subpopulations. Cells were stained with Rh-123 and sorted into Rh-123^{lo} and Rh-123^{hi} subpopulations, with the dye uptake reflecting mitochondrial content and cellular metabolic state. This Rh-123^{lo} subpopulation is enriched for self-renewal and long-term reconstitution potential.
- The publication states that cells stained with Rh-123 were sorted into Rh-123^{lo} (30%) and Rh-123^{hi} (30%) subpopulations. This notation means that the lowest 30% of Rh-123 staining was designated as Rh-123^{lo} and the highest 30% of Rh-123 staining was designated as Rh-123^{hi}. The middle 40% of cells with intermediate staining were excluded from analysis to create clearly distinct subpopulations.

Results

- Separate CD34^{+/−}, Thy-1^{+/−}, Lin^{+/−} or Rh-123^{lo/hi} subpopulations from total human fetal BM cells were enriched to assess potential pluripotent progenitor activities, including Thy-1⁺CD34⁺ (0.1-0.5%), Thy-1⁺Lin[−] (0.1-0.5%), Thy-1⁺CD34⁺Rh-123^{lo} (0.03-0.15%), Lin[−]CD34⁺ (0.1-0.5%), Thy-1⁺Lin[−]CD34⁺ (0.05-0.1%), Lin⁺CD34⁺ (2-10%), CD34⁺ (2-10%) cells.

Reviewer's Notes:

- The CD34⁺ population was heterogeneous. Therefore, use of CD34, together with or without other HSC or cell-lineage markers can identify the HSCs or mature blood-cell subpopulations (e.g., CD33 or CD13 for myeloid cells and CD10 or CD19 for B lymphoid cells).
- Cells were stained with Rh-123 and sorted into Rh-123^{lo} and Rh-123^{hi} subpopulations, with the dye uptake reflecting mitochondrial content and cellular metabolic state. Rh-123^{lo} enrichment is used to assess self-renewal and long-term reconstitution potential.

- Separate sorted subpopulations of cells (described above) demonstrated varying frequencies of long-term culture capacity and multilineage differentiation potential upon co-culture with confluent mouse stromal cells.
 - Nearly all long-term culture-initiating cells resided within the Thy-1⁺CD34⁺Rh-123^{lo} subpopulation.
 - The highest frequencies of long-term culture initiating cells were observed in Thy-1⁺CD34⁺ (1/20), Thy-1⁺Lin⁻ (1/21), Thy-1⁺CD34⁺Rh-123^{lo} (1/25), Lin⁻CD34⁺ (1/27), and Thy-1⁺Lin⁻CD34⁺ (1/44) subpopulations.
 - Lower frequencies were detected in Lin⁺CD34⁺ (1/80), CD34⁺ (1/200), Thy-1⁻CD34⁺ (1/323), and Thy-1⁻Lin⁻CD34⁺ (1/768) subpopulations. The lowest frequency was found in CD34⁻ subpopulation (>1/50,000), compared to that of whole BM cells (>1/3000).
 - No culture capacity was detected in Thy-1⁺Lin⁺ (0/288) or Thy-1⁺CD34⁺Rh-123^{hi} (0/1483) subpopulations.
- In vivo experiments showed that Thy-1⁺CD34⁺ subpopulation exhibited robust multilineage hematopoietic differentiation, generating both B-lymphoid cells (expressing CD10 and CD19 markers) and myeloid cells (expressing CD15 and CD33 markers) when 10⁴ donor Thy-1⁺CD34⁺ cells were injected into human fetal bone segments transplanted in SCID-hu mice. In contrast, the Thy-1⁻CD34⁺ subpopulation did not exhibit hematopoietic multilineage differentiation potential.
- In vivo thymic reconstitution experiment demonstrated that a single injection of Thy-1⁺CD34⁺ subpopulation into the human fetal thymus fragments transplanted in SCID-hu mice, resulted in chimerism in 11 of 16 (69%) grafts at 4-6 weeks post-injection, with donor T cells persisting beyond 2 months in multiple grafts. By comparison, the Thy-1⁻CD34⁺ subpopulation demonstrated substantially reduced thymic reconstitution potential, generating positive grafts in 5 of 16 (31%) grafts at 4–6 weeks post-injection. Only one graft showed minimal long-term donor cell persistence.

Conclusions

Thy-1⁺CD34⁺ human fetal BM subpopulation was enriched for multipotent hematopoietic progenitors with a capacity for long-term engraftment and thymic and BM reconstitution in vivo.

Reviewer's Comment:

- The Thy-1⁺CD34⁺, Thy-1⁺Lin⁻, Thy-1⁺CD34⁺Rh-123^{lo}, Lin⁻CD34⁺ and Thy-1⁺Lin⁻CD34⁺ subpopulations initiated long-term cultures on confluent mouse stromal cells at significantly higher frequencies than CD34⁺ and Thy⁻CD34⁺ subpopulations. These data suggest that Thy-1⁺CD34⁺, Thy-1⁺CD34⁺Rh-123^{lo}, Lin⁻CD34⁺ and Thy-1⁺Lin⁻CD34⁺ subpopulations are enriched for HSCs. However, the publication only compared the ability of donor Thy-1⁺CD34⁺ and Thy⁻CD34⁺ subpopulations to engraft and differentiate into B-lymphoid and myeloid lineages in human fetal bone sections or to

reconstitute T cells in thymus fragments of SCID-hu mice. Therefore, based on the results of the publication, the in vivo capabilities of donor Thy-1⁺Lin⁻, Thy-1⁺CD34⁺Rh-123^{lo}, Lin⁻CD34⁺ and Thy-1⁺Lin⁻CD34⁺ subpopulations, for long-term engraftment and thymic and BM reconstitution compared to CD34⁺ subpopulations remain unclear.

Study #3

Korngold R and Sprent J. Lethal graft-versus-host disease after bone marrow transplantation across minor histocompatibility barriers in mice. Prevention by removing mature T cells from marrow. J Exp Med 1978; 148:1687-98

Objective: To investigate the mechanism of GVHD pathogenesis following allogeneic BMT in mice.

Methods

- B10.BR (donor)→CBA/J [H-2k] (Recipient) Mice (Allogeneic) and Reciprocal Transplantation: Lethally irradiated (750 rads), major histocompatibility complex (MHC)-identical (H-2^k) but minor histocompatibility antigen-mismatched mice served as recipients of BMT. Allogeneic donor BM cells were obtained from mouse femurs and tibias and were either unfractionated or depleted of mature T cells using anti-Thy-1.2 antiserum and complement.
- 10⁷ viable allogeneic BM cells per mouse were IV administered to recipients and mice were evaluated for 80 days or until death for mortality, clinical features of GVHD, chimerism and immunocompetence. The threshold for GVHD induction was also assessed by adding varying numbers of purified allogeneic T cells back into TCD-BM cells from the same donor mice and evaluating the resulting impact.
- Direct (IgM) and Indirect (IgG) Anti-sheep Erythrocytes (SRC) Plaque-Forming Cell (PFC) Assays: SRCs were used as the antigen. This method was detailed in a referenced publication¹⁴ as follows:
 - Stage 1: T Cell Activation: 5 × 10⁷ nylon-wool-purified T cells (>90% Thy 1.2-positive) from lymph nodes of unprimed (CBA × B6) F1 mice were administered IV with 0.5 ml of 25% SRC into intermediate host mice that has received irradiation (800 rads) the previous day.
 - Stage 2: Helper T Cell Collection: Five days post-administration, thoracic duct fistulas were inserted in the cell recipients. Thoracic duct lymphocytes (TDL) were collected overnight. TDL were >95% thy 1.2-positive cells of donor origin.

¹⁴ Sprent J. Restricted helper function of F1 hybrid T cells positively selected to heterologous erythrocytes in irradiated parental strain mice. II. Evidence for restrictions affecting helper cell induction and T-B collaboration, both mapping to the K-end of the H-2 complex. 1978; 147:1159-74.

- Stage 3: T-B Collaboration Assay: The activated T cells were administered IV with (i) B cells ($5-10 \times 10^6$ viable, anti-Thy 1.2-serum-treated spleen or TDL from SRC-primed mice), and (ii) 0.1 ml of 5% SRC into irradiated (750 rads) (CBA $\times$ B6) F1 recipient mice.
- Stage 4: Measurement: Direct (IgM) and indirect (IgG) anti-SRC PFC were measured in recipient spleens 7 days later.

Reviewer's Note:

- Thy 1.2-positive cells in mice are predominantly T lymphocytes. In this study, they represented helper T cells that recognized antigen in an MHC-restricted manner, became activated through interaction with microphages, provided essential help for Ab production by B cells, and demonstrated genetic restriction mapping to the H-2 complex. The use of Thy 1.2 as a marker was essential for purifying T cells and tracking donor cells throughout the complex multi-stage experimental system.

Results

- Unfractionated allogeneic BMT from donor mice caused lethal cGVHD in greater than 80% of irradiated recipients, with mortality occurring between days 20-80 post-transplantation. Additionally, reconstitution of TCD-BM with as few as 3×10^4 purified allogeneic T cells (representing 0.3% contamination of the BM inoculum) from the same donor was sufficient to induce nearly 80% mortality by day 80.
- TCD-BMT completely prevented GVHD in recipient mice. Recipients remained healthy for extended periods (>12 months in some groups) and demonstrated donor chimerism, with 95% of spleen cells being of donor origin. Additionally, these chimeric recipients maintained normal cellularity in peripheral and mesenteric lymph nodes (approximately 10^8 cells/mouse), normal proportions of T cells (approximately 50% Thy-1.2-positive), and robust primary Ab responses to SRC comparable to syngeneic mice controls.

Conclusions

GVHD following allogeneic BMT in mice was mediated primarily by mature donor T cells contaminating the BM inoculum rather than by the progeny of transplanted allogeneic donor stem cells. Allogeneic TCD-BMT can prevent GVHD while preserving capacity for *in vivo* engraftment and immune reconstitution.

Study #4

Thornton AM and Ethan M. Shevach EM. CD4⁺CD25⁺ Immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. J Exp Med 1998; 188: 287-196

Objective: To establish an *in vitro* model system that recapitulates the *in vivo* suppressive function of CD4⁺CD25⁺ T cells and to use this model to analyze the MOA by which these cells prevent conventional T cell activation and autoimmune responses.

Methods

- Cell Purification and Phenotypic Characterization of CD4⁺CD25⁺ Cells: Lymph nodes (axillary, inguinal, superficial cervical, mandibular, and mesenteric) were harvested from 8-10-week-old female mice. Single-cell suspensions were enriched for T cells, followed by FACS-based sorting to obtain CD4⁺CD25⁺ (Tregs) and CD4⁺CD25⁻ T cells (responder T cells). Purified CD4⁺CD25⁺ T cells were phenotypically characterized by flow cytometry.
- Functional Assays to Characterize CD4⁺CD25⁺ T Cells:
 - Proliferation Assays: The proliferative potential of CD4⁺CD25⁻ and CD4⁺CD25⁺ T cells was assessed with or without Transwell inserts using various stimulation conditions, including irradiated TCD-spleen cells as accessory cells (ACs), anti-CD3 Ab/ACs, plate-bound anti-CD3 Ab, anti-CD3 Ab/ACs/anti-CD28 Ab, IL-2, anti-CD3 Ab/ACs/IL-2, phorbol 12-myristate 13-acetate (PMA)/ionomycin, and PMA/IL-2.
 - Transwell Assays: Performed in 24-well plates with CD4⁺CD25⁻ cells, AC, and 0.5 µg/ml anti-CD3 in the presence or absence of CD4⁺CD25⁺ cells in the Transwell insert.
 - Cytokine Gene Expression: Expression of cytokine genes (IL-2, IL-4, IL-10, Fas ligand, and tumor necrosis factor-α) in unstimulated or anti-CD3/AC-stimulated CD4⁺CD25⁻ and CD4⁺CD25⁺ T cells were quantified by reverse transcription polymerase chain reaction (RT-PCR).
 - Cytokine Neutralization Studies: Effects of cytokines on CD4⁺CD25⁺ T cell suppressive activity were quantitated by adding neutralizing Abs (anti-IL-2, -IL-4, -IFN-γ, IL-10, -transforming growth factor [TGF]-β and -IL10/TGF-β Abs) to cocultures of CD4⁺CD25⁺ and anti-CD3/AC-stimulated CD4⁺CD25⁻ T cells, compared to cocultures without antibodies.
 - IL-2 Quantification: IL-2 protein levels produced by anti-CD3-activated CD4⁺CD25⁻ T cells in the presence or absence of CD4⁺CD25⁺ T cells were measured by an enzyme-linked immunosorbent assay. IL-2 mRNA levels were quantified by Northern blot analysis.
 - Cytokine-Deficient Mice Studies: CD4⁺CD25⁺ T cells from IL-4^{-/-} and IL-10^{-/-} mice, supplemented with IL-2 or anti-CD28 Ab, were used to define the requirements for CD4⁺CD25⁺ cells-mediated suppression and its mechanism.
 - Induced CD25 Expression: CD4⁺CD25⁻ cells were cultured with AC and Con A to induce CD25 expression, generating induced CD4⁺CD25⁺ cells. Both induced or wild-type (WT) CD4⁺CD25⁺ cells were then co-incubated with 0.5 µg/ml anti-CD3 and AC before mixing with freshly isolated CD4⁺CD25⁻ cells. The suppressive activity of induced or WT CD4⁺CD25⁺ cells was assessed by measuring CD4⁺CD25⁻ cell proliferation.

Results

- CD4⁺CD25⁺ T cells represented 5-8% of the total lymph node cells or 10-15 % of CD4⁺ T cells.
- Purified CD4⁺CD25⁺ T cells failed to proliferate in response to T cell receptor (TCR)-derived signals (anti-CD3/AC) with or without costimulation (anti-CD28) or IL-2 alone.
- RT-PCR analysis showed IL-2 mRNA was not detected in unstimulated or anti-CD3/AC-stimulated CD4⁺CD25⁺ cells but was readily detectable in stimulated CD4⁺CD25⁻ cells.
- Addition of neutralizing Abs to known suppressive cytokines (IL-2, IL-4, IFN- γ , IL-10, or TGF- β), alone or in combination (anti-IL-10/TGF- β), failed to abrogate CD4⁺CD25⁺ T cells suppressive activity.
- When cocultured with CD4⁺CD25⁻ T cells, CD4⁺CD25⁺ T cells significantly suppressed CD4⁺CD25⁻ T cell proliferation by specifically inhibiting IL-2 production in CD4⁺CD25⁻ T cells at the transcription level. This inhibition:
 - Was dependent on direct cell contact between CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells, as demonstrated by loss of suppressive activity when cells were physically separated using a Transwell system.
 - Could be overcome by addition of exogenous IL-2 or anti-CD28 mAb to CD4⁺CD25⁻ T cells.
 - Required TCR-mediated activation of both CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells.
- IL-4 and IL-10 were not required for CD4⁺CD25⁺ T cells-mediated suppressive activity, as CD4⁺CD25⁺ cells from IL-4^{-/-} and IL-10^{-/-} donors were as effective as WT cells in mediating suppression *in vitro*.
- Induction of CD25 expression on CD4⁺CD25⁻ T cells *in vitro* or *in vivo* did not generate suppressive activity of induced CD4⁺CD25⁻ T cells, compared with WT CD4⁺CD25⁺ T cells.

Conclusions

CD4⁺CD25⁺ T cells represent a distinct lineage of immunoregulatory T cells that suppress polyclonal T cell activation through a cell contact-dependent mechanism that inhibits IL-2 production in responder CD4⁺CD25⁻ T cells. Recent data highlight the importance of suppressor cells in mediating transplantation tolerance and preventing autoimmune disease induction. The role of CD4⁺CD25⁺ T cells in suppressing CD4⁺CD25⁻ T cell activation and proliferation is important in maintaining peripheral self-tolerance.

Reviewer's Comment:

- The effect of the MAC regimen on IL-2 levels in patients prior to TREGZI administration was not evaluated in this study. Because exogenous IL-2 or anti-CD28 mAb addition to CD4⁺CD25⁻ T cells can inhibit CD4⁺CD25⁺ T cell suppressive activity,

increased IL-2 production following the MAC regimen may inhibit Treg suppressive activity, increase CD4⁺CD25⁻ T cell proliferation and expansion, and enhance GVHD severity

Study #5

Fontenot JD, Gavin MA, and Rudensky AY. Foxp3 programs the development and function of CD4⁺CD25⁺ regulatory T cells. Nat Immunol 2003; 4: 330-336

Objective: To investigate the role of the Forkhead transcription factor, Foxp3, in the development and function of CD4⁺CD25⁺ regulatory T cells (Tregs).

Methods

- Foxp3 gene and protein expression was analyzed in freshly purified resting and activated mouse Tregs and CD4⁺CD25⁻ T cells (Tcons) using real-time quantitative PCR (qPCR) and Western blot analysis.

Reviewer's Note:

- Based on this publication, CD4⁺CD25⁺ regulatory T cells are designated as Tregs and CD4⁺CD25⁻ T cells as Tcons to simplify terminology for these two cell populations.
- The role of Foxp3 in Treg development and function was determined by evaluating total number by flow cytometry, proliferation and suppressive function by (³H) thymidine incorporation, and associated disease development by clinical observation and pathological examination following administration of TCD-BM, Tregs, or Tcons in Foxp3-null (Foxp3⁻; male), Foxp3-mutant scurfy (Foxp3^{sf}), C57BL/6 (B6), B6 congenic, mixed BM chimeric B6 Ty1.1⁺ and B6 Ly5.1⁺, and neonatal (one- to two-day-old male Ly5.2 B6 Foxp3^{sf}) mice.
 - Mixed BM Chimeric Mice: Lethally irradiated B6 Thy1.1⁺ congenic mice were reconstituted with TCD-BM from congenic B6 Ly5.1⁺ donor mice mixed at a 1:1 ratio with BM from either Foxp3⁻ or Foxp3⁺ mice. Six weeks after BM reconstitution, mixed BM chimeras were analyzed to determine Treg development and function.
 - Neonatal Transfer: One- to two-day-old male Ly5.2⁺ B6 Foxp3^{sf} mice (derived from breeding female B6 Foxp3^{+/sf} carriers with B6 males) received intraperitoneal (IP) administration of WT Ly5.1⁺B6 Tregs or Tcons (4 × 10⁵ cell per mouse). Recipient mice were monitored for lymphoproliferative syndrome and analyzed at day 21 of age. Donor cell recovery was calculated based on total lymphocytes recovered multiplied by the percentage of Ly5.1⁺ cells determined by flow cytometry.
- Peripheral Tcons transduced with retroviral vector expressing Foxp3 were used to determine whether Foxp3 confers suppressive function. Purified Tcons, transduced with retroviral vector expressing either Foxp3 (MigR1[Foxp3]) or empty vector (MigR1[empty]), were IV injected into the tail vein of 8-week-old RAG1^{-/-} recipient mice. Recipient mice were monitored for wasting disease and colitis development. At 15 days post-injection, transduced cells from lymph node and spleen from recipient mice were isolated and sorted by FACS. The mRNA

levels of indicated genes (Foxp3, CD25, TGF- β , IL-2 and IL-10) from retrovirally transduced cells were analyzed, compared with mRNA levels from purified WT Tcons and Tregs.

Results

- Tregs expressed high levels of Foxp3 protein, which increased upon activation. Tregs showed ~40-fold higher Foxp3 mRNA expression compared to Tcons. In vitro activation of Tcons did not upregulate Foxp3 mRNA.
- Selective deletion of FoxP3 in male mice (Foxp3⁻) resulted in a fatal lymphoproliferative autoimmune syndrome matching that was observed in Foxp3^{sf} mice.
 - Clinical signs emerged around day 12, including growth retardation and scaly, inflamed skin on the ears and tail. Mice reached moribund state at approximately 4 weeks of age. Pathological findings included severely enlarged spleens and lymph nodes, along with lymphocytic infiltrates of multiple organs. Foxp3⁻CD4⁺ T cells exhibited an activated phenotype compared to normal littermate controls (Foxp3⁺).
 - Flow cytometry analysis of lymph node cells from 28-day-old Foxp3⁻ versus Foxp3⁺ mice revealed a CD4⁺ T cell population with a wide range of CD25 expression. Tregs from Foxp3⁻ mice proliferated and showed no suppressive activity. In contrast, Tregs from Foxp3⁺ mice were hyporesponsive to TCR stimulation and suppressed the proliferation of normal Tcons.
 - Compared to ten-day-old Foxp3⁺ mice, Foxp3⁻ mice showed no external signs of disease but exhibited slightly enlarged lymph nodes. Foxp3⁻ mice revealed a diminished Treg population in both the lymph node and thymus (0.4% and 0.6%, respectively, in Foxp3⁻ mice, compared with 3.0% and 3.8% in Foxp3⁺ mice). These data support a role for Foxp3 in the development of CD4⁺CD25⁺ T cells.
- Six weeks after BMT, the mixed chimeric mice appeared healthy with no signs of disease. In chimeric mice containing Foxp3⁻ BM, all Tregs came from the normal Ly5.1⁺B6 donor cells in both the thymus and lymph nodes. In chimeric mice with normal Foxp3⁺ BM, both donor sources contributed equally to the Treg population. This demonstrated that Foxp3 is essential for regulatory T cell development.
- All B6 Foxp3^{sf} neonate males receiving an IV injection of Tcons developed clinical and pathological disease signs with identical kinetics to untreated B6 Foxp3^{sf} controls. Conversely, B6 Foxp3^{sf} neonate males receiving an IV injection of Tregs remained virtually disease-free as determined by gross phenotype and lymph node cellularity. These findings demonstrate that autoimmune disease in Foxp3⁻ mice results from the absence of Tregs and that Tregs can suppress the pathogenic T cells driving disease.

Retroviral-mediated ectopic expression of Foxp3 in purified peripheral Tcons (CD4⁺CD25⁻ T cells) conferred a suppressive phenotype (CD4⁺CD25⁻ to CD4⁺CD25⁺ T cells) and function, prevented wasting disease and colitis mediated by Tcons, and upregulated IL-10 expression by transduced CD4⁺CD25⁻ T cells in RAG1^{-/-} recipient mice.

Conclusions

Foxp3 is specifically expressed in Tregs and required for their development and function. Autoimmunity in Foxp3⁻ mice resulted from the absence of Tregs rather than intrinsic hyperactivity of Tcons. These findings established Foxp3 as a master regulator of Treg lineage commitment and provided a genetic basis for immune tolerance mechanisms.

Study #6

Liu W, Amy L Putnam AL, Zhou Xu-Yu, et al. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4⁺ T reg cells. J Exp Med 2006; 203:1701-1711

Objective: To identify novel cell surface biomarkers for selectively isolating human CD4⁺CD25⁺ regulatory T cells (Tregs) from human peripheral blood mononuclear cells (PBMCs).

Methods

- PBMCs were obtained from 10 healthy subjects (age range: 20-25 years, mean age: 29 years) and 16 patients with type 1 diabetes (T1D) (age range 16-56 years, mean age: 34 years, disease duration > 5 years). Samples were analyzed through flow cytometry to detect FoxP3 expression in different CD4⁺ T cell subsets and sorted to obtain CD4⁺ T cell subsets for flow cytometry and functional studies.
- To identify additional cell surface markers associated with Treg phenotype and function, microarray analysis was performed comparing mRNA expressed by CD4⁺CD25^{hi} T cells with CD4⁺CD25⁻ T cells isolated from three healthy donor PBMC samples. The cRNA was prepared from three donors' mRNA and tested on Affymetrix U133A GeneChips.
- mRNA isolated from three independent CD4⁺CD25^{hi} and CD4⁺CD25⁻ T cell preparations was analyzed by quantitative qPCR.
- Different T cell subsets, defined based on expression of CD4, CD25, and the interleukin-7 receptor α chain (CD127), were evaluated in vitro for: 1) FoxP3 expression by intracellular staining in flow cytometry-based assays, and 2) regulatory/suppressive function using in vitro mixed lymphocyte reaction (MLR) assays.
- Chromatin immunoprecipitation (ChIP) of FoxP3-bound genomic DNA from CD4⁺CD25^{high} Tregs, followed by microarray hybridization (ChIP-chip) of the immunoprecipitated DNA, were performed to examine FoxP3 binding to the CD127 promoter.

Results

- Flow cytometric analysis showed that the majority of human CD4⁺CD25^{hi} cells (top 2% of gate) were FoxP3⁺ (range: 84.5%-96.8% in three individuals); however, considerable numbers of FoxP3⁺ cells were CD25⁻ or even negative. Analysis of FoxP3 expression in the CD4⁺CD25⁻ T cell subset showed that <5% of the CD4⁺CD25⁻ T cells expressed FoxP3.
- FoxP3 bound directly to the CD127 gene promoter, as demonstrated by ChIP-chip and ChIP-qPCR analyses, supporting transcriptional repression as the mechanism for reduced CD127 expression in Tregs.

- The combination of CD4, CD25 and CD127 cell surface markers identified a substantially larger Treg population than previously recognized using CD25 alone. On average, 86.6% of CD4⁺CD25⁺CD127^{lo/-} cells were FoxP3⁺ (range: 67.4–93.6%) and highly suppressive in functional assays. In contrast, few FoxP3⁺ T cells were found in the CD4⁺CD127⁺ subset (2.5%; range 1.2%-5.0%) except for a small percentage among those that expressed CD25 (22.9%; 11.5%-39.2%).
- Microarray analysis revealed that among the genes differentially expressed between CD4⁺CD25^{hi} and CD4⁺CD25⁻ T cells, CD127 mRNA expression was inversely correlated with CD25 expression. CD127 mRNA was expressed 3.14-fold lower (range: 2.26–4.21-fold) in CD4⁺CD25^{hi} T cells compared to CD4⁺CD25⁻ T cells.
- As predicted by the gene expression studies, flow cytometry analysis showed that the majority of CD4⁺CD25⁺ cells, especially the CD4⁺CD25^{hi} T cells had low or absent CD127 expression. In contrast, the majority of the CD4⁺CD25⁻ T cells were CD127 bright (73.8%).
- CD4⁺CD127^{lo/-} T cells, independent of CD25 level expression, were functionally anergic (unresponsive to TCR stimulation) and effectively suppressed allogeneic T cell proliferation in MLR assays, with suppressive capacity comparable to or greater than that of conventional CD4⁺CD25^{hi} Tregs.
- The frequency of FoxP3⁺ T cells in various subsets was not significantly different between healthy subjects and patients with T1D. For example, CD4⁺CD25⁺CD127^{lo/-} cells from T1D patients had a mean of 66.1% FoxP3⁺ cells (SD=11.3%; range 53.7–82.8%) versus 65.5% in healthy controls (SD=8.66%; range 45.4–76.2%).

Conclusions

Low CD127 expression identified a functionally suppressive population of human Tregs enriched for FoxP3 expression. CD127 is a better cell surface marker compared to CD25 to identify CD4⁺FoxP3⁺ T cells, improving the identification, isolation, and quantification of human Tregs. The combination of CD4, CD25, and CD127 enables isolation of a highly purified, functionally suppressive Treg population that accounts for 7-8% of CD4⁺ T cells, significantly more than previously identified using CD25 alone.

Reviewer's Comment:

- This study demonstrated that the majority of CD4⁺CD25⁺CD127^{lo/-} cells are FoxP3⁺ and exhibit potent suppressive function in MLR assays. These findings support the rationale for manufacturing of Treg DP under this BLA.

Study #7

Hoffmann P, Ermann J, Edinger M, et al. Donor-type CD4⁺CD25⁺ regulatory T cells suppress lethal acute graft-versus-host disease after allogeneic bone marrow transplantation. J Exp Med 2002; 196:389-399

Objective: To determine whether freshly isolated, unmanipulated CD4⁺CD25⁺ regulatory T cells (Tregs) from allogeneic donor mice can prevent lethal acute GVHD (aGVHD) in a complete MHC class I and II mismatched recipient mice following BMT.

Methods

- Cell Isolation and In Vitro Suppression Assays: Tregs and CD4⁺CD25⁺ T cells (responder T cells) were freshly isolated from spleens and BM of donor B6 mice via FACS-based sorting. Irradiated TCD-splenocytes from BALB/c recipient mice, served as stimulator cells. Suppressive function of donor Tregs was assessed in vitro using MLR assays by quantifying proliferative response of responder T cells cultured for 5 days with stimulator cells (responder T cells and stimulator cells: 1 x 10⁵ cells, each) in the presence or absence of Tregs at various ratios.

Reviewer's Note:

- This publication uses male B6 (H-2^b or H-2K^b) WT and male B6.129P2-*Il10^{tm1Cgn}* (B6.IL-10^{-/-}) mice as the donor strains and male BALB/c (H-2K^d or H-2^d) WT mice as the recipient strains, representing a complete allogeneic MHC class I and II mismatch.
- In Vivo GVHD Study: Donor BM cells were isolated from femur and tibia of C57BL/6 mice and depleted of T cells to obtain TCD-BM cells. BALB/c recipients received lethal total body irradiation (TBI; 800 rads) and were co-transplanted IV with 2 × 10⁶ donor TCD-BM cells together with 4.5 × 10⁵ donor responder T cells, with or without donor Tregs at ratios of 1:10 or 1:1 within 24 hours after lethal TBI. Recipient survival, body weight, clinical signs of GVHD were monitored for 100 days. Histopathological examination of GVHD target organs (skin, liver, large intestine) was performed at days 49 and 100 post-transplantation.
- Role of IL-10 and Cellular Origin of Tregs: The role of IL-10 was investigated by comparing donor-type WT B6 versus donor-type B6 IL-10^{-/-} Tregs for protection against lethal GVHD in irradiated recipients. The requirement for donor versus host origin of Tregs was assessed by co-transplanting donor responder T cells with either donor-type (B6) or host-type (BALB/c) Tregs into irradiated BALB/c recipients.

Results

- Freshly isolated donor Tregs demonstrated dose-dependent suppression of allo-responses of responder T cells in vitro, achieving >90% inhibition in MLRs at a 1:1 ratio of Tregs to responder T cells (P = 0.005) and >50% inhibition at a 1:4 ratio (P = 0.03).
- All recipients of TCD-BM plus responder T cells developed lethal aGVHD and died within 29 days. Co-administration of TCD-BM with donor Tregs and responder T cells at a 1:1 ratio provided significant protection from lethal aGVHD, with 93% recipients surviving at 100 days compared to 0% survival in control mice receiving donor TCD-BM and responder T cells only (P < 0.0001). Surviving animals showed complete donor chimerism and minimal to no histopathological signs of GVHD by day 100. At a ratio of 1:10, which approximates the physiological ratio of Tregs to responder T cells in normal B6 spleens, no protective effect of Tregs were observed. Recipients administered donor TCD-BM alone showed 100% survival without GVHD.

- Tregs were highly enriched in donor BM, comprising ~41% of TCR $\alpha\beta^+$ NK1.1 $^-$ CD4 $^+$ T cells compared to ~13% in spleen. BM-derived Tregs shared phenotypic markers (CD44 $^{\text{high}}$, CD45RB $^{\text{low}}$, intracellular CTLA-4 $^+$, and bimodal CD62L expression) and functional suppressive capacity with splenic Tregs both in vitro and in vivo. BM-derived Tregs significantly protected recipients from lethal GVHD induced by peripheral blood (PB)-derived responder T cells (83% survival vs. 12% in controls, $P = 0.019$).
- Tregs from IL-10 $^{-/-}$ donors suppressed allo-responses in vitro comparably to WT Tregs (>90% inhibition at 1:1 ratio). However, in vivo, IL-10 $^{-/-}$ Tregs conferred significantly reduced protection from lethal aGVHD compared to WT Tregs, with only 40% of recipients surviving beyond 60 days with continued clinical signs of aGVHD, compared to 100% survival with WT Tregs ($P < 0.0001$). This indicates that protection against GVHD is partially dependent on IL-10 in vivo.
- In MLR assays, only donor-origin Tregs suppressed allo-responses (>95% inhibition), whereas host-origin Tregs failed to suppress and slightly enhanced proliferation. In vivo, donor-type (B6) Tregs protected 100% of recipients from lethal aGVHD ($P < 0.0001$ vs. controls), whereas host-type (BALB/c) Tregs failed to prevent aGVHD, with 90% of recipients dying within 78 days ($P = 0.28$ vs. control mice receiving only responder T cells).

Conclusions

Freshly isolated, unmanipulated donor Tregs were sufficient to prevent lethal aGVHD after allogeneic BMT from a complete MHC mismatched donor. This protective effect was dependent on the following: 1) a sufficient ratio of Tregs to CD4 $^+$ CD25 $^-$ T cells, 2) donor origin of the Tregs, and 3) IL-10 production by Tregs (partially dependent). These findings demonstrated that the proportion of donor Tregs relative to conventional CD4 $^+$ CD25 $^-$ T cells determined the severity and outcome of aGVHD.

Study #8

Edinger M, Hoffmann P, Joerg 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

Objective: To determine whether donor CD4 $^+$ CD25 $^+$ regulatory T cells (Tregs) can suppress GVHD while preserving the GVL effects of donor CD4 $^+$ CD25 $^-$ and CD8 $^+$ CD25 $^-$ T cells (Tcons) following allogeneic BMT.

Methods

- Using fully MHC-mismatched murine BMT models, lethally irradiated (800 rads) BALB/c recipients received B6 donor cells (5×10^6 TCD-BM cells with or without 5×10^5 Tcons: - CD4 $^+$ CD25 $^-$ and CD8 $^+$ CD25 $^-$ T cells at a physiological 2:1 ratio used as responder T cells, and 5×10^5 Tregs at a 1:1 ratio with Tcons). GVHD severity (appearance and body weight) and survival of recipients were assessed in vivo.

- The Tcons and Tregs were isolated from spleens of B6 donor mice. Alloreactive T-cell proliferation of Tcons (1×10^5 cells), was evaluated in vitro using MLRs: Tcons (1×10^5 cells) and irradiated allogeneic T cell-depleted BALB/c splenocytes (1×10^5 cells) mixed with Tregs (4:1 and 1:1 Tcons: Tregs ratios), or in vivo via bioluminescence imaging (BLI) of luciferase-expressing donor Tcons and flow cytometric quantification of donor T cells in lymphoid organs and GVHD target tissues of recipient mice on day 5 post-transplantation.
- In vivo GVL activity of Tcons was examined in luciferase-expressing A20 leukemia bearing mice (1×10^4 cells injected at time of TCD-BMT) and BCL1 lymphoma-bearing mice (2×10^3 tumor cells injected 9 days before irradiation and TCD-BMT) following IV administrations of donor cells (TCD-BMT alone or combined with the addition of Tcons alone, Tregs alone or Tcons plus Tregs at a 1:1 ratio [1×10^5 cells each]). Perforin- and Fas ligand-deficient donor Tcons were also used to evaluate the cytotoxic activity of Tcons.

Results

- After TCD-BM transplantation with 5×10^5 donor Tcons alone, all recipients died from aGVHD within 30 days. Co-transplantation of donor Tregs with donor Tcons at a 1:1 ratio provided significant protection from lethal aGVHD, with more than 70% of recipients surviving beyond 75 days compared with 0% survival in mice receiving donor Tcons alone ($P < 0.0001$).
- The administration of donor Tregs and Tcons suppressed early expansion of alloreactive donor T cells in *in vivo*, reducing donor $CD4^+$ and $CD8^+$ T-cell recovery by 64-76% in spleen, mesenteric lymph nodes, and liver in recipient mice compared to administration of Tcons alone on day 5 post-transplantation. Additionally, BLI showed that the administration of donor Tregs and Tcons resulted in a 93% reduction in luciferase-expressing donor effector T-cell expansion by day 7 in recipients compared to administration of Tcons alone.
- Despite suppression of Tcon proliferation, Treg co-administration did not impair cytotoxic function. In A20 leukemia and BCL1 lymphoma models, 67-90% of animals receiving both 5×10^5 Tcons and 5×10^5 Tregs survived long-term without tumor relapse, whereas animals receiving Tregs alone showed no GVL activity and died from tumor progression.
- Tcons from perforin-deficient donor mice failed to eradicate A20 leukemia in Treg-protected recipient mice, whereas Tcons from Fas ligand-deficient donors showed delayed but incomplete tumor control.

Conclusions

Donor Tregs effectively suppressed lethal GVHD while preserving GVL activity of Tcons in allogeneic BMT through limiting early donor T-cell expansion, without abrogating cytotoxic effector function. The GVL effects mediated by Tcon occurred primarily through perforin-dependent cytotoxicity, with a secondary contribution from Fas-FasL interactions. These findings support the therapeutic use of Tregs to improve allogeneic BMT outcomes without compromising anti-tumor immunity.

Study #9

Nguyen VH, Zeiser R, DaSilva DL, et al. In vivo dynamics of regulatory T-cell trafficking and survival predict effective strategies to control graft-versus-host disease following allogeneic transplantation. Blood 2007; 109:2649-2656

Objective: To monitor the *in vivo* trafficking, expansion, tissue localization, and persistence of donor Tregs following donor TCD-BMT, and to determine how these parameters inform the timing of administration and dose level of T regs to prevent GVHD while preserving GVL activity.

Methods

- FVB donor mice (H-2^d) constitutively expressing luciferase were used as Tcon and Treg donors.
- Major-MHC mismatched Balb/c mice (H-2^d) were used as recipients to assess survival and GVHD.
- *luc/ypf* A20 leukemia was administered to Balb/C recipients to assess GVL.
- Recipient mice received one of the following dosing regimens: Lethal irradiation (800 rads) only, lethal irradiation (800 rads) followed by donor TCD-BMT (5×10^6 cells), lethal irradiation (800 rads) followed by donor TCD-BMT (5×10^6 cells) with donor Tcons (1×10^6) on Day 0, and lethal irradiation (800 rads) by donor TCD-BMT with donor Tcons, and donor Tregs (1×10^5 to 1×10^6) administered at various time points (e.g., TCD-BMT with Tregs and Tcons on Day 0 or TCD-BMT with Tregs on Day 0 and Tcons on Day 2).
- Luciferase-expressing donor Tregs were noninvasively tracked over time *in vivo* using BLI.
- Donor Tcon and Treg proliferation, Treg persistence and distribution, GVHD-free survival, and GVL activity were assessed at several timepoints throughout the duration of the study.

Results

- Irradiated mice receiving TCD-BMT alone or irradiation only survived without GVHD for over 100 days, while those receiving Tcons on Day 0 had reduced median survival (40-45 days); however, co-administration of Tregs and Tcons (1:1 ratio) on Day 0 significantly reduced GVHD incidence, severity, and mortality.
- In a leukemia model, administering a 10-fold lower Treg dose (1×10^5 cells, 1:10 Treg:Tcon ratio) two days before Tcons preserved GVL activity while protecting against GVHD ($P=0.02$) and significantly improving survival compared to Tcons alone ($P=0.004$), with donor Tregs expanding rapidly in lymphoid organs within 24-48 hours and persisting up to 90 days in peripheral tissues.

Conclusions

Early donor Treg administration (before or at the onset of effector T-cell expansion) maximizes suppression of GVHD at low (near-physiologic) dose levels and preserves GVL effects.

Study #10

Nguyen VH, Shashidhar S, Chang DS, et al. The impact of regulatory T cells on T-cell immunity following hematopoietic cell transplantation. *Blood* 2008; 111:945-953

Objective: To determine whether administration of donor Tcons and Tregs after allogeneic HSC transplantation can preserve T-cell immune reconstitution and functional antiviral immunity in recipient mice while preventing GVHD.

Methods

- Mice (8 to 14 weeks old): WT and thymectomized Balb/c mice (H-2^d) served as recipients. FVB mice (H-2^q) and B6 mice (H-2^b) served as donors, representing complete allogeneic MHC class I and II mismatches.
- Thymectomy and Irradiation: Thymectomies were performed on Balb/c recipients at 4 weeks of age. Both WT and thymectomized Balb/c recipients were lethally irradiated with 800 rads TBI.
- Transplantation: Within 24 hours of irradiation, recipient mice received one of the following FVB donor cell regimens: TCD-BMT (5×10^6 cells) alone, TCD-BMT (5×10^6 cells) with Tcons (5×10^5 CD4⁺ and CD8⁺ T cells at a physiology ratio of 3:1), or TCD-BMT (5×10^6 cells) with Tcons (5×10^5 cells) plus Tregs (5×10^5 cells) at a 1:1 Treg:Tcon ratio.

Reviewer's Note:

- The donor Tcon dose level was determined through titration studies to establish a subacute GVHD model permitting long-term immune reconstitution studies.
- Monitoring: Mice were monitored for clinical GVHD development and weight changes throughout the study.
- Lymphoid Reconstitution: Peripheral blood lymphoid reconstitution was evaluated at days 14 and 40 post-transplantation, with complete blood counts and comprehensive lymphoid subset analysis performed at day 60.
- Donor Chimerism: Donor chimerism was confirmed by flow cytometry at days 30, 60, and 100 post-transplantation.
- TCR-V β Repertoire: TCR-V β repertoire diversity was assessed at day 30 by flow cytometry using mAbs against 15 different V β families and CDR3 size spectratyping analysis of 21 functional TCR-V β genes by RT-PCR.
- Histologic Analysis: Thymic and peripheral lymph node architecture was evaluated histologically using hematoxylin and eosin staining at days 30 and 60 post-transplantation. Splenic analysis included spleen-to-body weight ratios and cellular subset quantification.
- Murine Cytomegalovirus (MCMV) Infection: To assess functional antiviral immunity post-transplantation, groups of WT and thymectomized Balb/c recipients were lethally irradiated

and administered FVB donor cells as described in “Transplantation” above. Subsequently, these recipient mice were infected IP with lacZ-tagged MCMV (5×10^5 plaque-forming units [PFU]) at days 14, 30, or 63 post-transplantations. These time points represented incomplete, near complete, and complete lymphoid recovery, respectively.

- **Survival and Viral Load Monitoring:** Survival was monitored through day 100 post-transplantation. Viral loads were determined in kidneys, liver, lungs, and salivary glands by PFU assay on 3T3 cell monolayers.
- **Secondary Immune Responses:** Secondary immune responses were evaluated at day 100 using IFN- γ enzyme-linked immunosorbent spot (ELISPOT) assays with MCMV 1E1 peptide stimulation of sorted donor lymphocytes isolated from infected mice. Lymphocytes from uninfected mice were negative controls.
- **Thymectomy Model:** Thymectomized Balb/c mice were used to assess the contribution of thymic-derived versus extrathymic-derived T cells to immune reconstitution and anti-viral immunity.

Results

- Following transplantation, Tregs prevented Tcon-mediated GVHD-related mortality, achieving 90% survival versus 20% in Tcon-only recipients at day 100 post-transplantation ($P < 0.001$).
- Lymphoid reconstitution was significantly enhanced in Tcon plus Treg recipients, with increased lymphocyte subsets at day 14 ($P < 0.05$) and robust recovery of CD4⁺, CD8⁺, NK, and CD19⁺ cells by day 40, reaching levels comparable to controls. At day 60, Tcon plus Treg recipients maintained normal neutrophil counts and enhanced lymphocyte numbers, while Tcon-only recipients showed elevated neutrophils ($P = 0.004$) and persistent lymphopenia ($P = 0.014$).
- Histological analysis revealed preserved thymic cortical-medullary organization and normal lymph node architecture in Tcon plus Treg recipients, contrasting with thymic involution and lymph node hypoplasia in Tcon-only recipients.
- TCR-V β analysis at day 30 demonstrated diverse, polyclonal repertoires in Tcon plus Treg recipients versus oligoclonal profiles in Tcon recipients.
- MCMV infection at day 14 resulted in 50% survival in Tcon plus Treg recipients versus 0% in Tcon recipients ($P < 0.001$); day 30 infection yielded 67% versus 0% ($P < 0.001$); and day 63 infection produced 100% versus 40% survival ($P = 0.08$) post-transplantation.
- Viral clearance rates improved with later infection timing: on day 100 after HCT, Tcon plus Treg recipients achieved 100% viral-free survival following day 63 infection, compared to detectable viral levels in the kidneys or liver of 50% of Tcon plus Treg recipients following day 14 infection.

- ELISPOT analysis at day 100 confirmed robust antigen-specific memory responses (IFN- γ production) in Tcon plus Treg recipients infected with MCMV at day 14 and day 30 post-transplantation. In contrast, T cells from uninfected Tcon plus Treg recipients did not release IFN- γ .
- Thymectomized recipients showed reduced survival, higher viral loads, and lower antigen specific memory responses, confirming the critical role of thymic-derived T cells in long-term anti-MCMV immunity.
- Improved survival of Tcon plus Treg recipients resulted from better lymphoid reconstitution, as Tregs prevented GVHD-induced damage to lymphoid organs and accelerated donor lymphoid reconstitution with a diverse TCR-V β repertoire.

Conclusions

Administration of donor Tregs with Tcons at a 1:1 ratio enhanced immune reconstitution following TCD-BMT by preventing GVHD-induced damage of thymic cortical-medullary organization and normal lymph node architecture while accelerating donor lymphoid reconstitution with a diverse TCR-V β repertoire. The resultant enhanced lymphoid reconstitution in Treg and Tcon recipients protected them from lethal MCMV infection, as demonstrated by improved survival rates and viral clearance, and increased antigen specific memory responses following MCMV infection at three different time points post-transplantation.

Study #11

Lin KL, Fulton LM, Berginski M, et al. Intravital imaging of donor allogeneic effector and regulatory T cells with host dendritic cells during GVHD. Blood 2014; 123:1604-1614

Objective: To determine how allogeneic donor Tcons interact with recipient DCs in vivo during the early GVHD after allogeneic HSC transplantation (HSCT), and how Tregs modulate these Tcon-DC interactions.

Methods

- Generating Inducible Tregs (iTregs) In Vitro: CD4⁺ T cells were purified from murine B6 lymph nodes and spleens using negative selection by magnetic-activated cell sorting. CD4⁺CD25⁻ T cells were then cultured for 4 days in medium containing 100 U/ml of IL-2 and 10 ng/ml of TGF- β . iTreg Purity was greater than 85%.
- Transplantations and Intravital Imaging: CD11c-Diphtheria Toxin Receptor -Enhanced Green Fluorescent Protein mice (BALB/c background) or CD11c-YFP mice (B6 background) were lethally irradiated (800 or 950 rads) one day before transplantation.
 - Irradiated mice received donor TCD-BM cells ($4-5 \times 10^6$ cells), Tcons alone ($3-5 \times 10^6$ cells), in combination with the red fluorescent CellTracker CMTPX-labeled naive T cells and unlabeled naive T cells, Tcons with eTregs (3×10^6 Tcons plus 2×10^6 eTregs) or Tcons with iTregs (1:1 Tcon: iTreg ratio) from B6 mice.

Reviewer's Note:

- eTregs refer to natural Tregs isolated from donor mice.
- Different experimental groups received various combination of labeled Tcons, labeled Tregs, or labeled iTregs, with unlabeled control cells.
- At 2 to 8 hours and 18 to 24 hours post-transplantation, recipient mice were surgically exposed and imaged using multiphoton laser scanning microscopy (Olympus FV1000MPE). Tcon velocity, displacement ratio, and Tcon-DC contact duration were evaluated.

Reviewer's Note:

- Multiphoton laser scanning microscopy allowed visualization of donor Tcons (3×10^6 cells), Tcons with eTregs (2×10^6 eTregs administered at a 3:2 Tcon: eTreg ratio), Tcons with iTregs (administered at a 1:1 Tcon: iTreg ratio) interacting with recipient DCs, or Tcons with IL-10^{-/-} eTregs in lymph nodes during the first 2-24 hours after HSCT.
- Flow cytometry assessed DC surface activation/adhesion molecule expression and viability after transplantation.

Results

- Intravital imaging of popliteal lymph nodes showed that donor allogeneic Tcons established long-term interactions with recipient DCs as early as 2 hours after transplantation. The percentage of T cell-DC conjugates lasting > 30 minutes was modestly lower at 2 hours compared to later time points. These prolonged Tcon-DC interactions persisted for approximately 20-24 hours post-transplantation.
- Co-transplantation of unlabeled eTregs (2×10^6) with Tcons (3:2 Tcon: Treg ratio) significantly disrupted Tcon-DC interaction, and increased donor Tcon velocity by 6 to 7 hours post-transplantation compared to Tcons transferred alone ($P < 0.0001$). Less than 20% of Tcons contact with DCs for > 30 minutes with eTregs, compared to $\geq 30\%$ for 30 minutes without eTregs. At eight hours post-transplantation, approximately 30% of eTregs contacted host DCs for ≥ 30 minutes, decreasing to 15% by 21 hours.
- iTregs (1:1 Tcon:iTreg ratio) similarly reduced Tcon-DC contact time with more pronounced early effects compared to eTregs ($P < 0.0001$). However, at later timepoints, iTregs showed significantly lower velocity than eTregs ($P = 0.0348$, $P = 0.0401$, $P = 0.0127$), with reduced motility persisting beyond 21 hours post-transplantation.
- IL-10^{-/-} Tregs failed to disrupt donor Tcon-recipient DC interactions, indicating IL-10 is required for Treg-mediated interruption of stable contacts.
- Both eTregs and iTregs significantly downregulated expression of costimulatory molecule CD86 ($P = 0.0009$) and adhesion molecule CD54 ($P = 0.0111$) on recipient splenic DCs by day 2

post-transplantation and induced a 50% reduction in recipient DC numbers through increased DC death at days 1-2 post-transplantation.

- These data indicate Tregs use multiple mechanisms affecting recipient DC numbers and function to mitigate acute GVHD.

Conclusions

Allogeneic donor Tcons established prolonged contacts (≥ 30 minutes) with recipient DCs as early as two hours post-HSCT, persisting for approximately 20-24 hours. Tregs mitigated early GVHD responses through multiple complementary mechanisms: IL-10-dependent disruption of Tcon-DC interactions, downregulation of DC costimulatory (CD86) and adhesion (CD54) molecules, and promotion of recipient DC death at days 1 and 2 post-transplantation.

Study #12

Reviewer's Comments:

- Three PT information requests (IRs) #1-#3 were sent to the applicant to request additional information omitted from study report #OBS-24-001. The applicant provided information in response to these PT IRs, including study reports #RPT-0283 and #RPT-0289 that contributed to study report #OBS-24-001 (refer to Amendments #11, 18, and 23 of this BLA for details). Study #12 incorporated data from these two study reports to accurately analyze and interpret study results.
- In response to our PT IR #1, the applicant stated that the studies described in Module 4.2.1.1 (Study report #OBS-24-001) and summarized in Modules 2.4 and 2.6 were not intended to support the rationale for TREGZI or to examine its safety. Instead, these studies were conducted to elucidate the MOA of TREGZI, supporting the potency method described in Module 3.2.P.5.2.2 (Treg). Please refer to the CMC review memo for data requirements in support of this potency method. Data obtained from these studies demonstrated the biological activities and xenogeneic GVHD onset and severity in sub-lethally irradiated immunodeficient mice following administration of human donor Tcons alone or human donor Tcons/Tregs and these aspects of the study are summarized in Study #12.
- In response to our PT IR #2, the applicant identified two main differences between nonclinical product lots tested in study report #OBS-24-001. These nonclinical product lots consist of: (1) fresh (not the cryopreserved) human Tcons which expedited GVHD effects in mice by approximately 7 days; and (2) CD25-enriched Tregs that were used in four independent studies under Follow-on Study (G_02_1105, G_02_002027, G_02_0105 and G_01_0708), potentially reducing Treg potency as total CD4⁺/CD127^{-low} cells would be lower than the final clinical Treg DP. Refer to applicant's responses to our PT IR #2 for details.

Report Number		OBS-24-001 Reviewer's Note: ➤ Study report #OBS-24-001 summarized the study results from two separate study reports #RPT-0283 (Initial Study) and #RPT-0289 (Follow-on Study)		
Date Report Signed		August 9, 2024		
Title		Activity of Orca-T in a Xenogeneic Murine Model of GVHD		
Good Laboratory Practice Status		Not in compliance with good laboratory practice		
Testing Facility		Orca Biosystems Inc.; 3475 Edison Way, Suite B; Menlo Park, CA 94025		
Objectives		– To establish an <i>in vivo</i> GVHD bioassay, immunodeficient mice were humanized by transplanting human patient donor Tcons (detailed in Study report #RPT-0283). – To evaluate Treg function in a murine model of GVHD (detailed in Study report #RPT-0283). This assessment involves sub-lethally irradiated immunodeficient mice that were humanized by transplanting healthy human donor Tcons either alone or in combination with Tregs (detailed in Study report #RPT-0289)		
Study Animals	Strain/Breed	(b) (4)		
	Species	<i>Mus musculus</i>		
	Age	The average age was 13.5 weeks		
	Group Mean Body Weight	Group	Initial Study (grams/animal)	Follow-on Study (grams/animal)
		1	27.2	27
		2	27.2	27
		3	27.3	26.7
		4	27.1	26.8
		5	27.2	26.8
		6	–	26.8
7		–	27.1	
Numbers (No.)/Sex/Group	Group	Initial Study (Animals receiving lot #G. 02.1017)	Follow-on Study (Animals receiving lot #G.04.0105)	
		# of Females/ # of Males	# of Females/ # of Males	
	1	4/2	4/2	
	2	4/3	5/5	
	3	5/2	3/2	
	4	3/4	2/3	
	5	4/3	3/2	
	6	–	3/2	
7	–	2/8		
Reviewers' Note: ➤ This memo provides number of female and male mice per group only for groups that were administered lots #G_02_1017 (Tcons) and #G_040_0105 (Tcons alone, or in combination with Tregs). The information on the number of female and male mice per group was not available for some of the other lots. Study report #OBS-24-001 indicated group sizes ranged from 4-7 mice.				
Total #	> 500 mice across 18 independent studies			

Test Article(s)	– Tcons (Initial Study): - Lot #s: G_02_1017, G_02_1105, G_04_0105, G_02_0715 and G_02_0716. - Formulated with (b) (4) HSA in MEI or (b) (4) HSA in (b) (4) media. – Tcons with or without Tregs (Follow-on Study): - Lot #s: G-02_1105, G_04_0105, G_02_002027 and G_01_0708 - Formulated with (b) (4) HSA in MEI.																																															
Control Article(s)	Vehicle control: –Initial Study: (b) (4) HSA in MEI, or (b) (4) HSA in (b) (4) media –Follow-on Study: (b) (4) HSA in MEI																																															
Route of Administration	Retro-orbital IV administration																																															
Description of the Disease/Injury Model and Implant Procedure	– (b) (4) mice Reviewer's Comment: ➤ (b) (4) mice are severely immunodeficient due to three critical mutations that collectively eliminate adaptive and innate immune functions. (b) (4) (b) (4) (b) (4). This triple-mutant combination makes (b) (4) mice highly permissive for human cell engraftment, establishing them as an acceptable model for xenotransplantation studies. –Irradiation: Sub-lethally irradiated (b) (4) mice (200 rads on Day 0, prior to Tcon/Treg administration) were employed to enhance human cell engraftment.																																															
Randomization	Yes, based on body weight.																																															
Study Groups and Dose Levels	<table border="1"> <thead> <tr> <th rowspan="2">Group</th><th>Initial Study</th><th colspan="3">Follow-on Study</th></tr> <tr> <th>Tcons/mouse</th><th>Tcons/mouse</th><th>Tregs/mouse</th><th>Tcon: Treg ratio</th></tr> </thead> <tbody> <tr> <td>1</td><td>vehicle control</td><td>vehicle control</td><td>vehicle control</td><td></td></tr> <tr> <td>2</td><td>0.25 x 10⁶ cells</td><td>2 x 10⁶ cells</td><td>vehicle control</td><td></td></tr> <tr> <td>3</td><td>0.5 x 10⁶ cells</td><td>2 x 10⁶ cells</td><td>0.5 x 10⁶ cells</td><td>4:1</td></tr> <tr> <td>4</td><td>1 x 10⁶ cells</td><td>2 x 10⁶ cells</td><td>1 x 10⁶ cells</td><td>2:1</td></tr> <tr> <td>5</td><td>2 x 10⁶ cells</td><td>2 x 10⁶ cells</td><td>2 x 10⁶ cells</td><td>1:1</td></tr> <tr> <td>6</td><td>–</td><td>2 x 10⁶ cells</td><td>4 x 10⁶ cells</td><td>1:2</td></tr> <tr> <td>7</td><td>–</td><td>2 x 10⁶ cells</td><td>6 x 10⁶ cells</td><td>1:3</td></tr> </tbody> </table>				Group	Initial Study	Follow-on Study			Tcons/mouse	Tcons/mouse	Tregs/mouse	Tcon: Treg ratio	1	vehicle control	vehicle control	vehicle control		2	0.25 x 10 ⁶ cells	2 x 10 ⁶ cells	vehicle control		3	0.5 x 10 ⁶ cells	2 x 10 ⁶ cells	0.5 x 10 ⁶ cells	4:1	4	1 x 10 ⁶ cells	2 x 10 ⁶ cells	1 x 10 ⁶ cells	2:1	5	2 x 10 ⁶ cells	2 x 10 ⁶ cells	2 x 10 ⁶ cells	1:1	6	–	2 x 10 ⁶ cells	4 x 10 ⁶ cells	1:2	7	–	2 x 10 ⁶ cells	6 x 10 ⁶ cells	1:3
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5	2 x 10 ⁶ cells	2 x 10 ⁶ cells	2 x 10 ⁶ cells	1:1																																												
6	–	2 x 10 ⁶ cells	4 x 10 ⁶ cells	1:2																																												
7	–	2 x 10 ⁶ cells	6 x 10 ⁶ cells	1:3																																												
Dosing Regimen	Single administration of Tcons on Day 0 (Initial Study) or Tcons plus Tregs (Follow on Study) on Day 0 or Day 1																																															

Study Duration	- Initial Study: 127 days (lot #G_02_1017), 116 days (lot #G_02_1105), 111 days (lot #G_02_0715), 111 days (lot # G_02_0716) and 68 days (lot #G_04_0105) - Follow-on Study: 171 days (lot #G0_1_0708), 116 days (lot #G_02_1105), 92 days (lot #G_04_0105), and 68 days (lot #G_02_002027)
Scheduled Sacrifice Time Points	Reviewer's Note: Mice were euthanized at various time points. The study report did not specify the euthanasia procedure, or criteria and timepoints for most mice, except for mice that were euthanized for tissue collection (e.g., spleens). - For tissue collections: - Initial Study: - Spleen, liver, small and large intestines, and skin: Lot #G_04_0105: Groups 1 and 5 (Day 17 post-administration of vehicle control or Tcons) - Follow-on Study: - Spleen, liver, small and large intestines, and skin: Lot #G_04_0105: Groups 1, 2 and 7 (Day 17 post-administration of vehicle control, Tcons alone or Tcons combined with Tregs).

Reviewer's Comments:

- Study report #OBS-24-001 consists of summary data and results of 18 independent POC studies conducted by the applicant and submitted under Module 4 in this BLA under 'Initial Study' and 'Follow-on Study'. In this memo, based on review of all of the studies for quality and relevance, summaries of data and results from five independent studies were included under 'Initial Study' and four independent studies under 'Follow-on Study' were summarized in Study #12.
- The study report did not clearly specify how nonclinical dose levels were calculated, specifically whether the dose level was based on viable cell count or total cell count. Although the human Tregs and Tcons administered in the nonclinical studies were manufactured similarly to the intended clinical DP lots, this lack of clarity limits the ability to determine whether nonclinical dose levels (cells/mouse) reflect comparable biological activity to clinical Treg DP and Tcon DP dose levels in TREGZI. However, since these studies evaluated the ability of human Tcons to induce xenogeneic GVHD and the ability of Tregs co-administered with Tcons to mitigate Tcon-induced xenogeneic GVHD in sub-lethally irradiated immunodeficient mice, the data demonstrated the biological activities of Tregs and Tcons, as well as xenogeneic GVHD onset and severity in sub-lethally irradiated immunodeficient mice following administration of human donor Tcons alone or combined with human donor Tregs.

Key Evaluations and Assessments:

- Clinical xenogeneic GVHD manifestations (anemia, hunched posture, decreased mobility, and ruffled fur): Assessed daily

- Body weights: Assessed daily, and then every other day or every few days once GVHD was established.
- Survival: Assessed daily
- Mean spleen weights and histopathological analysis of liver, small and large intestines, spleen, and skin tissues to evaluate organ damage: Assessed at different time points based on prespecified days for tissue collection.

Reviewer's Note:

- The study report stated, “Due to donor-to-donor variability, studies characterizing organ damage and analysis of splenocytes were event-triggered at ~50% death of the Tcon-only cohorts (page 8). This reviewer interprets this to mean that animals from the control group and the 2×10^6 Tcon/mouse group were euthanized when half the animals in these groups (groups 1 and 5 in Initial Study and group 1, 2 and 7 in Follow-on Study) exhibited extreme weights loss and morbidity. This approach was implemented to characterize organ damage through gross and histopathological evaluations, as described in Study Reports #RPT-0283 (page 12) and #RPT-0289 (page 14).
- The applicant stated “A qualified pathologist was not used for evaluations and assessments were not masked. The evaluations were conducted by trained scientists at Orca Bioscience.” (refer to applicant’s response to our PT IR #2 for details). Therefore, although Study report #12 summarized the applicant’s pathological findings, the accuracy of gross and histopathological findings for the selected organs is unclear because these evaluations were not conducted by board-certified veterinary pathologists.

Results:

- Initial Study:
 - Dose-dependent relationship was observed between Tcon dose level and both GVHD onset and severity.
 - Groups receiving Tcons exhibited dose-dependent loss in mean body weight at the median survival timepoint, with the most severe weight loss (~ 30 % of loss in mean body weight from day 45 and thereafter) for two high dose level groups (1×10^6 Tcons/mouse and 2×10^6 Tcons).
 - Overall median survival duration exhibited Tcon dose-dependent decrease across all donor-derived lots.

○ Group 1 (vehicle control):	Undefined
○ Group 2 (0.25×10^6 Tcons):	66.75 days median survival
○ Group 3 (0.5×10^6 Tcons):	45 days median survival
○ Group 4 (1×10^6 Tcons):	28.5 days median survival
○ Group 5 (2×10^6 Tcons):	19 days median survival

- Spleen size and mean weight from animals receiving 2×10^6 Tcons/mouse were reduced compared to vehicle control animals, with mean weight reductions ranging from 1.64- to 3.4-fold (Lot #G_04_0105).
 - Histopathological examination revealed dose-dependent T cell infiltration in hepatic periportal regions and parenchyma, thickening of intestinal lamina propria, and atrophy of subcutaneous fat and hair follicles (Lot #G_04_0105).
- Follow-on Study:
- Tregs demonstrated dose-dependent inhibition of GVHD onset and severity. Groups receiving Tcons plus Tregs showed significant preservation of body weight compared to mice receiving Tcon-only with improved median survival ($P < 0.0001$), as following:
 - Group 1 (vehicle control): No body weight loss; undefined median survival
 - Group 2 (2×10^6 Tcons): ~ 30 % of body weight loss before day 20; 22.5 days median survival
 - Group 3 (2×10^6 Tcons/ 0.5×10^6 Tregs): ~ 30 % of body weight loss around day 20; 32.3 days median survival
 - Group 4 (2×10^6 Tcons/ 1×10^6 Tregs): ~ 30 % of body weight loss after day 20; 36.6 days median survival
 - Group 5 (2×10^6 Tcons/ 2×10^6 Tregs): ~ 30 % of body weight loss after day 45; 60.6 days median survival
 - Group 6 (2×10^6 Tcons/ 4×10^6 Tregs): <30% of body weight loss; 78.8 days median survival
 - Group 7 (2×10^6 Tcons/ 6×10^6 Tregs): <30% of body weight loss; 73.5 days median survival
 - Spleen mean weight from Group 2 mice were significantly reduced (2.15-fold) compared to Group 1 mice (Lot #G_04_0105). In contrast, mice in Tcon plus Treg groups showed preserved spleen weight:

○ Group 1 (vehicle control):	82.48 mg
○ Group 2 (Tcons):	38.4 mg (2.15-fold deduction)
○ Group 7 (Tcons and Tregs):	83.75 mg (1.02-fold increase)
 - Mice in Group 7 showed preserved spleen morphology, with significant reduction in CD8⁺ T cell infiltration ($p < 0.05$), indicating suppression of inflammatory xeroreactive CD8⁺ T cell expansion.
 - Histopathological examination exhibited decreased tissue damage in liver, intestine, and skin, with reduced T cell infiltration and better-preserved tissue architecture in liver, intestine, and skin in Group 7 mice receiving Tcons combined with Tregs compared to Group 2 mice receiving Tcons only (Lot #G_04_0105).

Reviewer's Comment:

- Data from the Follow-on Study demonstrated that co-administration of human Tcons (2×10^6 cells/mouse) with human Tregs (dose level range: 0 to 6×10^6 cells/mouse) delayed GVHD onset, reduced severity, and prolonged survival time in sub-lethally irradiated immunodeficient mice in a dose-dependent manner (median survival: 32.3 days at 2:1 Tcon:Treg ratio; 78.8 days at 1:2 ratio; and 73 days at 1:3 ratio). These data were communicated to the clinical reviewer (Ailian Xiong) for consideration regarding potential widening of Tcons DP and Tregs DP dose level ranges in TREGZI.

Conclusion:

The Initial Study established a reproducible murine GVHD model that demonstrated dose-dependent biological activity of human Tcon DP from multiple donors, with measurable outcomes including body weight loss, decreased survival, and histopathological changes that correlated with Tcon dose levels. The Follow-on Study demonstrated that Tregs co-administered with Tcons exhibited a clear dose-response relationship in GVHD prevention by delaying disease onset, prolonging survival, and reducing tissue damage through suppression of inflammatory CD8⁺ T cell responses. Together, these studies supported the therapeutic potential of Tregs in preventing GVHD following administration of human HSPC and Tcons.

Overview of Safety Pharmacology Studies

No nonclinical studies were conducted to evaluate safety pharmacology of TREGZI. Given the characteristics and biological activity of TREGZI, together with the clinical safety data for TREGZI, this omission is acceptable.

PHARMACOKINETIC STUDIES (Cell Distribution)

No nonclinical studies were conducted to evaluate cell distribution of TREGZI. Given the characteristics and biological activity of TREGZI, together with the clinical safety data for TREGZI, this omission is acceptable.

TOXICOLOGY STUDIES**Summary List of Toxicology Studies**

No toxicology studies were conducted for TREGZI. This omission is acceptable, as the applicant provided safety data from a Phase 1/2 clinical study with a similar clinical product to support initiation of the Phase 1b study with MAC-TREGZI transplantation in adult patients with leukemias or high/very high-risk myelodysplastic syndromes under parent IND #18773^{9,10}:

- Phase 1/2 clinical study with a similar clinical product (NCT01660607)⁹:
 - This clinical study included six patients with acute myeloid leukemia, chronic myelogenous leukemia, myelofibrosis, or acute lymphoblastic leukemia who received MAC prior to IV administration of HLA-matched CD34-selected HSPCs (dose level range: $3.6\text{--}15.9 \times 10^6$ CD34⁺ cells/kg) and CD4⁺CD25⁺CD127^{lo} Tregs (dose level range: $2.3\text{--}3.0 \times 10^6$ fresh Tregs/kg) on day 0, and CD3⁺ Tcons (dose level: 3.0×10^6 CD3⁺ Tcons/kg) on

day 2. Single-agent GVHD prophylaxis (sirolimus or tacrolimus) was initiated the day after Tcon administration.

- No development of acute or chronic GVHD was observed in any patient, nor were any reported dose-limiting toxicities, suggesting feasibility and safety of donor HSPCs administration combined with defined ratios of fresh Tregs to Tcons.
 - Dose level(s), dosing regimen, and route of administration used in this clinical trial are identical to the Phase 1b clinical studies with TREGZI.
- Additionally, safety data from the randomized Phase 3 clinical study with MAC- TREGZI (Precision-T) can be used to support the safety of TREGZI administered in adult patients with hematologic malignancies.

DART Studies:

No nonclinical studies were conducted to evaluate DART of TREGZI. Given the characteristics and biological activity TREGZI, together with the clinical safety data for TREGZI, this omission is acceptable.

Genotoxicity Studies:

No nonclinical studies were conducted to evaluate genotoxicity of TREGZI. Given the characteristics and biological activity of TREGZI, together with the clinical safety data for TREGZI, this omission is acceptable.

Carcinogenicity/Tumorigenicity Studies:

No nonclinical studies were conducted to evaluate carcinogenicity/tumorigenicity of TREGZI. Given the characteristics and biological activity of TREGZI, together with the clinical safety data for TREGZI, this omission is acceptable.

Other Safety/Toxicology Studies

No safety/toxicology studies were conducted to assess additional safety endpoints, such as immunotoxicity and local tolerance, for TREGZI. Given the characteristics and biological activity of TREGZI, together with the clinical safety data for TREGZI, this omission is acceptable.

KEY WORDS/TERMS: TREGZI, hematologic malignancies, HLA-matched donor, cell surface phenotypic markers, human CD34⁺(b) (4) cells (HSPC DP), human (b) (4) CD45⁺ cells (Tcon DP), Tcon diluent DP, human (b) (4) CD4⁺CD25⁺(b) (4) CD127^{-low} cells (Treg DP), allogeneic hematopoietic stem cell transplantation, mechanism of action, bone marrow, murine hematopoietic stem cells, murine CD4⁺CD25⁺ cells (murine Tregs), murine CD4⁺CD25⁻ cells (murine Tcons), FoxP3, pharmacology, toxicology, cell phenotypes, immune suppression, IL-2, IL-10, graft-versus-host disease, graft-versus-leukemia, body weight loss, organ damage, and median survival.

¹ If the application is a rolling submission, cite the dates that the P/T, CMC, and clinical modules were received.

² Cite the proposed product name and description stated in bold at the beginning of the draft Package Insert (PI).

³ Cite the proposed clinical indication stated under 'Indications and Usage' in the draft PI.

⁴ Provide a brief summary of the findings observed in the Pharmacology and Toxicology (including Pharmacokinetics) studies. The summary should reflect the potential text in Sections 8, 12, and 13 of the draft PI. In addition, all/some of this summary will be incorporated in the Summary Basis of Regulatory Approval (SBRA).

⁵ Provide 2-3 sentences describing any deficiencies identified, need for additional non-clinical data, and your conclusion regarding the data submitted.

⁶ Include a brief description of the product (a diagram can also be included) - tissue and/or donor source, final construct formulation to include solution/suspension buffer, matrix/scaffold, encapsulation device, etc. Include information stated under 'Dosage and Information' and 'Dosage Forms and Strengths' in the draft PI.

⁷ Provide a list of each pharmacology/proof-of-concept (POC) study conducted by the study sponsor or applicant and/or the studies cited from the scientific literature that are included in the application.

⁸ Briefly summarize the submitted relevant pharmacology/proof-of-concept (POC) studies conducted by the study sponsor or applicant and/or the studies cited from the scientific literature. The selected studies should help inform the text in Section 12.1 of the draft PI ['Mechanism of Action'].