Phase I trial of autologous regulatory T cells for immune aplastic anemia

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Abstract

Immune-mediated aplastic anemia (AA) is a bone marrow failure syndrome characterized by cytotoxic CD8-mediated autoimmune suppression of hematopoietic stem/progenitor cells resulting in varying degrees of peripheral blood cytopenias. Treatment with immunosuppressive therapy and hematopoietic stem cell transplantation are not applicable to all patients and effective responses occur in only a proportion of patients, highlighting the unmet need for alternative treatments. We have previously shown a reduction in the number and function of regulatory T cells (Treg) in AA patients and their functional restoration following *in vitro* expansion that laid the foundations for this phase I trial. Required numbers of Treg were collected from leukapheresis and expanded under Good Manufacturing Practice conditions from all six patients in the trial who were resistant/refractory to standard forms of treatment. The trial design included two doses of autologous Treg (5×10^6/kg) administered 2 weeks apart. Mass cytometry, single-cell sequencing and cytokine profiling were performed on blood samples collected at various timepoints. Treg were successfully expanded to required doses from all six patients with no adverse or immune-related events in any of the patients. Hematologic responses were observed in three patients. In addition, we were able to track the persistence of the expanded Treg *in vivo*, correlate clinical efficacy with the accumulation of clusters of post-infusion Treg, and identify phenotypic markers in the infusion product that correlate with *in vivo* expansion. Treg from AA patients are expandable, safe for infusion, and may help to modulate the abnormal immune milieu associated with AA, with induction of clinical response. (Clinical-Trials.gov identifier: NCT05386264; EudraCT number: 2021-000082-33).

Introduction

Immune-mediated reduction of bone marrow hematopoietic stem/progenitor cells (HSPC) in aplastic anemia (AA) leads to peripheral blood pancytopenia.[1,2] Immunosuppressive therapy with antithymocyte globulin and cyclosporine A ± eltrombopag or hematopoietic stem cell transplantation (HSCT) have been mainstays of treatment.[3-10] Immunosuppressive therapy leads to long-term hematologic responses in up to 70% of patients, however, it can be associated with significant morbidity, partial hematologic recovery and frequent relapses following its withdrawal.[11,12] HSCT is a curative option but is limited to eligible patients with suitable donors and its benefits are often offset by graft failure, early mortality from infections, graft-*versus*-host disease and other post-transplant complications. Thus, alternative therapeutic options are urgently needed.[13]

Immune dysregulation is pivotal in the pathogenesis of AA. Immune effector responses manifested by increases in $CD4^+$ Th1 cells, CD8 cytotoxic T cells, and Th17 cells, with secretion of tumor necrosis factor α (TNF-α) and interferon γ (IFN-γ), contribute to apoptosis of bone marrow progenitor cells in a FAS-L-dependent manner.[14-16] Reduction in regulatory T cells (Treg) and their *in vivo* functional abnormalities lead to an unrestricted inflammatory milieu in the bone marrow.[17] We have shown that Treg are reduced in AA

patients, but can be expanded *in vitro* in the presence of interleukin-2 (IL-2) (1,000 IU/mL) and rapamycin (100 nM) with functional restoration. The expanded Treg are ≥90% FOXP3$^+$, highly suppressive, and stable and they prevent graft-*versus*-host disease in an NSG mouse model.[18-20] These observations laid the foundation for the first in-human clinical trial of expanded autologous Treg in AA patients who are ineligible for or refractory to immunosuppressive therapy ± eltrombopag and allogeneic stem cell transplant. In this phase I trial we assessed the safety and efficacy of infused autologous expanded Treg from AA patients.

Methods

TIARA is a single institution phase I open-label study conducted at Kings College London and Kings College Hospital. The trial is approved by UK National Research Ethics Service (Reference 22/NE/0021), Medicines and Healthcare Products Regulatory Agency (MHRA) and registered with Clinical-Trials.gov identifier NCT05386264 and EudraCT number 2021-000082-33.

Six participants have been recruited as of 01/12/2024. Inclusion criteria were acquired idiopathic, non-severe, severe or very severe AA, lacking a matched related or unrelated donor, ineligible for HSCT, or failed or ineligible for immunosuppressive therapy with antithymocyte globulin + cyclosporine A ± eltrombopag. All six patients have received two doses of 5x10^6/kg of expanded autologous Treg 2 weeks apart (*Online Supplementary Figures S1* and *S2*). The primary endpoint was safety, which was evaluated as any adverse events. Secondary endpoints were response, as assessed by improvements in hematologic indices, and reduction in supportive care requirements.

Isolation and expansion of polyclonal regulatory T cells

In order to obtain the Treg, 2.2-2.9 (average 2.6) times the total body volume was processed to achieve an apheresis volume between 120-200 mL which was transferred to the Good Manufacturing Practice (GMP) facility at Guys Hospital. The leukapheresis product was subjected to volume reduction and platelets were removed using a Lovo® device (Fresenius Kabi). The cells were then incubated with CliniMACS® CD8 reagent from Miltenyi at room temperature for 30 minutes followed by depletion on a automated CliniMACS® Plus system (Miltenyi Biotec) followed by incubation of depleted cells with CliniMACS® CD25 reagent for 15 minutes at 4°C. The Treg were then purified again CliniMACS® Plus system. The isolated Treg were resuspended in TheraPEAK® X-VIVO® 15 (Lonza) media containing 5% AB serum (biowest®) followed by seeding in a suitably sized bioreactor depending on the cell count. The cell culture was stimulated with anti-CD3/CD28 coated beads (MACS GMP ExpAct Treg Kit, Miltenyi Biotec). Rapamycin (100 nM Rapamune©, Pfizer) and IL-2 (1,000 IU/mL; Proleukin© Novartis) were added to the cells alternatively. The cells were counted after every 7 days and, depending on the cell number, cells were restimulated again and fresh medium containing rapamycin and IL-2 was added. Cells could be expanded for a maximum of 30 days with a maximum of five stimulations, but the protocol included the option of early harvest if the cell dose was achieved earlier at either day 16 or 23.

Cell harvesting

Once the cell dose was achieved, the cells were resuspended in CliniMACS® buffer containing 0.5% human serum albumin (HSA; Biotest) and subjected to bead depletion using the CliniMACS® Plus system to remove the anti-CD3/CD28 beads added during stimulations. Cells were resuspended in Plasma-Lyte148 (Baxter) and 5% HSA pre-prepared freezing mixture (20% CryoSure-dimethylsulfoxide [WAK-Chemie] and 5% HSA) to the desired concentration (dose formulation/kg patient weight) of cells in a CryoMACS® (Miltenyi Biotec) freezing bag and then assessed for various release criteria specifications (*Online Supplementary Methods*).

Cytometry by time-of-flight

The cytometry by time-of-flight (CyTOF) data were acquired using a CyTOF-XT mass cytometer (Fluidigm) and normalized based on EQ6 beads (Y89, In115, Ce140, Tb159, Lu175 and Bi209). Data were processed and analyzed using Cytobank and FlowJo. Details are provided in the *Online Supplementary Methods*.

Single-cell sequencing

Single-cell analysis was performed using the BD Rhapsody™ Express Single-Cell Analysis System. Details are provided in the *Online Supplementary Methods*.

Response

The criteria for response in patients with severe AA and non-severe AA are as defined in the British Committee for Standards in Haematology guidelines.[21] As a standard, all patients are offered packed red cell transfusions when their hemoglobin is <80 g/L. Individual variations allow for patients to be transfused at a higher threshold (i.e. <90 g/L, in the case of respiratory or cardiac compromise, or significant symptoms from the effects of anemia). All patients adhered to the standard criteria for this transfusion threshold, except patient 4 who was transfused at <90 g/L. Pooled platelet transfusions are offered when the platelet count is <10x10^9/L, as a standard practice across all centers from which patients were recruited into this study. Higher thresholds are considered in the event of an active bleeding episode, or evidence of consumption (neutropenic fever, etc.). Platelet refractoriness is investigated, as per standard protocols, and when necessary and indicated HLA-matched platelets are administered.

Results

Patients

The clinical details of the six patients studied are listed in Table 1. The median age of the patients was 74 years (range, 67-79) and four of the participants were refractory to standard treatments. Single nucleotide polymorphism karyotypic analysis at baseline only showed del13q(12.3;22.2) in patient 1. Similarly, a myeloid gene panel consisting of 31 genes frequently mutated in AA and myeloid malignancies, performed at baseline only, was abnormal in patient 3 in that *DNMT3A* and *BCOR* mutations were present at a variant allele frequency of 12% and 6%, respectively.

Patients 1 and 6 were considered unfit for immunosuppressive therapy and opted for the clinical trial. The median follow-up is 261 days (105-554). Patient 1 died after 390 days (239-758) due to complications of severe AA and patient 3 went off trial and received an allogeneic HSCT from a voluntary unrelated donor 283 days after receiving both doses of Treg.

Cytokine analyses

Multiplex serum cytokine analyses were performed in all patients on days 0, 14 and 28. There were no significant difference between cytokine levels before and after the infusions (*Online Supplementary Methods*). Notably, patient 1 maintained high levels of IL-8 and IL-17α at all timepoints.[22]

In vitro expansion of autologous regulatory T cells

Leukapheresis was carried out without any conditioning

Table 1. Clinical characteristics of patients with aplastic anemia treated with expanded autologous T-regulatory cells.

Patient	Age, years, Sex	Disease severity	Previous treatment for aplastic anemia	Pre-Treg infusions	Post-Treg infusions	Response	Follow-up since first infusion of Treg, days
KCH-001	74F	Severe	No response to CsA, eltrombobag. Unsuitable for hATG	PRC transfusion every 2 weeks and platelet transfusion weekly	PRC and platelet transfusion, as before treatment with Treg	NR	239
KCH-002	74F	Non-severe	Sibling allogenic HSCT failed engraftment with CMV activation. Failed treatment with CsA and eltrombopag	PRC transfusion once every 4 weeks and platelet transfusion when required	Transfusion Independent and normalization of FBC. Infusion of second dose of expanded Treg	CR	758
KCH-003	71M	Very severe	Failed IST with 2 X hATG and CsA, eltrombopag	PRC transfusion weekly and platelet transfusion once-twice weekly	PRC weekly, platelet transfusion 3x/weekly, and prolonged hospitalization with N/IPA (neutrophils $0.1x10^9$/L throughout)	NR	283 (salvage, allogeneic HSCT from a volunteer unrelated donor)
KCH-004	79F	Non-severe	No response to erythropoietin, CsA, tacrolimus and eltrombopag. Unsuitable for hATG	PRC transfusion every week	Transfusion independent and complete normalization of blood counts	CR	737
KCH-005	67F	Severe	Loss of response to CsA, eltrombopag and tacrolimus. Patient declined hATG	PRC transfusion once every 2 or 3 weeks and platelet transfusion weekly	Weekly PRC and platelet transfusion	NR	253
KCH-006	76M	Severe	No response to CsA, eltrombopag. Abnormal liver function precluded use of eltrombopag. Unsuitable for hATG	PRC transfusion every week and platelet transfusion once every 2 weeks	Transfusion independent	NR*	497

*Patient 6 did not satisfy the criteria for complete or partial response in the RACE trial; however, he has remained transfusion independent since treatment with Treg cells. T-reg: regulatory T cells; F: female; CsA: cyclosporine A; hATG: horse antithymocyte globulin; PRC: packed red cells; NR: no response; HSCT: hematopoietic stem cell transplant; CMV: cytomegalovirus; FBC: full blood count; CR: complete response; M: male; IST: immunosuppressive therapy; N/IPA: neutropenic fever/invasive pulmonary aspergillosis.

and Treg were successfully expanded from all six patients from a starting median number of 92.43×10^6 cells (24.32×10^6 -181×10^6 cells) to 4.3×10^9 cells (2.5×10^9 -6.8×10^9 cells) between 16-23 days and frozen. The frozen expanded product was thawed prior to infusion and met all the release criteria specifications (Table 2).

Safety and adverse events

Both doses of Treg infusions were well tolerated without any adverse events, except for one patient who developed antibiotic-responsive fever (38°C) within 24 hours of the first infusion. The second dose of Treg was administered without any side effects. There was no evidence of cytokine release syndrome in any of the patients following infusion of either dose of Treg.

Hematologic response

The hematologic responses are shown in Figure 1. Improvements in hematologic parameters (partial response) were observed in three patients (patients 2, 4 and 6) after infusion of both doses of Treg. The criteria for response were the same as those used in the RACE trial protocol.[5] Patients 1, 3 and 5 did not show any improvements in blood counts and continued to require packed red cells and platelet transfusions. No correlation between response and presence of a paroxysmal nocturnal hemoglobinuria clone was ascertained, possibly due to the small cohort of patients.

Patient 2 became transfusion-independent with a gradual increase in platelet count from 10×10^9/L at baseline prior to the first infusion to 41×10^9/L at 12 months and hemoglobin levels rose from 91 g/L to 122 g/L. The absolute reticulocyte count increased from 17.1×10^9/L to 27.3×10^9/L at 6 months (Figure 1A). A subsequent increase in neutrophil and lymphocyte cell counts from 0.98×10^9/L to 2.07×10^9/L and 1.6×10^9/L to 2.83×10^9/L, respectively, was observed at 12 months.

Patient 4 had a platelet count that increased from 77×10^9/L to 139×10^9/L at day 28, then dropped to 41×10^9/L at 6 months after a viral infection, before rising to 96×10^9/L at day 263. Hemoglobin levels improved from 71 g/L to 84 g/L, sustained at day 263. The neutrophil count increased from 1.12×10^9/L to 1.86×10^9/L at day 28. Lymphocyte and absolute reticulocyte counts increased from 1.3×10^9/L to 1.9×10^9/L and 75×10^9/L to 104×10^9/L, respectively, at day 57, but later decreased after an infection (Figure 1B). The patient achieved 112+ days of red cell transfusion independence. Although transfusion dependence resumed, the frequency of transfusions of packed red blood cells decreased to every 8 weeks and platelet count remained above 100×10^9/L at the last follow-up.

Patient 6 showed a consistent reduction in transfusion requirements after the first 3 months following both infusions. The patient needed ten packed red cell units in the first 3 months which has reduced to needing only four units in the following 3 months. Patient 6 has been transfusion independent for >8 months, fulfilling the criteria for a partial response as defined above.

Patient 3 eventually underwent HSCT from a voluntary unrelated donor, after a full 10/10 HLA matched donor became available through a donor registry. The transplant was subsequent to the patient's participation in this study.

Mass cytometry immune monitoring

To characterize how the infusion of *in vitro* expanded Treg influenced the composition of peripheral blood CD4$^+$ T cells, mass cytometry analysis on peripheral blood mononuclear cells was carried out at baseline (day 0) and on days 14, 28, and 57 after infusion. We applied uniform manifold approximation and projection (UMAP) to visualize CD4$^+$ T cells across the different timepoints after infusion. This analysis revealed an accumulation of cells after infusion

Table 2. Collection and expansion of autologous T-regulatory cells.

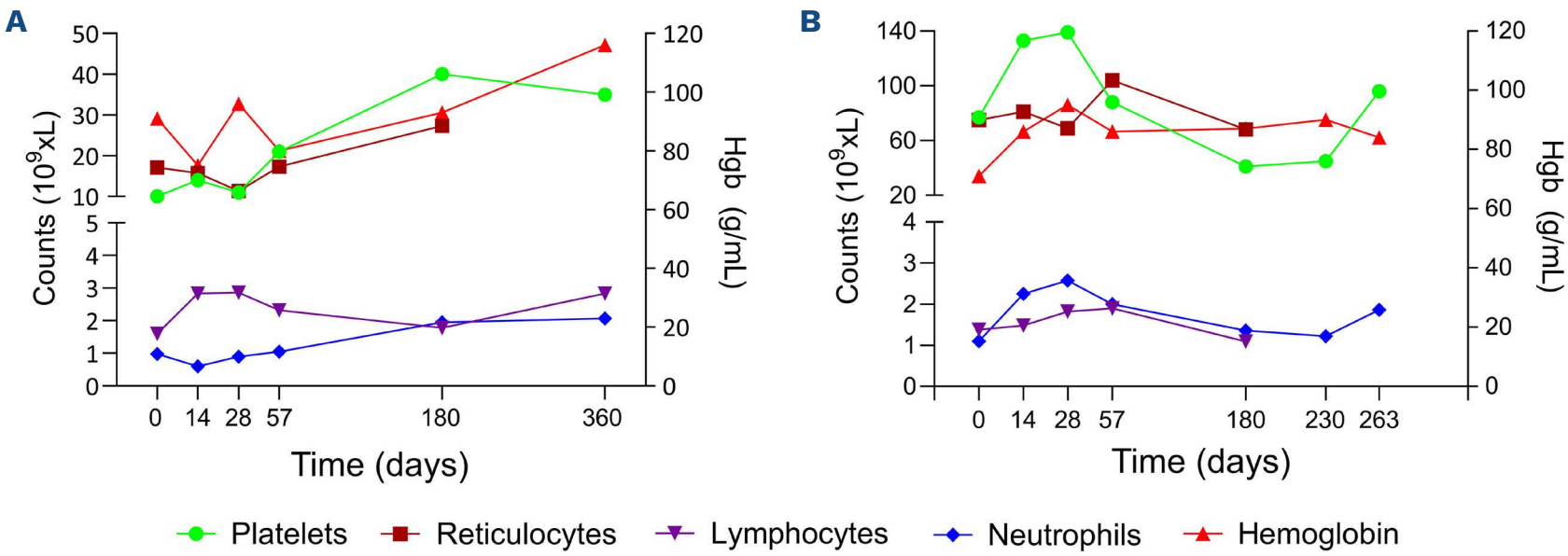
Patient	TBV processed	Isolated Treg cell number from leukapheresis	Treg cell number after expansion	N of cells per infusion on days 0 and 14*	Viability %	CD4$^+$, CD25$^+$ CD127^{low}, FoxP3$^+$ phenotype %	Suppression, 1:1, 1:5, 1:10 %	CD8$^+$ cells, %
KCH-001	2.9	181.01x10^6	4.95x10^9	307.5x10^6	97.3	73.91	73.85, 72.65, 72.18	3.56
KCH-002	2.4	24.32x10^6	4.16x10^9	248x10^6	97.92	65.11	86.40, 97.60, 94.25	4.22
KCH-003	2.5	68.79x10^6	3.16x10^9	420x10^6	98.7	78.69	92.95, 90.70, 97.3	3.99
KCH-004	2.7	163.77x10^6	6.81x10^9	317x10^6	98.20	85.48	93.52, 90, 75.14	3.57
KCH-005	2.2	90.06x10^6	4.44x10^9	390x10^6	98.10	46.64	83.57, 90.78, 75.55	5.25
KCH-006	2.9	94.8x10^6	2.52x10^9	357x10^6	98.70	47.16	94.08, 85.38, 75.31	4.80

*Patients received the same dose on day 0 and day 14. Regulatory T cells (Treg) were expanded to the required cell dose/patient kg body weight and met all the release criteria specifications, which included viability ≥60%, phenotype ≥30%, suppression ≥60%, and CD8$^+$ cells ≤10%. Expanded Treg were able to suppress proliferation of effector T cells at all T_{reg}:T_{eff} ratios. TBV processed: the number of times the patients total blood volume was processed during apheresis; N: number.

in a specific UMAP region characterized by low IL7R and high CD25, FOXP3, and CTLA4 expression, consistent with the Treg phenotype (Figure 2A, B).

Next, we used Phenograph, a clustering algorithm, to define phenotypically distinct subsets of $CD4^+$ T cells and track their frequencies over time to identify clusters expanding after infusion. Seven out of the 22 clusters we identified showed hallmarks of Treg (i.e., $FOXP3^+CD25^+IL7R^-$) (*Online Supplementary Figures S3* and *S4*). Two of these (Treg $FOXP3^{dim}$, and Treg $FOXP3^{dim}CD161^+$), which we broadly defined as *expanding* Treg, were initially present at a frequency lower than 0.5% but showed a marked increase after infusion in all treated patients, peaking on day 28 and remaining elevated on day 57. The frequency of the other five Treg clusters which we broadly defined as *pre-existent* Treg, remained stable throughout treatment and represented classical $CCR4^+CLA^+$ Treg, $CD45RA^+$ Treg, proliferating $Ki67^+$ Treg, $CD103^+$ Treg, and a subset of $CCR4^{dim}$, CLA^- Treg. Among the non-Treg clusters identified, only one, characterized by high expression of both CD25 and IL7R, showed a substantial increase in some of the treated patients (Figure 2D and *Online Supplementary Figure S2E*). No consistent changes were observed in Th1, Th17 or Th2 cell clusters. Effector Th1 cells decreased after treatment in patients 2 and 6, but increased in patient 4 (*Online Supplementary Figure S3D*).

The most pronounced accumulation of expanding Treg was observed in the two patients showing the best hematologic response, in whom the combined frequency of these cells increased from 0.5% at baseline to 14.37% and 30.72% of total $CD4^+$ cells at day 28 in patient 2 and 4, respectively. A measure of increased potential suppressive capacity, based



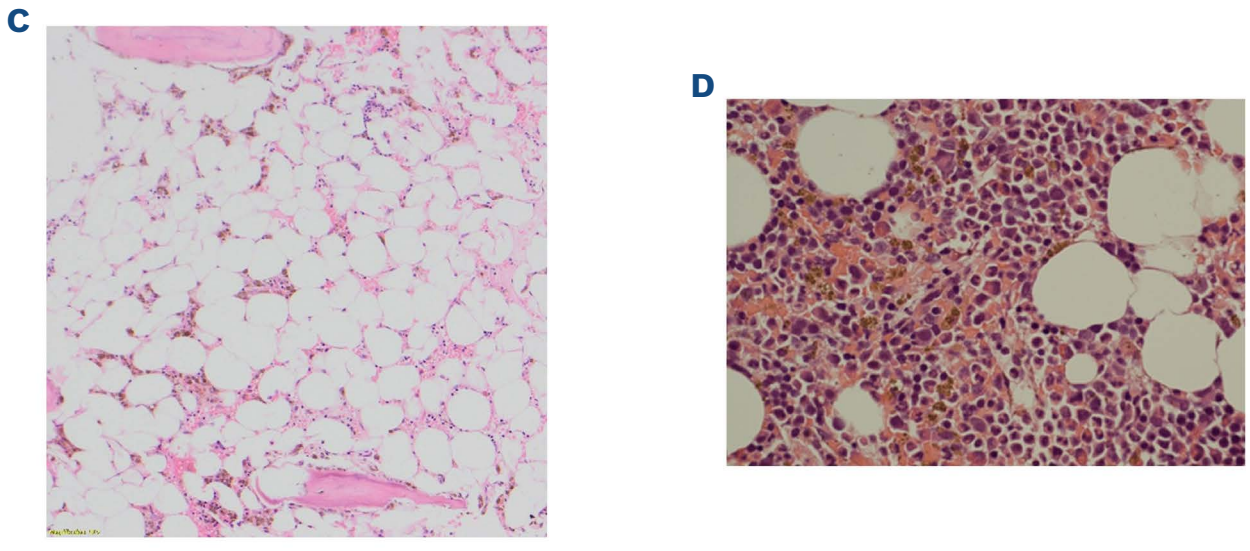
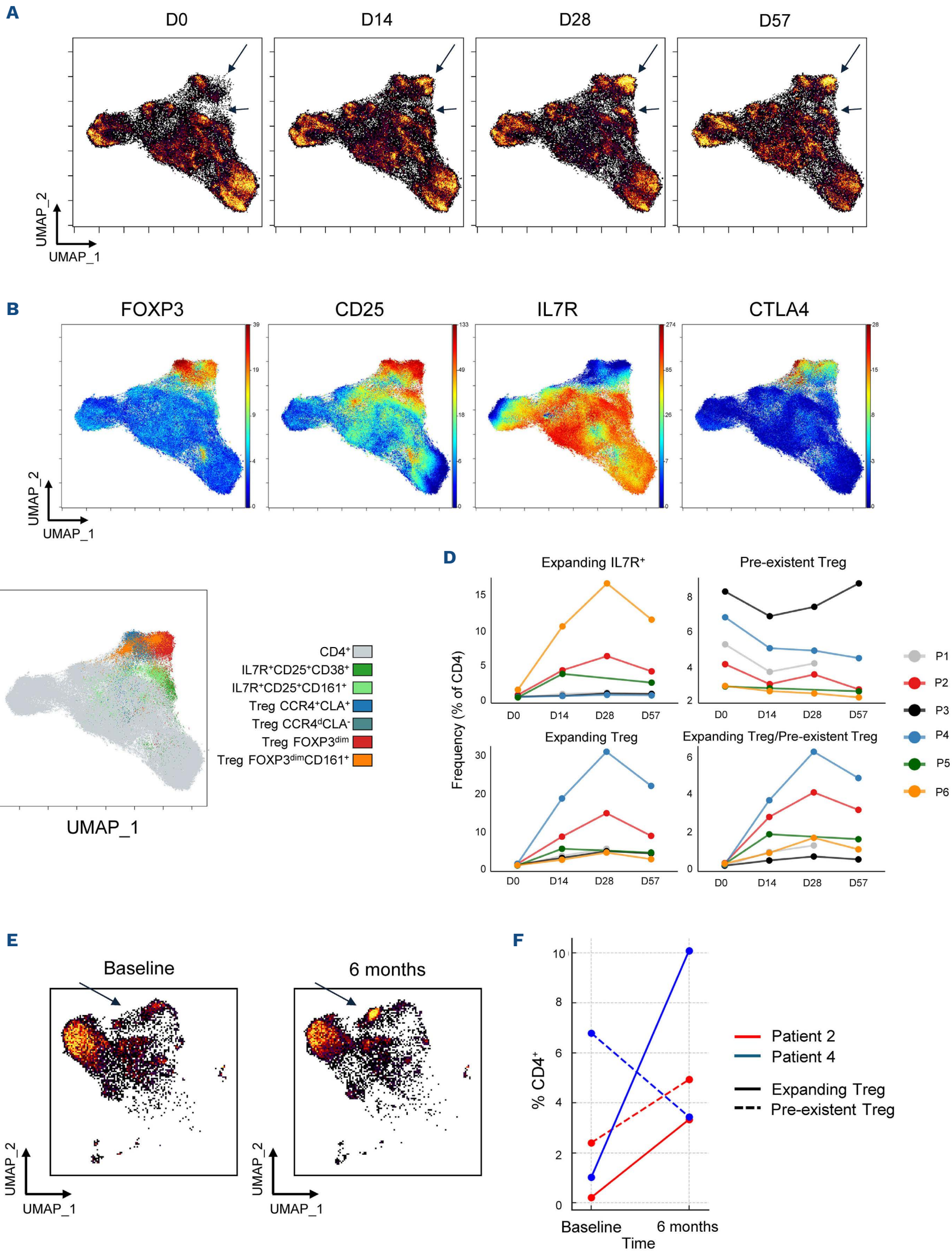


Figure 1. Changes in blood counts in two patients and appearance of the bone marrow in patient 2 after infusion of two doses of expanded autologous regulatory T cells. (A) Peripheral blood counts in patient 2 after infusion of two doses of expanded autologous regulatory T cells (Treg) at day 0 and day 14. This patient attained transfusion independence after day 28. Subsequent increases in platelet, neutrophil, lymphocyte and absolute reticulocyte counts were also observed. (B) Patient 4 also showed improvement in peripheral cell counts after infusion of two doses of expanded autologous Treg. However, a viral infection resulted in a drop of platelet count at day 57. Improvements in blood counts were observed again at day 263. Throughout this period the patient only required one transfusion. (C, D) Bone marrow micrograph at magnification × 10 before (C) and × 40 after (D) infusion of two doses of Treg in patient 2 showing a markedly hypocellular bone marrow before treatment and a normocellular marrow with maturation in all three cell lineages after treatment, consistent with a hematologic response. The data shown are from day 0 (baseline) and 14, 28, 57, 180, 230 and 263 days after infusion of expanded autologous Treg. Hgb: hemoglobin; ARC: absolute reticulocyte count.



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Figure 2. Immune monitoring analysis on peripheral blood and bone marrow samples using mass cytometry. (A) Uniform manifold approximation and projection (UMAP) plot showing the heterogeneity of CD4$^+$ T cells across all patients at four timepoints: day (D) 0, D14, D28, and D57. Arrows indicate the cell populations accumulating after infusion. (B) Colormap representing the expression levels of FOXP3, CD25, IL7R, and CTLA4 within CD4$^+$ T cells. (C) Overlay showing the clusters mapped onto the UMAP plot. (D) Combined frequency of expanding regulatory T cells (Treg) (Treg FOX3^{dim} and Treg FOXP3^{dim} CD161$^+$), expanding IL7R$^+$ cells (IL-7R$^+$CCR4$^+$CD38$^+$ or IL7R$^+$CCR4$^+$), pre-existent Treg (all other Treg clusters) and ratio of expanding Treg to pre-existent Treg at different timepoints in all patients. (E) UMAP plot showing the heterogeneity of bone marrow resident CD4$^+$ T cells in patient 4 at baseline and 6 months after infusion. The arrows indicate expanding Treg accumulating after infusion. (F) Frequencies of expanding Treg at baseline and 6 months in patients 2 and 4.

on the ratio of the combined frequency of the expanding *versus* the stable/pre-existent Treg, also showed that both these patients had the largest ratios at all the different timepoints following infusion. For these two patients, we had access to bone marrow samples collected at baseline and at 6 months after infusion. In both patients we identified a population of expanding Treg which were 10-fold more frequent at 6 months compared to baseline (Figure 2E, F and *Online Supplementary Figure S4*).

We then set out to further define the differences between expanding, pre-existent Treg and the IL7R$^+$CD25$^+$ cells expanding after infusion. Compared to the pre-existent Treg, both subsets of Treg expanding after infusion showed similar expression of CTLA4, but exhibited lower FOXP3, TOX and TIGIT and lacked CD39 expression (Figure 3A, B). The expanding IL7R$^+$CD25$^+$CD4$^+$ T cells did not express any markers typically associated with Treg, such as FOXP3, CTLA4 or CD39, nor markers of Th1 cells, such as CXCR3, T-bet or Eomes.[23]

Lack of CD39 and lower expression of FOXP3 compared to pre-existent Treg was also observed in expanding Treg identified in the bone marrow of patients 2 and 4 at 6 months after treatment (Figure 3C and *Online Supplementary Figure S4*).

Antibody sequencing, single-cell T-cell receptor sequencing and single-cell RNA sequencing

To elucidate protein and transcriptional signatures of expanding Treg, we performed single-cell RNA sequencing, antibody sequencing, and single-cell T-cell receptor (TCR) sequencing on peripheral blood collected at different times after infusion from patients 1, 2, and 4.[24] In all three patients, we identified clusters of Treg, defined by high expression of FOXP3 and CD25 and low expression of IL7R (Figure 3D, and *Online Supplementary Figures S4-S6*). Tracking their frequency over time, we identified Treg clusters present at baseline with stable frequencies during treatment (pre-existent Treg) and clusters with frequencies that increased after treatment (expanding Treg). Both sets of clusters shared the expression of a core set of genes associated with differentiation into Treg and suppressive functions, such as increased expression of FOXP3, IKZ2F, F5, LGALS3 and CTLA4 (Figure 3E). However, compared to pre-existent Treg, expanding Treg showed lower expression of several genes associated with Treg, including CD74, TIGIT, LGALS1, DUSP4, and MHC class II genes. In contrast, expanding Treg consistently expressed elevated levels of genes involved in cell cycle and proliferation, i.e., YBX3, MYC, and FOSB (Figure 3F). T-bet expression was not observed in the expanding Treg at any timepoint.

To determine the clonal relationship between the infusion product, pre-existent and expanding Treg we measured the overlap of their TCR repertoire. In patients 2 and 4, the repertoire of the clusters showing the largest fold-change after infusion also showed the largest overlap with the infusion product as measured by the Morisita index (Figure 4A). The TCR repertoire overlap between the infusion product and circulating Treg subsets increased after infusion and was more pronounced in expanding Treg than in pre-existent Treg (Figure 4B, C). In addition, there was minimal overlap between the infusion product and pre-existent Treg, and between pre-existent and expanding Treg, suggesting that the *in vitro* expansion of Treg might favor the expansion of less abundant clonotypes of Treg.

Our analysis revealed great heterogeneity in the magnitude of Treg expansion after infusion. To explore potential correlations between the phenotype of the infusion product and the expansion of Treg after infusion we performed mass cytometry analysis of the infusion products of all six patients. Patient 4, who demonstrated the largest post-infusion expansion, had a homogeneous cell product where all cells were FOXP3$^+$, CD27$^+$ and PD1$^-$ (Figure 4D-F). In contrast, the infusion products from other patients were more heterogeneous, containing varying proportions of PD1$^+$ cells. Within the infusion product, PD1$^+$ cells showed reduced expression of CD27 and FOXP3. PD1 is a marker of cell activation and exhaustion, and the lower expression of FOXP3 and CD27 on PD1$^+$ cells suggests that these cells might represent over-stimulated Treg with an attenuated phenotype during *in vitro* expansion. Notably, the two patients with the higher frequency of FOXP3$^-$ cells (patients 5 and 6) showed the highest proportions of expanding IL7R$^+$ cells after infusion (*Online Supplementary Figure S7*). However, patient 6 has shown a partial response with reduction in transfusion requirements.

Discussion

Immunosuppressive therapy with antithymocyte globulin and cyclosporine A has been the cornerstone of treatment

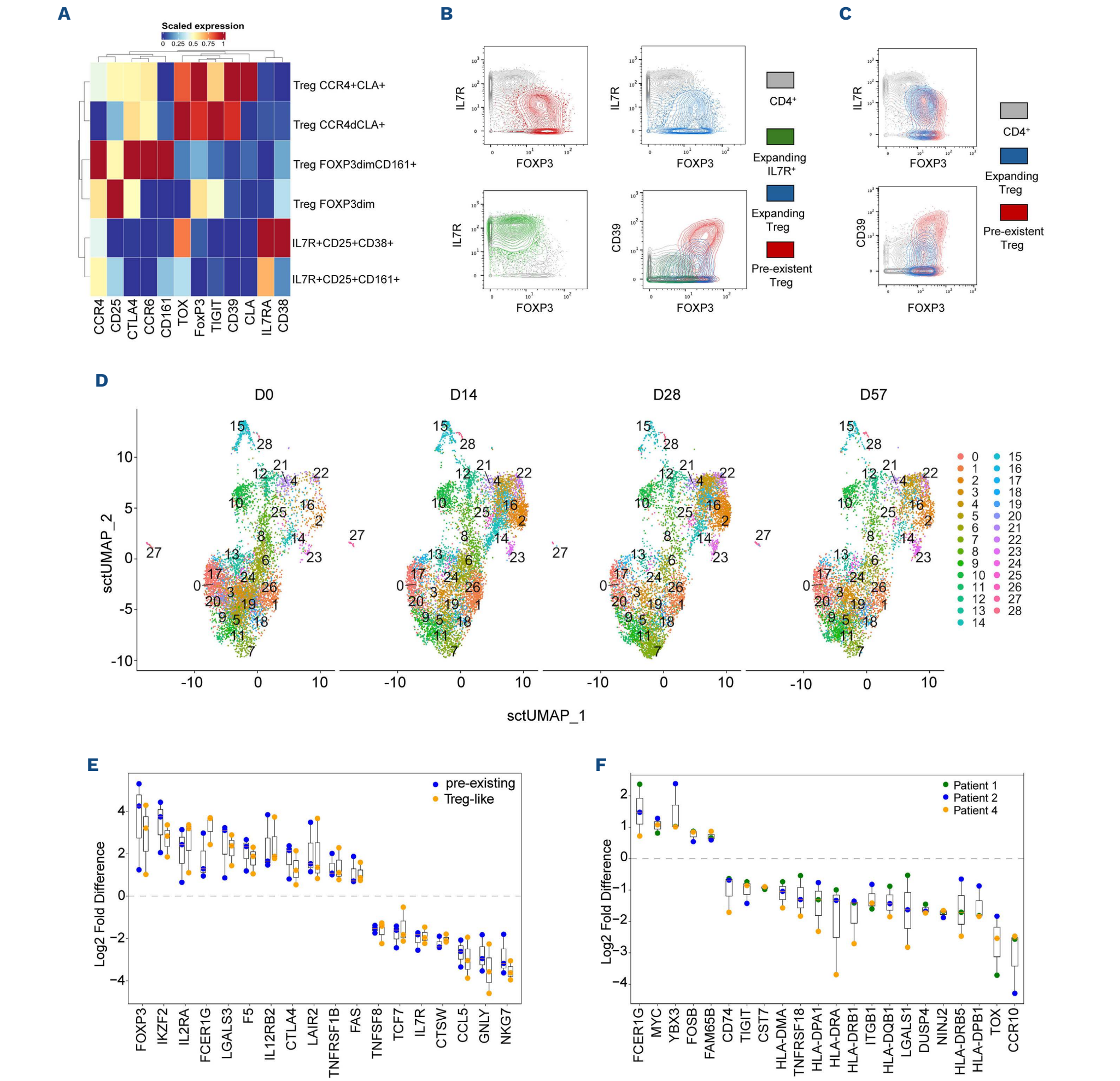


Figure 3. Immune-monitoring on peripheral blood and bone marrow samples and single-cell analysis. (A) Heatmap displaying the median expression levels of selected markers in the CD4⁺ T-cell clusters shown in Figure 2C. (B) Contour plot illustrating the expression patterns of FOXP3, IL7R, and CD39 in total CD4⁺ cells, expanding regulatory T cells (Treg) (Treg Foxp3^{dim} and Treg Foxp3dimCD161⁺ defined using Boolean gating), expanding IL7R⁺ cells (IL7R⁺CCR4⁺CD38⁺ or IL7R⁺CCR4⁺) and pre-existent Treg (all other Treg clusters). (C) Contour plots showing the expression of IL7R, FOXP3, and CD39 in total CD4⁺, pre-existent Treg and expanding Treg in the bone marrow 6 months after infusion. (D) Uniform manifold approximation and projection plot illustrating the heterogeneity of CD4⁺ T cells, as defined by single-cell RNA sequencing, from patient 4 at different timepoints. (E) Boxplot showing the $\log_2$ fold-change in expression compared to non-Treg cells in pre-existent (blue) and expanding Treg (yellow) cells. Genes with at least a 1.4-fold difference in all three patients are shown. (F) Boxplot showing the $\log_2$ fold-change in expanding compared to pre-existent Treg. Genes with at least a 1.4-fold difference in all three patients are shown. scUMAP: single-cell uniform manifold approximation and projection.

for patients with AA. Addition of eltrombopag to immunosuppressive therapy improves the speed of response in all subtypes of AA.[5] However, only 70% of patients respond to this treatment and relapses are frequent. HSCT provides a curative therapy but requires a suitable donor and can be associated with significant morbidity. Expanded Treg have been extensively used in the context of organ transplantation and have been considered as a useful therapeutic

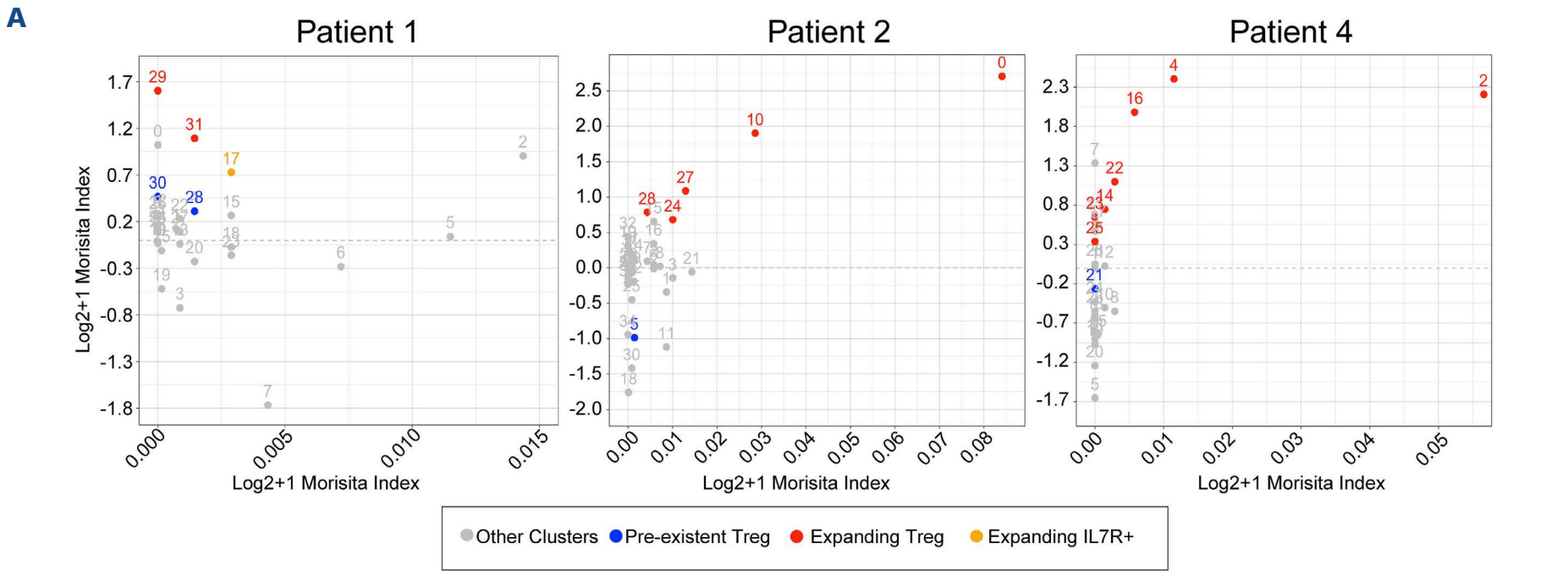


Figure 4. T-cell receptor repertoire overlap between the infusion product and regulatory T-cell clusters. (A) Scatter plot showing, for each cluster, the fold-change in frequency at day 14 compared to baseline, and the Morisita index of the repertoire overlap with the infusion product for patients 1, 2 and 4. (B) Morisita index matrices showing the overlap between each group of clusters and the infusion product at different timepoints for patient 4. (C) Morisita index matrices showing the overlap between each group of clusters and the infusion product across all three patients and across all the different time points. Treg: regulatory T cells; IP: infusion product; NA: not available.

approach to minimize the effects of immunosuppression.[25-30] Trials conducted in the context of expanded autologous Treg have shown the safety and efficacy of these expanded cells. We have previously shown that Treg are reduced in the peripheral blood of AA patients and the reduction of these cells correlates with the severity of AA. Our previous results showed that Treg from AA patients are expandable *in vitro* and retain their suppressive function while maintaining polyclonality.[19,20] These observations led us to conduct this phase I clinical trial to assess the safety and kinetics of infused autologous Treg, expanded under GMP conditions, in AA patients. Treg could be expanded *in-vitro* for all six patients in the study and expanded sufficiently to administer the stipulated trial dosage of $5x10^6$/kg body weight on two occasions 2 weeks apart. Three of the patients showed a hematologic improvement and in one patient who responded previously hypocellular marrow became normocellular. Two of the responders had non-severe AA. The two patients who showed the best response had significantly increased numbers of circulating Treg after infusion compared to the non-responders. In our patients infused Treg peaked at day 28. Importantly, we demonstrated that the expansion of Treg was sustained over time, with detectable levels persisting in the bone marrow up to 6 months after infusion. We acknowledge the heterogeneity of the disease status and the small cohort size of the patients treated. This however, is often the case with phase I studies in which patients who receive the treatment often have refractory, resistant disease and have received several lines of previous therapy. All of our patients had relapsed and/or had refractory disease, with at least transfusion dependency. All patients received at least cyclosporine and/or eltrombopag and cyclosporine alternatives (tacrolimus in patients 4 and 5), as a backbone of immunosuppressive therapy. Horse antithymocyte globulin treatments can be associated with significant toxicity, particularly in the elderly, and all of the patients in this cohort, except patient 5, were more than 70 years of age with comorbidities. Patient 5 refused horse antithymocyte globulin treatment because of personal beliefs regarding animal welfare. Patient 2 was enrolled, because of loss of graft function/relapse of AA after a HSCT. Salvage treatment with eltrombopag failed to improve her blood counts, and she remained transfusion-dependent.

Previous studies conducted in the context of HSCT using HLA disparity as markers demonstrated persistence of expanded Treg for up to 14 days, while Treg labeled with deuterium in type 1 diabetes and kidney transplant recipients showed a peak in circulation 7-14 days after infusion and 20% of these cells were still detectable in the circulation after 1 year.[31-33] Comparative analysis of expanding Treg revealed distinct phenotypic differences from pre-existent Treg, including lower expression of FOXP3, TOX, and TIGIT, and a lack of CD39 expression. These differences suggest that, although effective in suppressing the immune response, Treg expanding after infusion might not be able to deploy the full immune-suppressive capabilities of regulatory T cells. Significant heterogeneity was present in the infusion products. Some products contained subsets of $PD1^+FOXP3^{low}$ T cells, suggesting variations in the degree of Treg activation and stability which may impact the subsequent expansion and effectiveness of the infused Treg, pointing to a potential area for optimization. Standardizing the expansion protocols to reinforce the Treg phenotype, potentially by minimizing the presence of $PD1^+FOXP3^{low}$ cells, could improve the consistency and efficacy of Treg-based therapies. In a recent study by Kadia *et al.*, infusion of expanded Treg derived from cord blood into four patients with AA led to hematologic responses in two patients.[34] It is noteworthy that, like expanded autologous Treg, cord blood-derived Treg were also well tolerated and were not associated with any untoward effect.

Our study provides proof of concept of the safety and efficacy of autologous Treg, which can be successfully expanded from AA patients, and provides key insights into the fate and heterogeneity of *in vitro*-expanded Treg suggesting that refining the expansion protocols to maintain a stable and potent Treg phenotype could significantly enhance the clinical responses in AA. Given the safety and encouraging hematologic responses we plan to extend the trial to a further cohort of patients treated with higher doses ($1x10^7$/kg) of expanded Treg, which will allow evaluation of the *in vivo* kinetics and effectiveness of infused cells and provide insights into criteria for a phase II trial.

Disclosures

GJM received funding from Novartis and Bristol Myers Squibb. GL is a founder of and consultant for Quell Therapeutics. SK has received research support and honoraria from Novartis, Alexion, Beckman Coulter, MorphoSys and Pfizer.

Contributions

NM carried out the expansion of cells under Good Manufacturing Practice conditions, performed experiments, analyzed and interpreted data and wrote the paper. SGa was involved in recruiting the patients and contributed to writing some sections of the paper. MR performed single-cell sequencing experiments and analyzed the data. AD helped in designing the trial. SGe, MMK, AK, SK, AM and GL contributed to writing the paper. LD and HC were involved with patients' recruitment and leukapheresis. JL helped in managing the trial. GN analyzed and interpreted the data and wrote the paper, GJM conceived the idea, supervised the project, interpreted the results and wrote the paper.

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Data-sharing statement

The authors confirm that the data supporting the findings

of this study are available within the article and its Online Supplementary Material. Raw data that support the findings of this study are available from the corresponding author upon reasonable request.

References

1. Young NS. Aplastic anemia. N Engl J Med. 2018;379(17):1643-1656.
2. Giudice V, Selleri C. Aplastic anemia: pathophysiology. Semin Hematol. 2022;59(1):13-20.
3. Marsh JC, Bacigalupo A, Schrezenmeier H, et al.; European Blood and Marrow Transplant Group Severe Aplastic Anaemia Working Party. Prospective study of rabbit antithymocyte globulin and cyclosporine for aplastic anemia from the EBMT Severe Aplastic Anaemia Working Party. Blood. 2012;119(23):5391-5396.
4. Marsh JC, Pearce RM, Koh MB, et al.; British Society for Blood and Marrow Transplantation, Clinical Trials Committee. Retrospective study of alemtuzumab vs ATG-based conditioning without irradiation for unrelated and matched sibling donor transplants in acquired severe aplastic anemia: a study from the British Society for Blood and Marrow Transplantation. Bone Marrow Transplant. 2014;49(1):42-48.
5. Peffault de Latour R, Kulasekararaj A, Iacobelli S, et al.; Severe Aplastic Anemia Working Party of the European Blood and Marrow Transplant Group. Eltrombopag added to immunosuppression in severe aplastic anemia. N Engl J Med. 2022;386(1):11-23.
6. Desmond R, Townsley DM, Dumitriu B, et al. Eltrombopag restores trilineage hematopoiesis in refractory severe aplastic anemia that can be sustained on discontinuation of drug. Blood. 2014;123(12):1818-1825.
7. Patel BA, Groarke EM, Lotter J, et al. Long-term outcomes in patients with severe aplastic anemia treated with immunosuppression and eltrombopag: a phase 2 study. Blood. 2022;139(1):34-43.
8. Rosenfeld SJ, Kimball J, Vining D, Young NS. Intensive immunosuppression with antithymocyte globulin and cyclosporine as treatment for severe acquired aplastic anemia. Blood. 1995;85(11):3058-3065.
9. Alvarado LJ, Huntsman HD, Cheng H, et al. Eltrombopag maintains human hematopoietic stem and progenitor cells under inflammatory conditions mediated by IFN-gamma. Blood. 2019;133(19):2043-2055.
10. Young N, Griffith P, Brittain E, et al. A multicenter trial of antithymocyte globulin in aplastic anemia and related diseases. Blood. 1988;72(6):1861-1869.
11. Scheinberg P, Wu CO, Nunez O, Young NS. Predicting response to immunosuppressive therapy and survival in severe aplastic anaemia. Br J Haematol. 2009;144(2):206-216.
12. Maciejewski JP, Kim S, Sloand E, Selleri C, Young NS. Sustained long-term hematologic recovery despite a marked quantitative defect in the stem cell compartment of patients with aplastic anemia after immunosuppressive therapy. Am J Hematol. 2000;65(2):123-131.
13. Marsh JC, Kulasekararaj AG. Management of the refractory aplastic anemia patient: what are the options? Hematology Am Soc Hematol Educ Program. 2013;2013:87-94.
14. Young NS, Maciejewski J. The pathophysiology of acquired aplastic anemia. N Engl J Med. 1997;336(19):1365-1372.
15. Long J, You X, Yang Q, et al. Bone marrow CD8(+) Trm cells induced by IL-15 and CD16(+) monocytes contribute to HSPC destruction in human severe aplastic anemia. Clin Immunol. 2024;263:110223.
16. Alexander WS, Roberts AW, Nicola NA, Li R, Metcalf D. Deficiencies in progenitor cells of multiple hematopoietic lineages and defective megakaryocytopoiesis in mice lacking the thrombopoietic receptor c-Mpl. Blood. 1996;87(6):2162-2170.
17. Solomou EE, Rezvani K, Mielke S, et al. Deficient CD4+ CD25+ FOXP3+ T regulatory cells in acquired aplastic anemia. Blood. 2007;110(5):1603-1606.
18. Kordasti S, Marsh J, Al-Khan S, et al. Functional characterization of CD4+ T cells in aplastic anemia. Blood. 2012;119(9):2033-2043.
19. Kordasti S, Costantini B, Seidl T, et al. Deep phenotyping of Tregs identifies an immune signature for idiopathic aplastic anemia and predicts response to treatment. Blood. 2016;128(9):1193-1205.
20. Lim SP, Costantini B, Mian SA, et al. Treg sensitivity to FasL and relative IL-2 deprivation drive idiopathic aplastic anemia immune dysfunction. Blood. 2020;136(7):885-897.
21. Kulasekararaj A, Cavenagh J, Dokal I, et al. Guidelines for the diagnosis and management of adult aplastic anaemia: a British Society for Haematology guideline. Br J Haematol. 2024;204(3):784-804.
22. Feng X, Scheinberg P, Wu CO, et al. Cytokine signature profiles in acquired aplastic anemia and myelodysplastic syndromes. Haematologica. 2011;96(4):602-606.
23. Giudice V, Feng X, Lin Z, et al. Deep sequencing and flow cytometric characterization of expanded effector memory CD8(+)CD57(+) T cells frequently reveals T-cell receptor Vbeta oligoclonality and CDR3 homology in acquired aplastic anemia. Haematologica. 2018;103(5):759-769.
24. Hao Y, Hao S, Andersen-Nissen E, et al. Integrated analysis of multimodal single-cell data. Cell. 2021;184(13):3573-3587.e29.
25. Bluestone JA, Tang Q. T(reg) cells-the next frontier of cell therapy. Science. 2018;362(6411):154-155.
26. Sanchez-Fueyo A, Whitehouse G, Grageda N, et al. Applicability, safety, and biological activity of regulatory T cell therapy in liver transplantation. Am J Transplant. 2020;20(4):1125-1136.
27. Brook MO, Hester J, Petchey W, et al. Transplantation Without Overimmunosuppression (TWO) study protocol: a phase 2b randomised controlled single-centre trial of regulatory T cell therapy to facilitate immunosuppression reduction in living donor kidney transplant recipients. BMJ Open. 2022;12(4):e061864.
28. Bernaldo-de-Quiros E, Camino M, Martinez-Bonet M, et al. First-in-human therapy with Treg produced from thymic tissue (thyTreg) in a heart transplant infant. J Exp Med. 2023;220(12):e20231045.
29. Sawitzki B, Harden PN, Reinke P, et al. Regulatory cell therapy in kidney transplantation (the ONE study): a harmonised design and analysis of seven non-randomised, single-arm, phase 1/2A trials. Lancet. 2020;395(10237):1627-1639.
30. Romano M, Fanelli G, Albany CJ, Giganti G, Lombardi G. Past, present, and future of regulatory T cell therapy in transplantation and autoimmunity. Front Immunol. 2019;10:43.

31. Brunstein CG, Miller JS, Cao Q, et al. Infusion of ex vivo expanded T regulatory cells in adults transplanted with umbilical cord blood: safety profile and detection kinetics. Blood. 2011;117(3):1061-1070.
32. Bluestone JA, Buckner JH, Fitch M, et al. Type 1 diabetes immunotherapy using polyclonal regulatory T cells. Sci Transl Med. 2015;7(315):315ra189.
33. Chandran S, Tang Q, Sarwal M, et al. Polyclonal regulatory T cell therapy for control of inflammation in kidney transplants. Am J Transplant. 2017;17(11):2945-2954.
34. Kadia TM, Huang M, Pemmaraju N, et al. Phase 1 study of CK0801 in treatment of bone marrow failure syndromes. NEJM Evid. 2024;3(6):EVIDoa2300362.