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

Nazia Matto*[1], Shreyans Gandhi*[1,2], Marta Rzepkowska[1], Sila Gerlevik[1], Mohammad M Karimi[3], Austin Kulasekararaj[1,2], Abdel Douiri[4], Anji Miller[5], Lynne Duran[2], Haili Cui[2], Jen Lewis[2] Shahram Kordasti[6], Giovanna Lombardi[7], Giorgio Napolitani[1], Ghulam.J. Mufti[1,2]

1. Department of Haematology Medicine, School of Cancer and Pharmaceutical Sciences, SGDP Centre, Denmark Hill, Kings College London, London, United Kingdom.
2. King's College Hospital NHS Foundation Trust, London United Kingdom.
3. School of Cancer and Pharmaceutical Sciences, Comprehensive Cancer Centre, Kings College London, London, United Kingdom.
4. Division of Population Health Sciences Kings College London, London, United Kingdom.
5. LifeArc, Lynton House, 7-12 Tavistock Square, London, United Kingdom.
6. System Cancer Immunology, Comprehensive Cancer Centre, King's College London, London, United Kingdom.
7. Immunoregulation Laboratory, MRC Centre for Transplantation, School of Immunology & Microbial Sciences, King's College London, London, United Kingdom.

AUTHORSHIP

*Both are first authors.

NM carried out the expansion of cells under GMP experiments, performed experiments, analysed and interpreted data and wrote the paper. SG was involved in recruiting the patients and contributed in writing some sections of the paper. MR performed single cell sequencing experiments and analysed the data. AD helped in designing the trial. SG, MMK, AK, SK, AM, GL contributed in writing the paper. LD and HC were involved with patient recruitment and leukapheresis. JL helped in managing the trial. Gna analysed and interpreted the data and wrote the paper, G.J.M conceived the idea, supervised the project, interpreted the results and wrote the paper.

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Correspondence Author

Prof G. J. Mufti

ghulam.mufti@kcl.ac.uk

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Data availability

The authors confirm that the data supporting the findings of this study are available within the article and its Supplementary material. Raw data that support the finding of this study are available from the corresponding author upon reasonable request.

Abstract

Background

Immune mediated aplastic anaemia (AA) is a bone marrow failure syndrome characterised by cytotoxic CD8 mediated autoimmune suppression of haematopoietic stem progenitor cells (HSPCs) resulting in varying degrees of peripheral blood cytopenias. Treatment with immunosuppressive therapy and haematopoietic stem cell transplantation are not applicable to all the patients and effective responses occur in a proportion of patients only, highlighting the unmet need for alternative treatments. We have previously shown a reduction in number and function of regulatory T cells (Tregs) in AA patients and their functional restoration following *in vitro* expansion that laid the foundations for this Phase 1 trial. Required number of Tregs were collected from leukapheresis and expanded under GMP conditions from all 6 patients in the trial who were resistant/refractory to standard form of treatments. The trial design included 2 doses of autologous Tregs (5×10^6 /kg) administered 2 weeks apart. Mass cytometry, single cell sequencing and cytokine profiling was performed on blood samples collected at various timepoints. Tregs were successfully expanded to required doses from all 6 patients with no adverse or immune related events in any of the patients. Haematological responses were observed in 3 patients. In addition, we were able to track the persistence of the expanded Tregs *in vivo*, correlate clinical efficacy with the accumulation of clusters of Tregs post-infusion, and identify phenotypic markers in the infusion product that correlate with *in vivo* expansion. Tregs from AA patients are expandable, safe for infusion, and may help modulate the abnormal immune milieu associated with AA, with induction of clinical response.

INTRODUCTION

Immune mediated reduction of bone marrow haematopoietic stem/progenitor cells (HSPCs) in aplastic anaemia (AA) leads to peripheral blood pancytopenia (1, 2). Immunosuppressive therapy (IST) with antithymocyte globulin and ciclosporin A ± Eltrombopag or haematopoietic stem cell transplantation (HSCT) have been mainstay of treatment (3-10). IST leads to long term haematological responses in up to 70% of patients, however IST can be associated with significant morbidity, partial haematological 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 increase in the $CD4^+$ Th1 cells, CD8 cytotoxic T cells, Th17 cells, secretion of tumour 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 (Tregs) and their *in vivo* functional abnormalities lead to unrestricted inflammatory milieu in the bone marrow(17). We have shown that Tregs are reduced in AA patients, but can be expanded *in vitro* in the presence of IL-2 (1000IU/mL) and Rapamycin (100nM) with functional restoration. The expanded Tregs are ≥90% $FOXP3^+$, highly suppressive, and stable and they prevent graft vs host disease in an NSG mouse model (18-20). These observations laid the foundation for the first in human clinical trial of expanded autologous Tregs in AA patients who are ineligible or refractory to IST+/- Eltrombopag and allogenic stem cell transplant.

In this phase 1 trial we assessed the safety and efficacy of infused autologous expanded Tregs from AA patients.

Methods

TIARA is a single institution phase 1 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).

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 IST with ATG + CiclosporinA +/- Eltrombopag. All six patients have received two doses of $5x10^6$/kg of expanded autologous Tregs two weeks apart (Figures S1 and S2). Primary endpoint was safety which was evaluated as any adverse events. Secondary endpoints were response as assessed by improvements in haematological indices and reduction in supportive care requirements.

Isolation and Expansion of Polyclonal Tregs

2.2 – 2.9 (avg 2.6) times total body volume (TBV) was processed to achieve an apheresis volume between 120-200mL which was transferred to the GMP facility at Guys Hospital**.** The leukapheresis product was subjected to volume reduction and platelets were removed using the 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 automated CliniMACS® Plus system (Miltenyi Biotec) followed by incubation of depleted cells with CliniMACS® CD25 reagent for 15minutes at 4°C. Tregs were then purified again on the

automated CliniMACS® Plus system. The isolated Tregs were resuspended in TheraPEAK® X-VIVO® 15 (Lonza) media containing 5% AB serum (biowest®) followed by seeding in a suitable size 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 (100nM Rapamune©, Pfizer) and IL-2 (1000IU/mL; Proleukin© Novartis) were added to the cells alternatively. Cell count was performed after every seven days and depending on the cell number, cells were re-stimulated again and fresh media containing Rapamycin and IL-2 was added. Cells could be expanded to a maximum of 30 days with a maximum of five stimulations, but the protocol has the option of early harvest if the cell dose was achieved earlier at either day 16, 23 and 30.

Harvest

Once the cell dose was achieved the cells were resuspended in CliniMACS® buffer containing 0.5% HSA (Biotest) and subjected to bead depletion using the CliniMACS® Plus system to remove anti-CD3/CD28 beads added during stimulations. Cells were resuspended in Plasma-Lyte148 (Baxter) and 5% HSA.pre-prepared freezing mixture [20% CryoSure-DMSO (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 (Supplementary methods).

CyTOF

Data for CyTOF was acquired using 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. The details are in the supplementary methods.

Single cell sequencing

Single cell analysis was performed using the BD Rhapsody™ Express Single-Cell Analysis System. The details are in the supplementary methods.

Response

The criteria for response in SAA and NSAA, are as defined in the BCSH guidelines (21). As a standard, all patients are offered packed red cell (PRC) transfusions when haemoglobin < 80 g/L. Individual variations allow for patients to be transfused at a higher threshold (i.e < 90 g/L, if respiratory or cardiac compromise, or significantly symptomatic from effects of anaemia. All patients adhered to the standard criteria for this transfusion thresholds, except patient 4 who was transfused at < 90 g/L.

Pooled platelet transfusions are offered when platelets < 10 x 10^9/L, as a standard practise across all the centres from where patients were recruited into this study. Higher thresholds are considered, in the event of an active bleeding episode, or evidence of consumption i.e neutropenic fevers etc. Platelet refractoriness is investigated, as per standard protocols, and where necessary and indicated HLA matched platelets are administered.

RESULTS

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

Patients 1 and 6 were considered unfit for IST and opted for the clinical trial. The median follow up is 261 days (105-554). Patient 1 deceased 239 days due to SAA complications and Patient 3 went off trial and received an allogeneic haematopoietic stem cell transplant from a voluntary unrelated donor 283 days after receiving both doses of Tregs.

CYTOKINE ANALYSIS

Multiplex serum cytokine analysis was performed in all patients at Day 0, 14 and 28. There were no significant difference between cytokine levels pre and post infusion (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 and Tregs 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 hrs of the first infusion. The second dose of Tregs was administered without any side effects. There was no evidence of cytokine release syndrome (CRS) in any of the patients following infusion of both doses of Tregs.

HEMATOLOGIC RESPONSE (See Fig1)

Improvements in haematological parameters, Partial Response (PR) was observed in three patients (Patient 2, 4 and 6) after infusion of both doses. Criteria for response was the same as followed in the RACE trial protocol (5). Patient 1, 3 and 5 did not show any improvements in blood counts and continued to require PRC and platelet transfusions. No correlation between response and presence of a PNH clone was ascertained possibly due to the smaller cohort of patients.

Patient 2 became transfusion independent with an increase in platelet count from 10×10^9/L at baseline prior to 1st infusion to a gradual increase to 41×10^9/L at 12 months and haemoglobin (Hgb) levels from 91g/L to 122g/L. Absolute reticulocyte count (ARC) increased from 17.1×10^9/L to 27.3×10^9/L at six months (Figure 1a). A subsequent increase in neutrophil and lymphocyte cell count 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 platelet count increased from 77×10^9/L to 139×10^9/L at Day 28, then dropped to 41×10^9/L at six months after a viral infection, before rising to 96×10^9/L at day 263. Hgb levels improved from 71g/L to 84g/L, sustained at day 263. Neutrophil count increased from 1.12×10^9/L to 1.86×10^9/L at Day 28. Lymphocyte and ARC counts increased from 1.3×10^9/L to 1.9×10^9/L and 75×10^9/L to 104×10^9/L at Day 57, but later decreased after infection (Figure 1b). The patient achieved 112+ days of red cell transfusion independence. Although transfusion dependence resumed, the frequency of transfusion for PRC decreased to every 8 weeks and platelet count remained above 100×10^9/L at the last follow-up.

Patient 6 has shown a consistent reduction in transfusion requirements after the first three months following both infusions. The patient needed 10 PRC units in the first three months which has

reduced to needing only 4 units in the following three months. Patient 6 has been transfusion independent > 8 months, equating a PR as defined above.

Patient 3 eventually received a voluntary unrelated allogeneic stem cell transplantation (VUD Allo SCT), after a full 10/10 HLA matched donor became available through donor registry, subsequent to their participation in this study.

IMMUNE-MONITORING

Mass Cytometry

To characterize how the infusion of *in vitro* expanded Tregs influenced the composition of peripheral blood $CD4^+$ T cells, Mass Cytometry analysis on peripheral blood mononuclear cells (PBMCs) was carried out at baseline (D0) and on days 14, 28, and 57 post-infusion. We applied Uniform Manifold Approximation and Projection (UMAP) to visualize $CD4^+$ T cells across the different time points post-infusion. This analysis revealed an accumulation of cells after infusion in a specific UMAP region characterized by low IL7R and high CD25, FOXP3, and CTLA4 expression, consistent with Tregs phenotype (Figure 2a and 2b).

Next, we used Phenograph, a clustering algorithm, to define phenotypically distinct subsets of $CD4^+$ T cells and tracked their frequencies over time to identify clusters expanding post-infusion. 7 out of the 22 clusters we identified showed hallmarks of Tregs (i.e. $FOXP3^+CD25^+IL7R^-$)(Figures S3 and S4). Two of these (Tregs $FOXP3^{dim}$, and Tregs $FOXP3^{dim}CD161^+$), which we broadly defined as **expanding Tregs**, were initially present at a frequency lower than 0.5% but showed a marked increase post-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 Tregs** remained stable throughout treatment and represented classical $CCR4^+CLA^+$

Tregs, CD45RA^{+} Tregs, proliferating Ki67^{+} Tregs, CD103^{+} Tregs, and a subset of CCR4^{dim}, CLA^{-} Tregs. 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 (Figures 2d and S2e). No consistent changes were observed in Th1, Th17 or Th2 cell clusters. Effector Th1 cells decreased post treatment in Patient 2 and 6, but increased in Patient 4 (Fig S3 panel d).

The most pronounced accumulation of expanding Tregs was observed in the two patients showing the best haematological response, where 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 on the ratio of the combined frequency of the expanding vs the stable/pre-existent Tregs, also showed that both the patients had the largest ratio at all the different time points post infusion. For these two patients, we had access to bone marrow samples collected at baseline and at six months post-infusion. In both patients we identified a population of expanding Tregs which were at a frequency 10-fold higher at six months compared to baseline (Figures 2e-f and S4).

We then set out to further define the differences between expanding, pre-existent Tregs and the IL7R^{+}CD25^{+} cells expanding post-infusion. Compared to the pre-existent Treg both subsets of Tregs expanding post-infusion showed similar expression of CTLA4, but exhibited lower FOXP3, TOX, TIGIT and lacked CD39 expression (Figures 3a, b). The expanding IL7R^{+}CD25^{+}CD4^{+} T cells, did not express any markers typically associated with Tregs, 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 Tregs, was also observed in expanding Tregs identified in the bone marrow of Patients 2 and 4 six months post-treatment (Figures 3c and S4).

Ab-Seq, sc-TCR and scRNA sequencing

To elucidate protein and transcriptional signatures of expanding Tregs, we performed scRNAseq, Abseq, and scTCRseq on peripheral blood collected at different times post-infusion in Patients 1, 2, and 4 (24). In all three patients, we identified clusters of Tregs, defined by high expression of FOXP3, CD25 and low expression of IL7R (Figures 3d, and S4, 5, 6). Tracking their frequency over time, we identified Treg clusters present at baseline with stable frequency during treatment (pre-existent Tregs) and clusters with frequency increased post-treatment (expanding Tregs). Both set of clusters shared the expression of a core set of genes associated with differentiation into Tregs and suppressive functions, such as increased expression of FOXP3, IKZ2F, F5, LGALS3 and CTLA4 (Figure 3e). However, compared to pre-existent Tregs, expanding Tregs showed lower expression of several genes associated with Tregs, including CD74, TIGIT, LGALS1, DUSP4, and MHC Class II genes. In contrast, expanding Tregs consistently expressed elevated levels of genes involved in cell cycle and proliferation i.e YBX3, MYC, and FOSB (Figure 3f). Tbet expression was not observed in the expanding Tregs at any time points.

To determine the clonal relationship between the infusion product, pre-existent and expanding Tregs we measure the overlap of their TCR repertoire. In Patients 2 and 4, the repertoire of the clusters showing the largest fold change post-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 post-infusion and was more

pronounced in expanding vs pre-existent Tregs (Figures 4b and c). In addition, there was minimal overlap between the infusion product and pre-existent Tregs, and between pre-existent and expanding Tregs, suggesting that the *in vitro* expansion of Tregs might favour the expansion of less abundant clonotypes of Tregs.

Our analysis revealed great heterogeneity in the magnitude of Treg expansion post infusion. To explore potential correlations between the phenotype of the infusion product and the expansion of Tregs after infusion we performed Mass cytometry analysis of the infusion products of all six patients. Patient 4, who demonstrated the largest expansion post-infusion, had a homogeneous cell product where all cells were $FOXP3^{+}$, $CD27^{+}$and $PD1^{-}$ (Figures 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 activation and exhaustion, and the lower expression of FOXP3 and CD27 on $PD1^{+}$ cells suggests that these cells might represent over-stimulated Tregs with attenuated phenotype during *in vitro* expansion. Notably, the two patients with the higher frequency of $FOXP3^{-}$cells (Patient 5 and 6) showed the highest proportion of expanding $IL7R^{+}$ cells post-infusion (Fig S7). However, Patient 6 has shown a partial response with reduction in transfusion requirements.

DISCUSSION

IST with Antithymocyte Globulin (ATG) and Ciclosporin A(CSA) has been the cornerstone of treatment for patients with AA. Addition of Eltrombopag to IST 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 Tregs have extensively been used in the context

of organ transplantation and have been considered as a useful therapeutic approach to minimise the effect of immunosuppression (25-30). Trials conducted in the context of expanded autologous Tregs have shown the safety and efficacy of these expanded cells. We have previously shown that Regulatory cells 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 Tregs from AA patients are expandable in vitro and retain the suppressive function while maintaining the polyclonality (19,20). These observations led us this phase 1 clinical trial to assess the safety and kinetics of infused autologous Tregs in AA patients expanded under GMP conditions . Tregs could be expanded *in-vitro* in all 6 patients and expanded sufficiently to administer the trial stipulated dosage of $5x10^6$/kg body weight on two occasions two weeks apart. Three of the patients showed a haematological improvement and in one responder patient 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 Tregs post infusion compared to the nonresponders. In our patients infused Tregs peaked at day 28. Importantly, we demonstrated that the expansion of Tregs sustained over time, with detectable levels persisting in the bone marrow up to six months post-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 1 studies where patients who receive the treatment often have refractory resistance disease and have received several lines of previous therapy . All patients had relapsed and/or refractory disease, with at least transfusion dependency. All patients received at least Ciclosporin and/or Eltrombopag and CsA alternatives (Tacrolimus in patient 4 and 5), as a backbone of IST. Horse Anti-thymocyte globulin (hATG), treatments can be associated with significant toxicity, particularly in the elderly and all of the patients in this

cohort , bar patient No.5 were more than 70 years of age with co-morbidities. Patient 5, refused hATG treatment due to personal choice about her beliefs with animal welfare. Patient 2 was enrolled, because of loss of graft function/relapse of aplastic anaemia after a stem cell transplant. Salvage treatment with Eltrombopag, failed to improve blood counts, and she remained transfusion dependent.

Previous studies conducted in the context of HSCT using HLA disparity as markers demonstrated persistence of expanded Tregs for up to 14 days, while Tregs labelled 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 circulation after one year (31-33).

Comparative analysis of expanding Tregs revealed distinct phenotypic differences from pre-existent Tregs, 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, Tregs expanding post-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 infused Tregs, 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 Tregs derived from cord blood (CB) in four patients with AA lead to haematological responses in two patient (34). It is noteworthy that like expanded autologous Tregs, CB derived Tregs were also well tolerated and not associated with any untoward effect.

Our study provides proof of concept of safety and efficacy of autologous Tregs that can be successfully expanded from AA patients and provides key insights into the fate, heterogeneity, of *in vitro* expanded Tregs 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 haematological responses we plan to extend the trial to a further cohort of patients treated with higher doses ($1x10^7$/kg) of expanded regulatory T cells, that will allow evaluation of the invivo kinetics and effectiveness of infused cells and provide insights into criteria for phase 2 trial.

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Table 1: Clinical characteristics of patients treated with Expanded Autologous T-regulatory cells

	Age (y) Sex	Disease severity	Previous treatment for Aplastic Anemia	Pre-Treg* infusions	Post-Treg infusions	Response	Follow-up days since first infusion of Tregs
KCH-001	74F	SAA	No response to CsA*, Eltrombobag. Unsuitable for hATG*	PRC* transfusion every two weeks and platelet transfusion weekly	PRC and platelet transfusion, as before pre-treatment with Tregs	NR	239
KCH-002	74F	NSAA	Sibling allogenic HSCT* failed engraftment with CMV* activation. Failed treatment with CsA and Eltrombopag	PRC transfusion once every four weeks and platelet transfusion when required.	Transfusion Independent and normalization of FBC*. Infusion of second dose of expanded Tregs	CR	758
KCH-003	71M	VSAA	Failed IST with 2 X hATG and CsA, Eltrombopag	PRC transfusion weekly and platelet transfusion once-twice weekly	PRC weekly, Platelet Tx 3x/weekly, and prolonged hospitalization with N/IPA (Neutrophil < 0.1 throughout)	NR	283 (Salvage, Allo HSCT from a volunteer unrelated donor
KCH-004	79F	NSAA	No response to Erythopoietin, CsA, Tacrolimus and Eltrombopag. Unsuitable for hATG.	PRC transfusion every week.	Transfusion independent and complete normalization of blood counts	CR	737
KCH-005	67F	SAA	Loss of response to CsA, Eltrombopag and Tacrolimus. Patient declined hATG	PRC transfusion once every two-three week and platelet transfusion weekly.	Weekly PRC and platelet transfusion	NR	253
KCH-006*	76M	SAA	No response to CsA, Eltrombopag. Abnormal liver function preclude use of Eltrombopag. Unsuitable for hATG	PRC transfusion every week and platelet transfusion once every two weeks.	Transfusion independent	NR*	497

*hATG: horse anti-thymocyte globulin, CsA: Ciclosporin A, FBC: Full blood count, PRC: Packed red cells, T-reg: Regulatory T cells, HSCT: Haematopoietic stem cell transplant. Patient 6 didn't satisfy CR/PR criteria as in Race trial however he has remained transfusion independent since treatment with Treg cells.

Table 2: Collection and Expansion of autologous T-regulatory cells.

	Total Blood Volume (TBV) processed	Isolated Treg cell number from leukapheresis	Treg cell number after expansion	No of cells per infusion on Day 0 and Day 14	Viability (%)	Phenotype (%) ($CD4^+$, CD25+ CD127 low, $FoxP3^+$)	Suppression (1:1,1:5,1:10) %	$CD8^+$(%)
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 same dose on Day 0 and Day 14. T-regs were expanded to the required cell dose/patient kg body weight and met all the release criteria specifications, that included viability ≥60%, phenotype ≥30%, suppression ≥60%, and CD8+ ≤10%. Expanded Tregs were able to suppress proliferation of effector T cells at all T_{reg}:T_{eff} ratio. TBV processed refers to number of times the patients total blood volume is processed during apheresis.

FIGURE LEGENDS

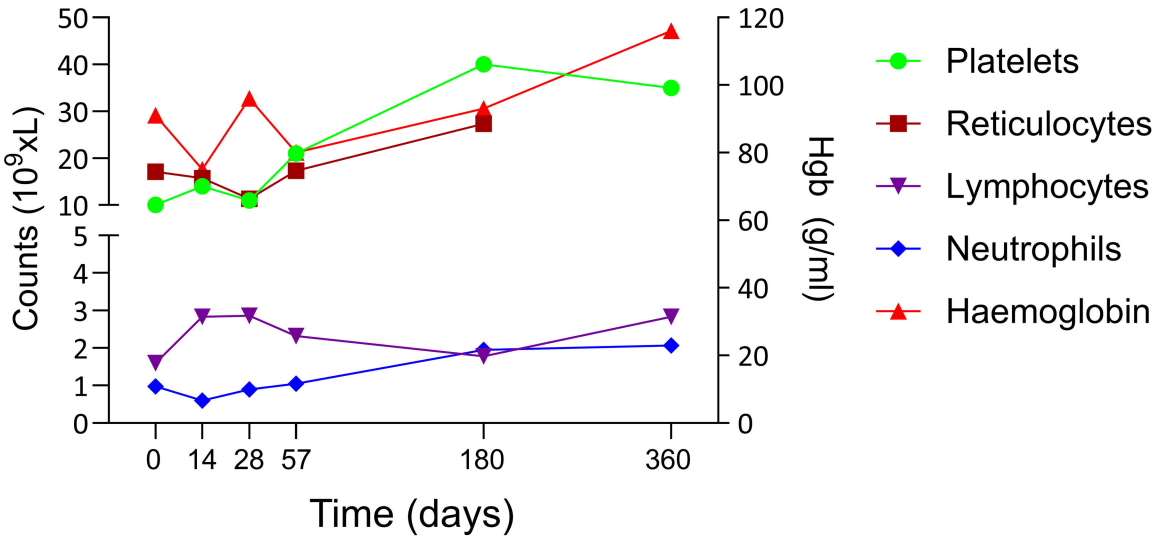
Figure 1: Changes in blood counts in two patients and bone marrow appearance (Patient 2) after infusion of two doses of expanded autologous Tregs
(a) Peripheral blood counts in Patient 2 after infusion of two doses of expanded autologous Tregs at Day 0 and Day 14. This patient attained transfusion independence after Day 28. A subsequent increase in platelet, neutrophil lymphocyte and Absolute Reticulocyte count was also observed. (b) Patient 4 also showed improvement in peripheral cell counts after infusion of two doses of expanded autologous Tregs however a viral infection resulted in a drop of platelet count at Day 58. Improvements in blood counts were observed again at Day 263. Throughout this period the patient only required transfusion once. (c) Bone marrow micrograph at magnification × 10 before (c) and × 40 (d) after infusion of two doses of Tregs in Patient 2 showing a markedly hypocellular bone marrow pre-treatment and a normocellular marrow with maturation in all three cell lineages post-treatment consistent with haematological response. Shown are data from Day 0 (baseline), 14, 28, 57, 180, 230 and 263 days after infusion of expanded autologous Tregs. Hgb (Haemoglobin), ARC (Absolute Reticulocyte count).

Figure 2: **Immune monitoring analysis on peripheral blood and bone marrow samples using Mass cytometry**
(a) UMAP plot showing the heterogeneity of $CD4^+$ T cells across all patients at four time points: D0, D14, D28, and D57. Arrows indicate the cell populations accumulating post-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 Tregs (Tregs FOX3dim and Tregs FOXP3dim $CD161^+$), expanding $IL7R^+$ cells ($IL7R^+CCR4^+CD38^+$ or $IL7R^+CCR4^+$), pre-existent Tregs (all other Treg clusters) and ratio of expanding Tregs vs pre-existent Tregs at different time point in all patients. (e) UMAP showing the heterogeneity of bone marrow resident $CD4^+$ T cells in Patient 4 at baseline and six months post infusion. Arrow indicate expanding Tregs accumulating post-infusion. (f) Frequency of expanding Tregs at baseline and six months in Patients 2 and 4.

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 Tregs (Tregs Foxp3dim and Tregs$Foxp3dimCD161^+$ defined using Boolean gating), expanding $IL7R^+$ cells ($IL7R^+CCR4^+CD38^+$ or $IL7R^+CCR4^+$) and pre-existent Tregs (all other Treg clusters). (c) Contour plots showing the expression of IL7R, FOXP3, and CD39 in total $CD4^+$, pre-existent Tregs and expanding Tregs in the bone marrow six months post infusion. (d) UMAP plot describing the heterogeneity of $CD4^+$ T cells from Patient 4 at different time points as defined by scRNAseq. (e) Boxplot showing the Log2 FC in expression compared to non-Treg cells in pre-existent (blue) and expanding Tregs (yellow) cells. Shown are genes with at least a 1.4 fold difference in all three patients. (f) Boxplot showing the Log2 FC in expanding compared to pre-existent Tregs. Shown are genes with at least a 1.4-fold difference in all three patients.

Figure 4: TCR overlap between the infusion product and Treg clusters: (a) Scatter plot showing for each cluster the fold change frequency at day14 compared to baseline, and the Morisita index of the repertoire overlap with the infusion product for Patients 1, 2 and 4. (b) Morisita index matrix showing the overlap between each group of clusters and the infusion product at different time points for Patient 4. (c) Morisita index matrixes showing the overlap between each group of clusters and the infusion product across all three patients and across all the different time points.

a


Counts (10^9xL)
Hgb (g/ml)
Time (days)
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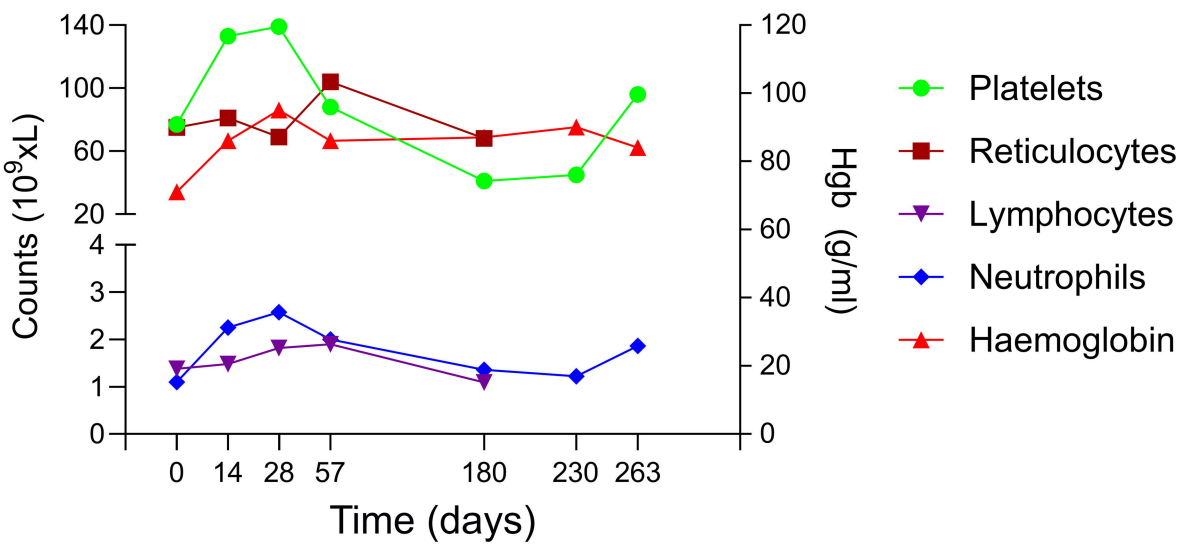
Platelets
Reticulocytes
Lymphocytes
Neutrophils
Haemoglobin

b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Time (days)
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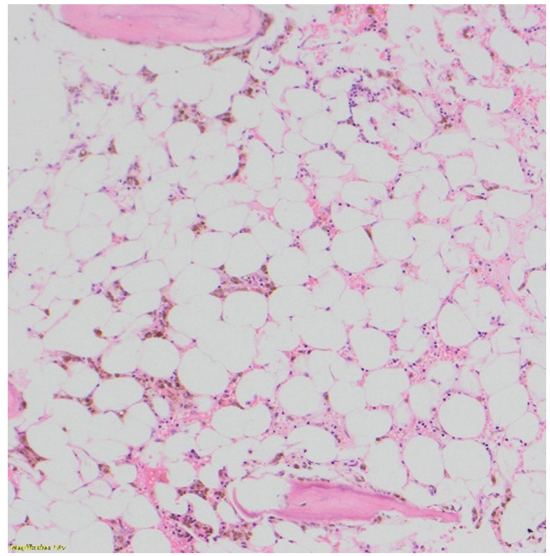
Platelets
Reticulocytes
Lymphocytes
Neutrophils
Haemoglobin

c



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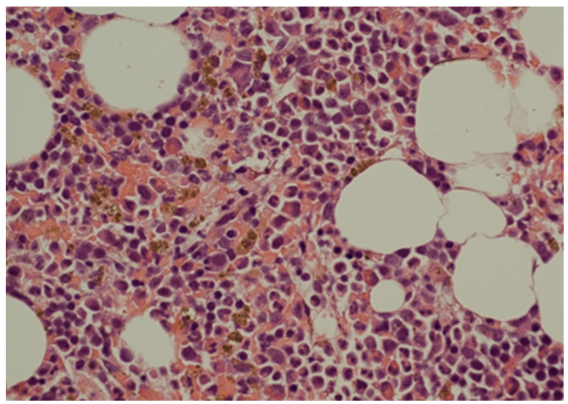
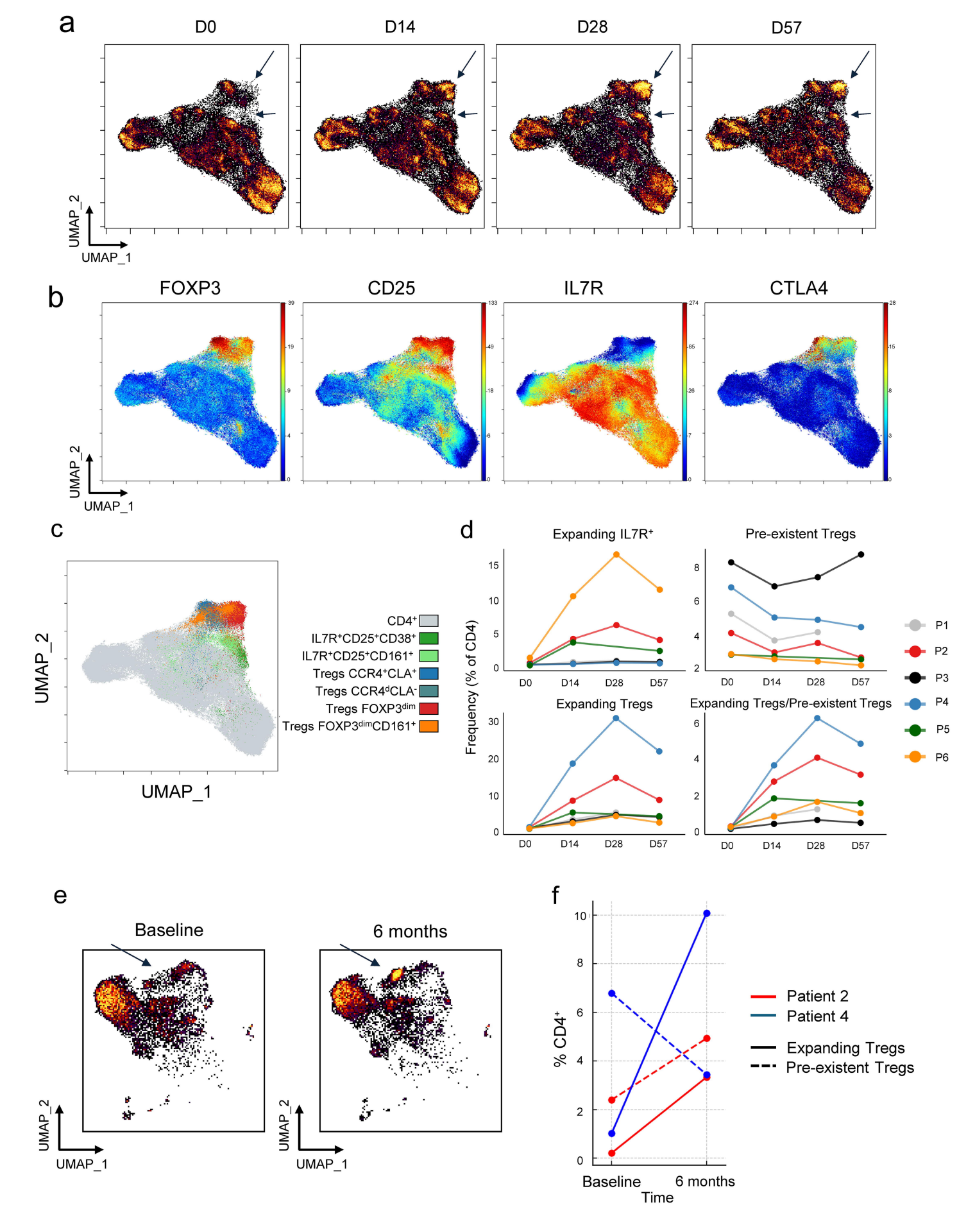
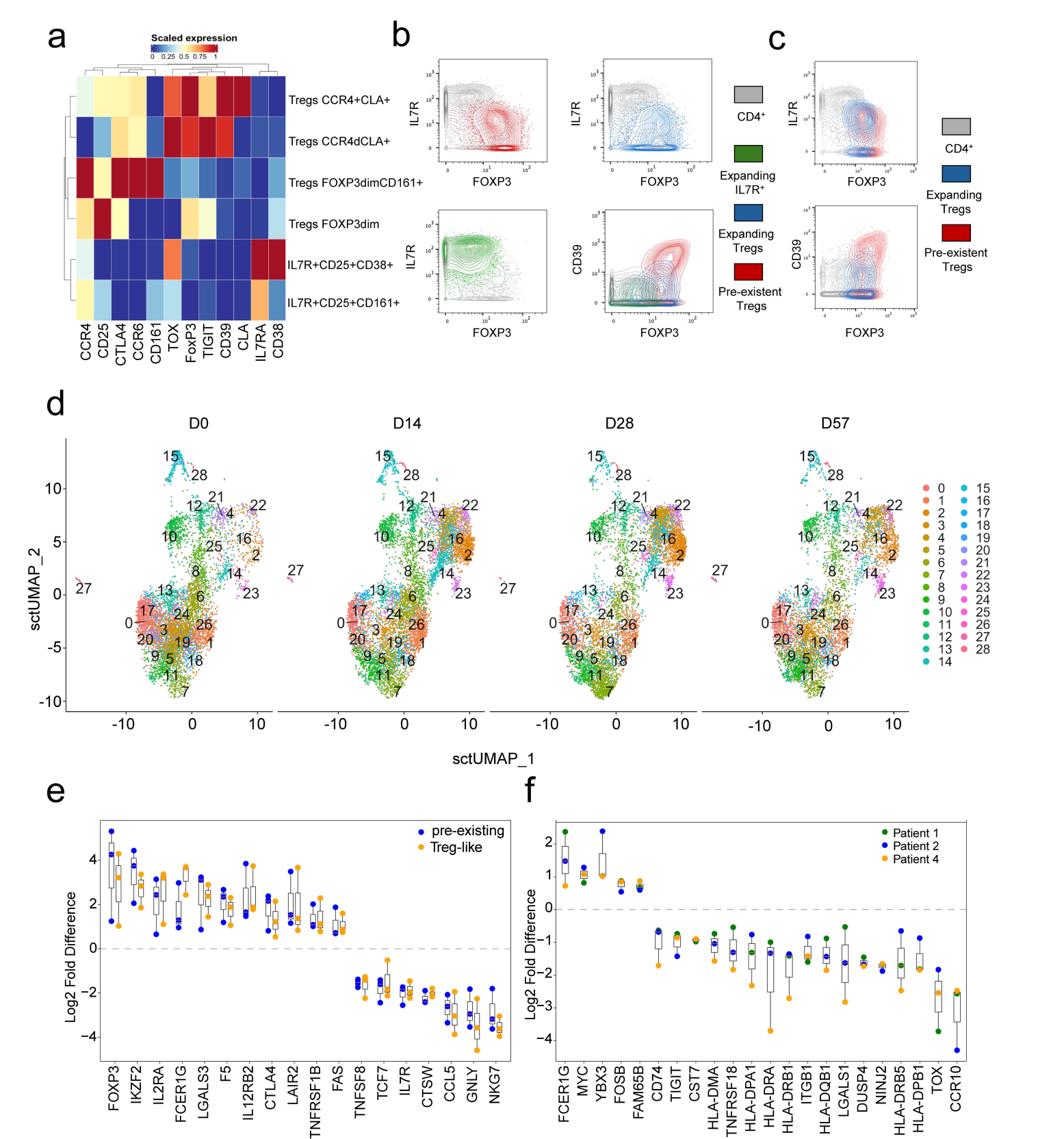



a
D0
D14
D28
D57
UMAP_2
UMAP_1
b
FOXP3
CD25
IL7R
CTLA4
UMAP_2
UMAP_1
c
UMAP_2
UMAP_1
CD4+
IL7R+CD25+CD38+
IL7R+CD25+CD161+
Tregs CCR4+CLA+
Tregs CCR4dCLA-
Tregs FOXP3dim
Tregs FOXP3dimCD161+
d
Expanding IL7R+
Pre-existent Tregs
Expanding Tregs
Expanding Tregs/Pre-existent Tregs
Frequency (% of CD4)
D0
D14
D28
D57
P1
P2
P3
P4
P5
P6
e
Baseline
6 months
UMAP_2
UMAP_1
f
% CD4+
Baseline
6 months
Time
Patient 2
Patient 4
Expanding Tregs
Pre-existent Tregs


a
Scaled expression
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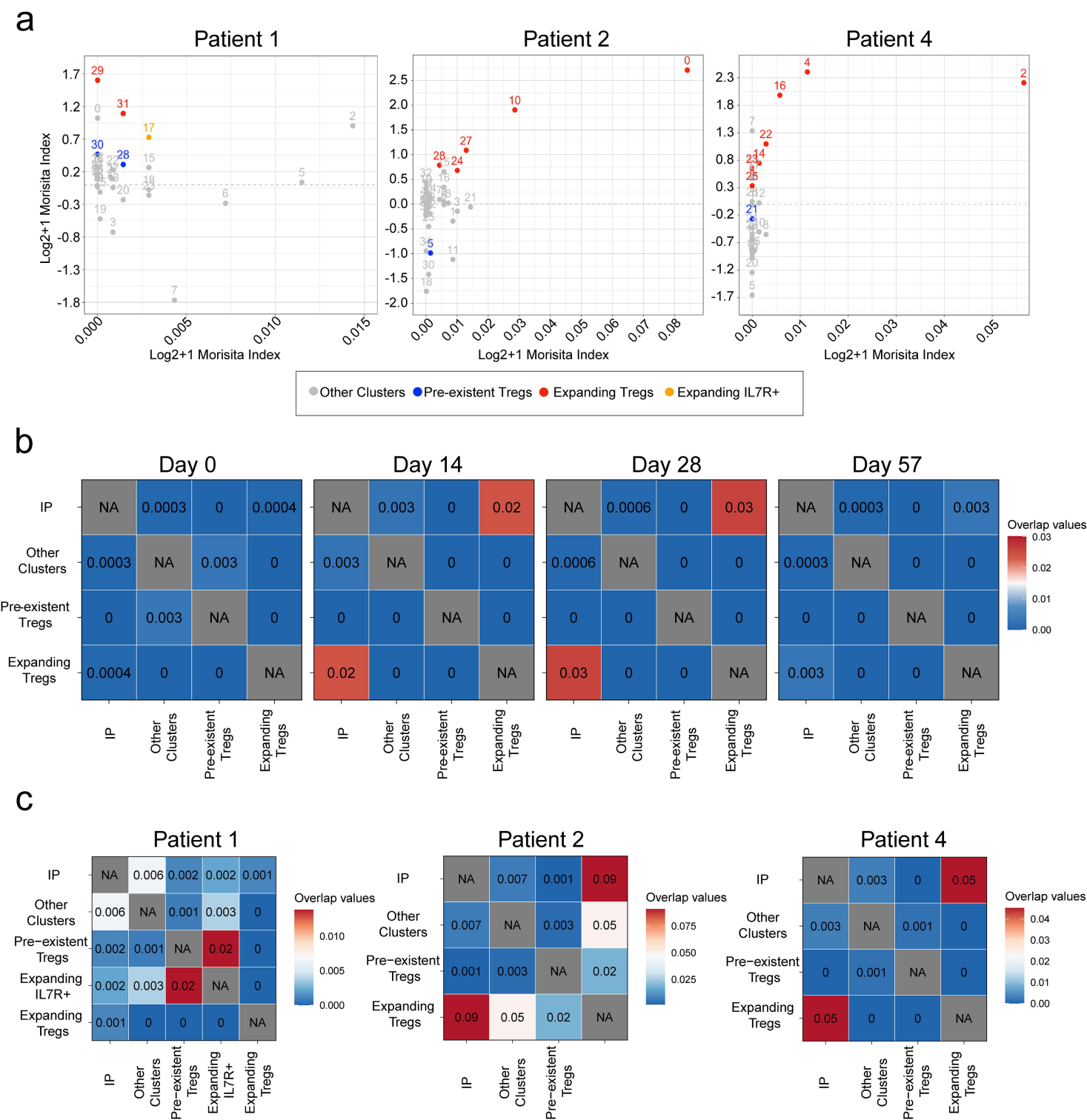
Tregs CCR4+CLA+
Tregs CCR4dCLA+
Tregs FOXP3dimCD161+
Tregs FOXP3dim
IL7R+CD25+CD38+
IL7R+CD25+CD161+
CCR4 CD25 CTLA4 CCR6 CD161 TOX FoxP3 TIGIT CD39 CLA IL7RA CD38
b
IL7R
FOXP3
CD39
CD4+
Expanding IL7R+
Expanding Tregs
Pre-existent Tregs
c
IL7R
FOXP3
CD39
CD4+
Expanding Tregs
Pre-existent Tregs
d
D0
D14
D28
D57
sctUMAP_2
sctUMAP_1
e
pre-existing
Treg-like
Log2 Fold Difference
FOXP3 IKZF2 IL2RA FCER1G LGALS3 F5 IL12RB2 CTLA4 LAIR2 TNFRSF1B FAS TNFSF8 TCF7 IL7R CTSW CCL5 GNLY NKG7
f
Patient 1
Patient 2
Patient 4
Log2 Fold Difference
FCER1G MYC YBX3 FOSB FAM65B CD74 TIGIT CST7 HLA-DMA TNFRSF18 HLA-DPA1 HLA-DRA HLA-DRB1 ITGB1 HLA-DQB1 LGALS1 DUSP4 NINJ2 HLA-DRB5 HLA-DPB1 TOX CCR10


a
Patient 1
Patient 2
Patient 4
Log2+1 Morisita Index
Log2+1 Morisita Index
Log2+1 Morisita Index
Log2+1 Morisita Index
Other Clusters
Pre-existent Tregs
Expanding Tregs
Expanding IL7R+
b
Day 0
Day 14
Day 28
Day 57
IP
Other Clusters
Pre-existent Tregs
Expanding Tregs
Overlap values
c
Patient 1
Patient 2
Patient 4
IP
Other Clusters
Pre-existent Tregs
Expanding IL7R+
Expanding Tregs
Overlap values

SUPPLEMENTARY DATA

Contents

SUPPLEMENTARY METHOD

CyTOF

A panel of antibodies tagged with a rare metal isotope based on surface markers, transcription factors and cytokines were designed. Cells were first barcoded, pooled together and then stained with Cell-ID Rhodium-103 (Fluidigm) to distinguish live and dead cells. Intracellular staining was performed after fixation and permeabilization using FoxP3/Transcription Factor Staining Buffer Set (eBioscience™). Before data acquisition, stained cells were DNA stained using Cell-ID Intercalator-Ir (Fluidigm).

Single cell sequencing

Cell Preparation

Patient samples were enriched for T cells using the Pan T Cell Isolation Kit (Miltenyi Biotec). The enriched cell populations were co-stained using the following antibodies: CD4, CD8, CD25, CD127(obtained from, BD® AbSeq Antibody-Oligos, and BD® SampleTag (ST) antibodies BD® Single-Cell Multiplexing Kit, BD Biosciences). The staining protocol followed the manufacturer's guidelines. The AbSeq panel used consisted of the BD® AbSeq Immune Discovery Panel, supplemented with 11 additional single AbSeq oligonucleotides (BD Biosciences).

The cells were sorted into distinct populations: total CD4+ T cells, CD8+ T cells, and regulatory T cells (Tregs). The sorted cell populations were pooled and analysed using the BD Rhapsody™ Express Single-Cell Analysis System., The retrieved beads were washed, and reverse transcription was performed using the BD Rhapsody™ cDNA Kit (BD Biosciences).

Library Preparation and Sequencing

Following reverse transcription, AbSeq, mRNA, SampleTag (ST), and T cell receptor (TCR) libraries were prepared using the BD Rhapsody™ The mRNA libraries were constructed using a custom gene panel (ID: 0002174, BD Biosciences) as well as the BD Rhapsody™ Immune Response Panel HS (BD Biosciences).

The quality of the libraries was evaluated using the Agilent TapeStation 4150 system. The libraries were sequenced on the NovaSeq X Plus (Illumina) or NovaSeq 6000 platforms (Illumina) with paired-end 150 bp reads (PE150).

Data Processing and Analysis

The raw sequencing data was uploaded to the Seven Bridges Genomics platform. Sequencing reads were demultiplexed, aligned to the reference genome, and filtered using the BD Rhapsody™ Sequence Analysis Pipeline v2.2. Generated molecular count matrices per cell, were imported into Seurat v5 for downstream analysis.

Single cells were selected on SampleTag (ST) identifiers, and datasets integrated using canonical correlation analysis (CCA). Uniform Manifold Approximation and Projection (UMAP) embeddings were computed and TCR sequences were incorporated into the Seurat metadata, and clonotype overlap between samples was analyzed (Satija Lab)(17).Clonotypes were defined based on cells sharing identical CDR3α and CDR3β sequences.

Cytokine Estimation

Cytokine Analysis was performed by Synnovis using their test code CY-19, CY-20, CY-21 and CY-23. All cytokine levels were measured in pg/ml

1.	IL-1β, IL-6, IL-8, TNF-α	CY-19
2.	IL-2RA, IL-10, 1L-17α INF-γ	CY-20
3.	IL-15, GM-CSF, MCP-1 VEGF	CY-21
4.	IL-15, IL-13	CY-23

Patient 1

Day	IL-1β	IL-6	IL-8	TNF-α	IL-2Ra	IL-10	IL-17a	INF-γ	IL-15	GM-CSF	MCP-1	VEGF	IL-5	IL-13
0	<0.80	4.62	119	12.8	2354	7.62	6.15	1.88	4.14	<1.40	480	45.6	2.21	<7.20
14	<0.80	5.96	151	10.7	3145	7.35	8.34	0.91	4.3	<1.40	674	42.8	1.74	141
28	<0.80	10.9	174	10.6	3192	7.06	13.6	1.08	4.24	<1.40	643	56.8	1.1	35.1

Patient 2

Day	IL-1β	IL-6	IL-8	TNF-α	IL-2Ra	IL-10	IL-17a	INF-γ	IL-15	GM-CSF	MCP-1	VEGF	IL-5	IL-13
0	<0.80	2.86	20.4	14.2	2277	3.76	<2.10	1.63	3.11	<1.40	399	49.2	0.45	<7.2
14	<0.80	2.11	34.4	19	2868	4.45	<2.10	1.31	4.52	<1.40	414	55.8	0.46	<7.2
28	<0.80	2.31	26.9	15.5	2729	4.23	<2.10	0.82	4.72	<1.40	430	48.3	0.28	<7.2

Patient 3

Day	IL-1β	IL-6	IL-8	TNF-α	IL-2Ra	IL-10	IL-17a	INF-γ	IL-15	GM-CSF	MCP-1	VEGF	IL-5	IL-13
0	<0.80	8	51.8	5.63	2132	2.18	<2.10	5.58	5.34	<1.42	911	67.6	0.95	<7.20
14	<0.80	5.13	46.4	5.16	1959	1.92	<2.10	2.05	4.88	<1.74	1042	57.6	0.4	<7.20
28	<0.80	4.47	41.2	5.08	1829	2.04	<2.10	1.21	4.57	<1.40	874	69.8	0.49	<7.20

Patient 4

Day	IL-1β	IL-6	IL-8	TNF-α	IL-2Ra	IL-10	IL-17a	INF-γ	IL-15	GM-CSF	MCP-1	VEGF	IL-5	IL-13
0	<0.80	4.66	23.2	10.4	1501	3.45	<2.10	0.92	3.22	<1.40	510	171	0.92	<7.20
14	<0.80	21.7	98.9	11.7	1558	3.56	<2.10	0.83	2.73	<1.40	446	214	0.33	<7.20
28	<0.80	24.3	31	11.1	1792	3.24	<2.10	0.64	2.79	<1.40	430	223	0.56	<7.20

Patient 5

Day	IL-1β	IL-6	IL-8	TNF-α	IL-2Ra	IL-10	IL-17a	INF-γ	IL-15	GM-CSF	MCP-1	VEGF	IL-5	IL-13
0	<0.80	10.3	100	8.11	3223	3.61	<2.10	1.57	5.74	<1.40	583	54	1.9	<7.20
7	<0.80	7.92	39.4	7.13	3417	3.63	<2.10	1.51	4.96	<1.40	480	40.2	1.8	<7.20
14	<0.80	12	65.4	7.41	3522	3.5	<2.10	2.66	6.88	2.7	556	54.9	1.52	<7.20

Patient 6

Day	IL-1	IL-6	IL-8	TNF-α	IL-2Ra	IL-10	IL-17a	INF-γ	IL-15	GM-CSF	MCP-1	VEGF	IL-5	IL-13
8	<0.80	4.24	31.10	7.88	2570	8.26	<2.10	0.99	2.62	<1.40	326	42.80	0.67	17.30
14	<0.80	4.67	37.40	8.87	2757	13.30	<2.10	1.04	2.93	<1.40	511	55.30	1.07	17.70
29	<0.80	10.10	47.90	12.30	3518	15.10	<2.10	7.64	3.76	<1.40	448	43.60	1.26	14

SNP- Array

The sample was analysed by CytoscanHD microarray which can detect copy number aberrations (CNA) at resolution of 25kb for losses and 50kb for additions and analysed using Chromosome Analysis Suite v4.3 with GRCh37/arr [hg 19] genome build. In the absence of a germline sample.
1.CNA Length>5Mb will be reported. CNA length<5Mb only reported if an overlap with genes forming part of the Sanger Cancer Gene list (www.sanger.ac.uk).
2.Copy-neutral loss of heterozygosity (CN-LOH) reported if extends/includes the telomeres or if >10Mb and interstitial (Schoumans et al, 2016).
3. CN-LOH, balanced translocations and low-level mosaicism (<10% of the cells) maybe undetectable.
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Myeloid Gene Panel

The sequencing method uses QiaSeq targeted amplicon and an Illumina NextSeq 550. Targets covered: ANKRD26 (all coding and 3 UTR); ASXL1(exon 12); BCL2 (all coding); BCOR (all coding); CALR (exon 9); CBL (exons 7+8+9); CEBPA (all coding); CSF3R (exons 13 to 18); CUX1 (all coding); DDX41 (all coding); DNMT3A (all coding); ETV6 (all coding); EZH2 (all coding); FLT3 (exons 14+15+20); GATA1 (all coding); GATA2 (all coding); GNB1 (all coding); HRAS (exons 2+3); IDH1 (exon 4); IDH2 (exon 4); IKZF1 (exons all coding); JAK2 (all coding); KIT (exons 2, 8 to 11, 13+17); KMT2A (all coding); KMT2C (all coding); KRAS (exons 2+3); MPL (all coding); NF1 (all coding); NFE2 (all coding); NPM1 (exon 12); NRAS (exons 2+3); PHF6 (all coding); PPM1 D (all coding); PTPN11 (exons 3+13); RAD21 (all coding); RUNX1 (all coding); SETBP1 (exon 4); SF3B1 (exons 12 to 16); SH2B3 (all coding); SRSF2 (exon 1); STAG2 (all coding); STAT5B (all coding); TET2 (all coding); TP53 (all coding); U2AF1 (exons 2+6); UBA1 (all coding); WT1 (exons 7+9); ZRSR2 (all coding). For regions with read depth >400x, the limit of detection = 5% clone size. For sub-optimal coverage (200-399x), this is 10%.

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PNH CLONE ANALYSIS (PNH)

PNH clone was detected in 4 patients at a median size of 19.39% (0.39-31.39) in the granulocytes 15.6% (1.85-31.07) in the monocytes, 2.8% (0.25-5.82) in red blood cells. Type II red cell clones were 0.3% (0.22-0.38) and Type III clones 2.6% (0.08-5.44).

GMP TESTING

Suppression assay

Suppression assay is a GMP in-house validated test. To assess the suppressive activity of expanded Tregs, effector T cells (Teff) were stained with a fluorescent proliferation dye (CellTrace, Invitrogen) and co-cultured with allogenic expanded Tregs (1:1, 1:5 and 1:10 ratio) stained with PKH-26 (Sigma) for 5 days in the presence of anti CD3/CD28 beads. After 5 days of culture, cells were harvested and stained with Live/ Dead Blue Dead cell stain. The samples were run on MACSQuant Analyser and data were analysed using FlowJo, software.

Mycoplasma detection

Mycoplasma detection using qPCR is an in-house validated test performed at the GMP facility at Guys using validation protocol CRF-PRO-103

Mycoplasma detection is based on the MycoSEQ qPCR method (Life Technologies 4460627). Validation is based upon Ph. Eur. 2.6.7 recommendations. Information provided by the supplier demonstrates that the primers used in the PCR assay provide specific detection of mycoplasma species (M. argini, M. fermentans, M. hominis, M. hyorhinis, M. orale, M. pneumoniae, M. salivarium and S. citri), but do not lead to amplification of other DNA templates (CHO, Human, Mouse, B. cerus, B. subtilis, C. albicans, C. perfringens, C. sporogenes, E. coli, E. hirae. L. delbrueckii, M. luteus, S. aureus and S. epidermidis).

Endotoxin

The endotoxin assay by Limulus Amebocyte Lysate (LAL; Ph. Eur. 2.6.14) has been validated by Scottish National Blood Transfusion Service (SNBTS).

Sterility

The sterility assay was performed by rapid test method (Ph. Eur. 2.6.27). The rapid test method (Ph. Eur. 2.6.27) has been validated in collaboration with Scottish National Blood Transfusion service under Protocol VD NAT 837 (Validation of BacT/ALERT Testing for Treg Products (TR005). Method suitability was conducted in collaboration with the SNBTS. The inocula (~100 cfu) were identified using the Vitek 2 automated laboratory System.

Phenotype

This is a qualified in-house GMP quality control assay. A sample of bulk Drug Product was stained with a fluorescent Live/Dead marker, and fluorescent antibodies against cell-surface markers CD4, CD8, CD25, $CD127^{low}$ and then permeabilized and stained using fluorescent anti-Foxp3 antibodies. The samples were run on MACSQuant Analyser and data were analysed using FlowJo, software.

SUPPLEMETARY TABLES

Table S1: Antibody panel used for CyTOF

Channel	Target	Supplier	Catalogue Number
	FITC anti iNKT cell	BioLegend	342906
153Eu	Va7.2 (3C10)	Standard Biotools	3153024B
159Tb	CD197 (CCR7)	BioLegend	353237
141Pr	CD196 (CCR6)	Standard Biotools	393502
146Nd	CXCR5 (J252D4)	BioLegend	356902
209Bi	CD16 (3G8)	Standard Biotools	3148004B
89Y	CD45 (H130)	Standard Biotools	3089003B
106Cd	CD8a	BioLegend	301002
110Cd	CD3 (UCHT1)	BioLegend	300438
113Cd	CD244 (2-69)	BioLegend	393502
144Nd	CD38 (HIT2)	Standard Biotools	3144014B
145Nd	TCRgd (REA591)	Miltenyi	130-122-291
154Sm	CD161 (HP-3G10)	BioLegend	339902
158Gd	CD194 (CCR4)	Standard Biotools	3158032A
165 Ho	CD127 (IL-7Ra)	Standard Biotools	3165008B
166 Er	CD25 (IL-2R)	BioLegend	356102
167 Er	TIGIT (A15153G)	BioLegend	372702
169 Tm	TIM-3	BioLegend	345019
173 Yb	HLA-DR (L243)	Standard Biotools	3173005B
174 Yb	CXCR3	BioLegend	353733
175 Lu	CD279 (PD-1)	Standard Biotools	3175008B
111Cd	CD4 (RPA-T4)	BioLegend	300570
112Cd	CD56 (HCD56)	BioLegend	318302
147 Sm	CD28	BioLegend	302902
148 Nd	CD45RA	BioLegend	304102
151 Eu	CD103 (Ber-ACT8)	BioLegend	350202
155 Gd	CD27 (L128)	Standard Biotools	3155001B
172 Yb	CCR5	BioLegend	359102
115 In	CD57	BioLegend	359602
150 Nd	CD39	BioLegend	328202
152 Sm	CD31 (WM59)	BioLegend	303102
156 Gd	CD35 (E11)	BioLegend	333402
160 Gd	CD21 (544408)	Bio-Techne	MAB4909
164 Dy	CD95 (Fas)	Standard Biotools	3164008B
176Yb	CLA	BioLegend	321302

Internal Staining

Channel	Target	Supplier	Catalogue Number
139 La	FITC	BioLegend	408305
162 Dy	TCF1	BioLegend	655202
163 Dy	TOX (REA473)	Miltenyi	130-126-455
161 Dy	CD152 (CTLA-4)	Standard Biotools	3161004B
171 Yb	FoxP3 (150D)	eBioscience	14-4776-82
142 Nd	Eomes	eBioscience	14-4877-82
149 Sm	BCL2	BioLegend	658702
170 Er	Tbet	BioLegend	644825
168 Er	Ki67	BioLegend	644825

Table S2: Reagents used for CyTOF staining

	Reagents	Catalogue Number	Supplier
1.	Benzonase	2896216	Biolegend
2.	Heparin	H3149	
3.	Maxpar® Cell staining Buffer	2302177-01	Fluidigm
4.	Rhodium	230ID4D-24	Fluidigm
5.	Iridium	201192B	Fluidigm
6.	Trustain	14-9165-42	Invitrogen
7.	eBioscience™FOXP3/Transcription Factor Staining Buffer kit	2518973	Invitrogen
8.	Paraformaldehyde 16%	YJ380494	Thermo Scientific
9.	Maxpar® Cell Acquistion Solution Plus	201244	Standard Biotools
10.	β2-microglobulin Antibody	316302	Biolegend

Table S3: Reagents used for single cell sequencing

Reagent	Supplier	Catalogue #
BD Horizon™ APC-R700 Mouse Anti-Human CD4	BD Biosciences	564975
BD Pharmingen™ PE Mouse Anti-Human CD8	BD Biosciences	555367
BD Horizon™ BB515 Mouse Anti-Human CD25	BD Biosciences	565096
PE/Cyanine7 anti-human CD127 (IL-7Rα) Antibody	BioLegend	351320
Pan T Cell Isolation Kit, human	Miltenyi Biotec	130-096-535
CD4+ T Cell Isolation Kit, human	Miltenyi Biotec	130-096-533
MACS® BSA Stock Solution	Miltenyi Biotec	130-091-376
autoMACS® Rinsing Solution	Miltenyi Biotec	130-091-222
Hoechst 33258	Invitrogen™	H1398
BD Pharmingen™ Human BD Fc Block™	BD Biosciences	564220
BD Pharmingen™ Stain Buffer (BSA)	BD Biosciences	554657
BD Rhapsody Custom Panel 100– 199 genes (ID:0002174)	BD Biosciences	633778
BD Rhapsody™ Cartridge Kit	BD Biosciences	633733

BD Rhapsody™ cDNA Kit	BD Biosciences	633773
BD Rhapsody™ Enhanced Cartridge Reagent Kit	BD Biosciences	664887
BD Rhapsody™ Immune Response Panel HS	BD Biosciences	633750
BD Rhapsody™ Targeted mRNA and AbSeq Amplification Kit	BD Biosciences	633774
BD Rhapsody™ TCR/BCR Amplification Kit.	BD Biosciences	665345
BD® AbSeq Immune Discovery Panel	BD Biosciences	625970
BD® Single-Cell Multiplexing Kit	BD Biosciences	633781
BD™ AbSeq Oligo Mouse Anti-Human CD103	BD Biosciences	940067
BD™ AbSeq Oligo Mouse Anti-Human CD194	BD Biosciences	940047
BD™ AbSeq Oligo Mouse Anti-Human CD195	BD Biosciences	940050
BD™ AbSeq Oligo Mouse Anti-Human CD38	BD Biosciences	940013
BD™ AbSeq Oligo Mouse Anti-Human CD39	BD Biosciences	940073
BD™ AbSeq Oligo Mouse Anti-Human CD45RO	BD Biosciences	940022
BD™ AbSeq Oligo Mouse Anti-Human CD69	BD Biosciences	940019
BD™ AbSeq Oligo Mouse Anti-Human TCR γδ	BD Biosciences	950057
BD™ AbSeq Oligo Mouse Anti-Human Vδ2 TCR	BD Biosciences	940297
BD™ AbSeq Oligo Mouse Anti-Human Vγ9 TCR	BD Biosciences	940295
BD™ AbSeq Oligo Mouse Anti-Human CD95	BD Biosciences	940037
AMPure XP Reagent, 60 mL	Beckman Coulter	A63881
High Sensitivity D5000 ScreenTape	Agilent Technologies	5067-5592
High Sensitivity D5000 Reagents	Agilent Technologies	5067-5593
High Sensitivity D1000 ScreenTape	Agilent Technologies	5067-5584
High Sensitivity D1000 Reagents	Agilent Technologies	5190-6504
Qubit™ 1X dsDNA High Sensitivity (HS) and Broad Range (BR) Assay Kits	Invitrogen™	Q33231

Table S4: Reagents used for expansion of cells under GMP conditions.

	Reagents	Catalogue Number	Supplier
1.	CliniMACS CD8 Reagent, CE	200-070-115	Miltenyi Biotec
2.	CliniMACS CD25 Reagent, CE	200-070-131	Miltenyi Biotec
3.	CliniMACS Tubing Set LS, CE	200-073-204	Miltenyi Biotec
4.	CliniMACS Tubing Set, CE	200-073-104	Miltenyi Biotec
5.	CliniMACS PBS/EDTA Buffer CE 3×1000mL	200-070-025	Miltenyi Biotec
6.	MACS GMP Rapamycin	170-076-308	Miltenyi Biotec
7.	MACS GMP ExpAct Treg kit	170-076-119	Miltenyi Biotec
8.	Aldesleukin/Proleukina	31644/0003	Clinigen
9.	CryoSure-DMSO	WAK-DMSO-70	Wak-Chemie-Medical-GmbH
10.	Plasma-Lyte148	PL 00116/0332	Baxter
11.	Human AB serum	S4190	biowest®
12.	Human serum Albumin (HSA) 20%	PL04500/0012	Biotest
13.	TheraPEAK® X-VIVO® 15	BEBP02-054Q	Lonza

Table S5: Consumables used for expansion of cells within the GMP conditions.

1.	LOVO Med cell processing kit	Part No. R6R4905	Fresenius Kabi
2.	CliniMACS tubing set TS	161-01	Miltenyi Biotec
3.	CliniMACS tubing set LS	162-01	Miltenyi Biotec
4.	Sepax Processing Kit	CT 60.1	Cytiva
5.	G-Rex Bioreactors	P/N 80040S P/N 81100CS	Wolf Wilson

Table S6: Batch Release criteria specifications.

	Identity/Purity	**Patient 1**	**Patient 2**	**Patient 3**	**Patient 4**	**Patient 5**	**Patient 6**
1.	ExpAct Treg bead concentration	≤100 beads per 3×10^6 cells	≤100 beads per 3×10^6 cells	≤100 beads per 3×10^6 cells	65 beads per 3×10^6 cells	65 beads per 3×10^6 cells	92.5 beads per 3×10^6 cells
2.	Mycoplasma-PCR	Not detected	Not detected	Not detected	Not detected	Not detected	Not detected
3.	Sterility BacT/ALERT	No growth after 5 days	No growth after 5 days	No growth after 5 days	No growth after 5 days	No growth after 5 days	No growth after 5 days
4.	Endotoxin	≤7 IU/mL	≤7 IU/mL	≤7 IU/mL	≤7 IU/mL	≤7 IU/mL	<1 IU/mL

Table S7: Collection and Expansion of autologous T-regulatory cells.

	Total Blood Volume (TBV) processed	Isolated Treg cell number from leukapheresis	Treg cell number after expansion	No of cells per infusion on Day 0 and Day 14*	Viability (%)	Phenotype (%) (CD4+,CD25+,CD127 low FoxP3+)	Suppression (1:1, 1:5, 1:10) (%)	CD8+ (%)
KCH-001	2.9	181.07×10^6	4.95×10^9	307.5×10^6	97.3	73.91	73.85, 72.65 72.18	3.56
KCH-002	2.4	24.32×10^6	4.16×10^9	248×10^6	97.92	65.11	86.40, 97.60 94.25	4.22
KCH-003	2.5	68.79×10^6	3.16×10^9	420×10^6	98.7	78.69	92.95, 90.70 97.3	3.99
KCH-004	2.7	163.77×10^6	6.81×10^9	317×10^6	98.20	85.48	93.52, 90 75.14	3.57
KCH-005	2.2	90.06×10^6	4.44×10^9	390×10^6	98.10	46.64	83.57, 90.78 75.55	5.25
KCH-006	2.9	94.8×10^6	2.52×10^9	357×10^6	98.70	47.16	94.08,85.38, 75.31	4.80

*Patients received same dose on Day 0 and Day 14.T-regs were expanded to the required cell dose/patient kg body weight and met all the release criteria specifications, that included viability ≥60%, phenotype ≥30%, suppression ≥60%, and CD8+ ≤10% Expanded Tregs were able to suppress proliferation of effector T cells at all T_{reg}:T_{eff} ratios. TBV processed refers to number of times the patients total blood volume is processed during apheresis.

SUPPLEMETARY FIGURES

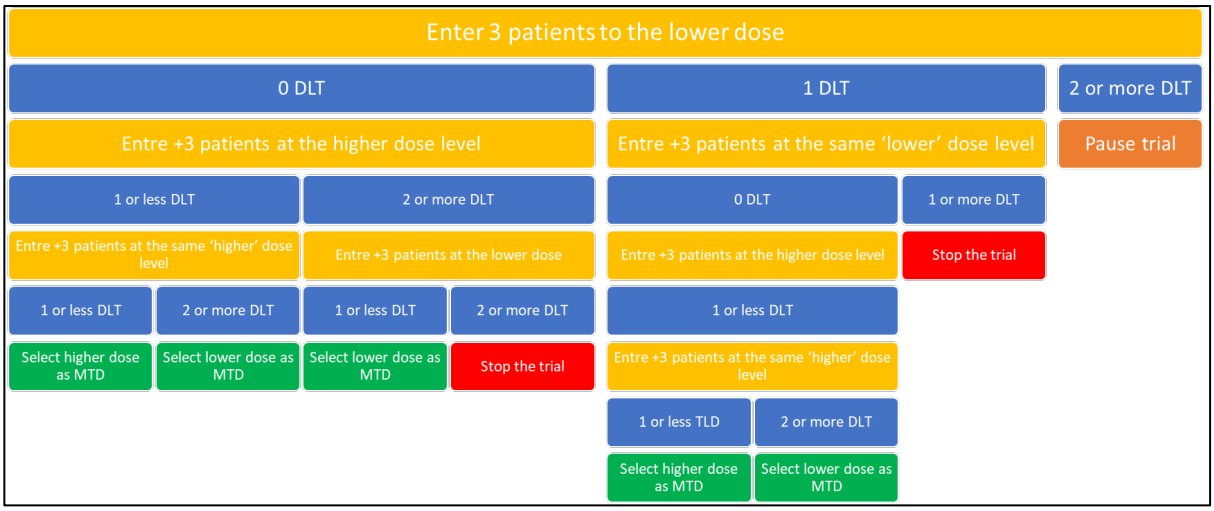

Figure S1: 3+3 Dose escalation design

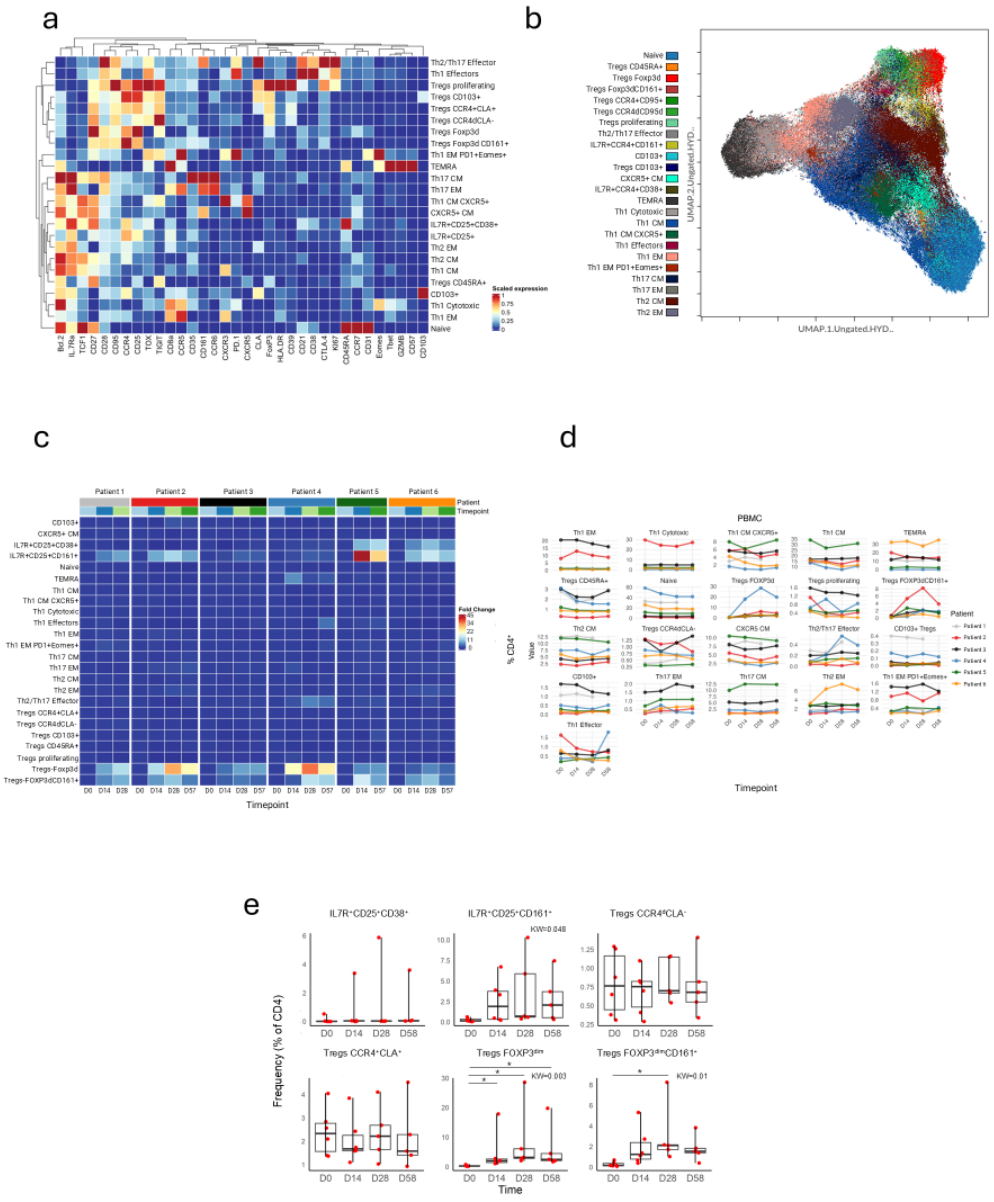

Figure S2: Immune monitoring analysis on peripheral blood samples using mass cytometry (a) Heatmap displaying the median expression levels of CD4$^+$ T cell clusters identified in the peripheral blood of treated patients by mass cytometry and phenograph analysis. (b) UMAP visualization of CD4$^+$ T cell clusters identified in panel a. (c) Heatmap showing the fold change across different time points of the clusters described in panel a and b in the six treated patients. (d) Plots describing changes in the frequency of the different cluster across different time points in the six patients. (e) Boxplots illustrating the frequency of selected CD4+ T cell clusters at different time points. The Kruskal-Wallis test (KW) was used for statistical analysis, and * indicates a p-value < 0.05 after Bonferroni correction for multiple testing. Shown are the only statistically significant comparisons identified

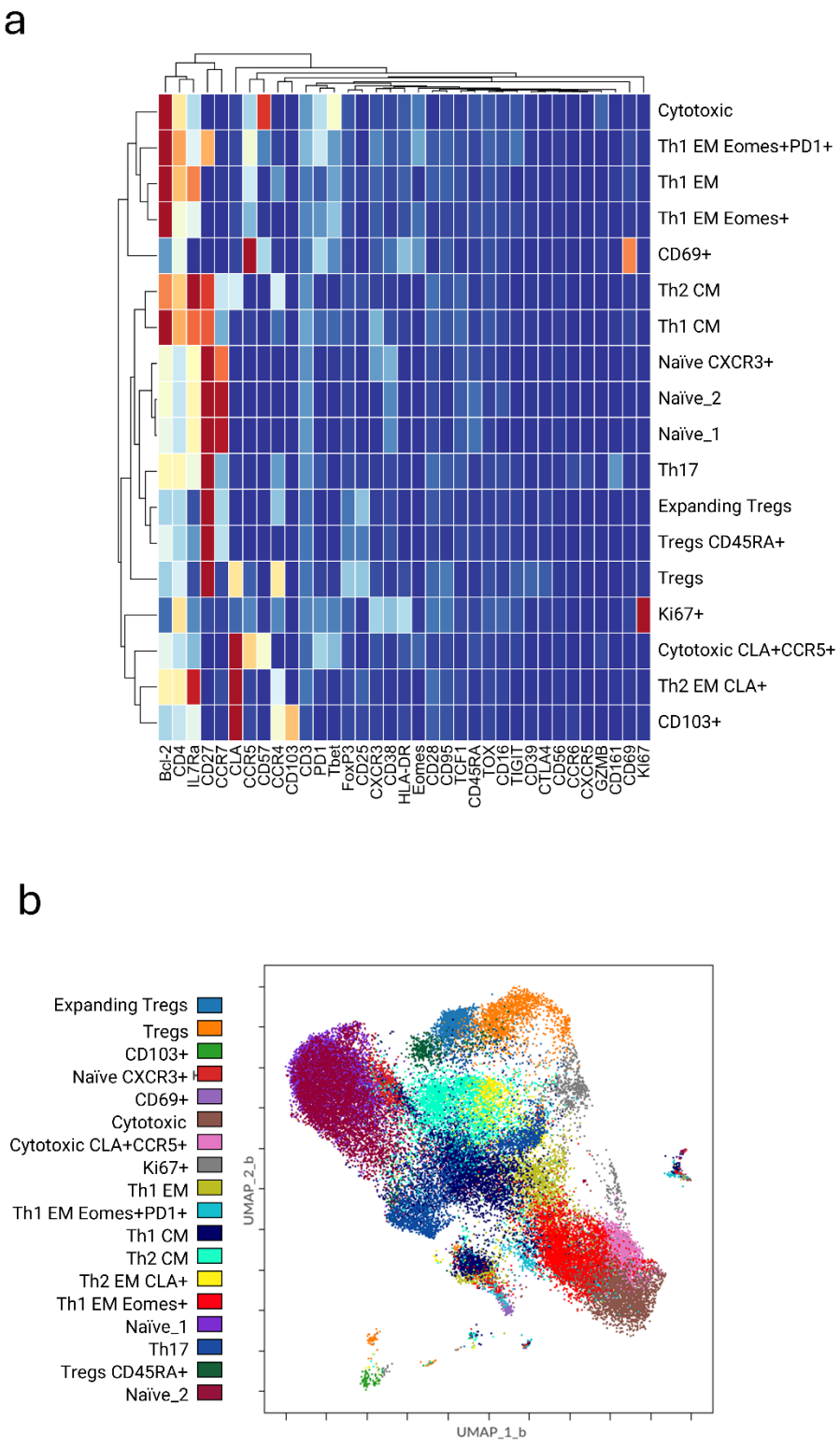

Figure S3: Mass Cytometry Analysis of peripheral blood CD8+ T cells in infused patients (a) Heatmap displaying the median expression levels of $CD4^+$ T cell clusters identified by Phenograph analysis of mass cytometry samples collected from the bone marrow of Patients 2 and 4 at baseline and six months post infusion. (b) UMAP visualization of $CD4^+$ T cell clusters described in panel a.

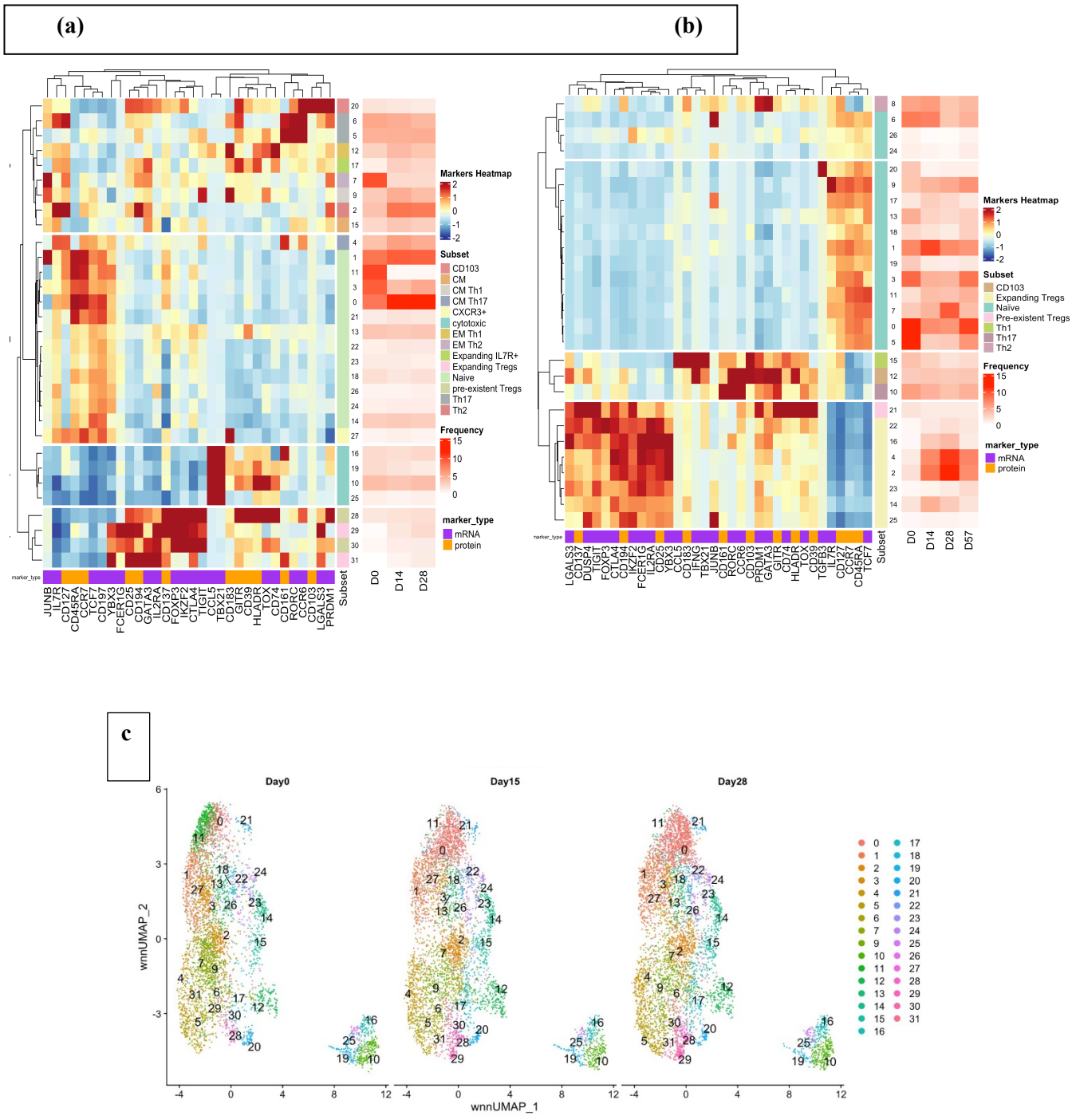

Figure S4: scRNA analysis performed on peripheral blood samples of Patient1 (a) UMAP plot describing the heterogeneity of CD4^{+} T cells from Patient 1 and Patient 4 clusters at different time points as defined by scRNAseq. (c) Combined heatmap showing the expression of selected transcript and protein markers in Patient 1 clusters and their frequency at different time points.

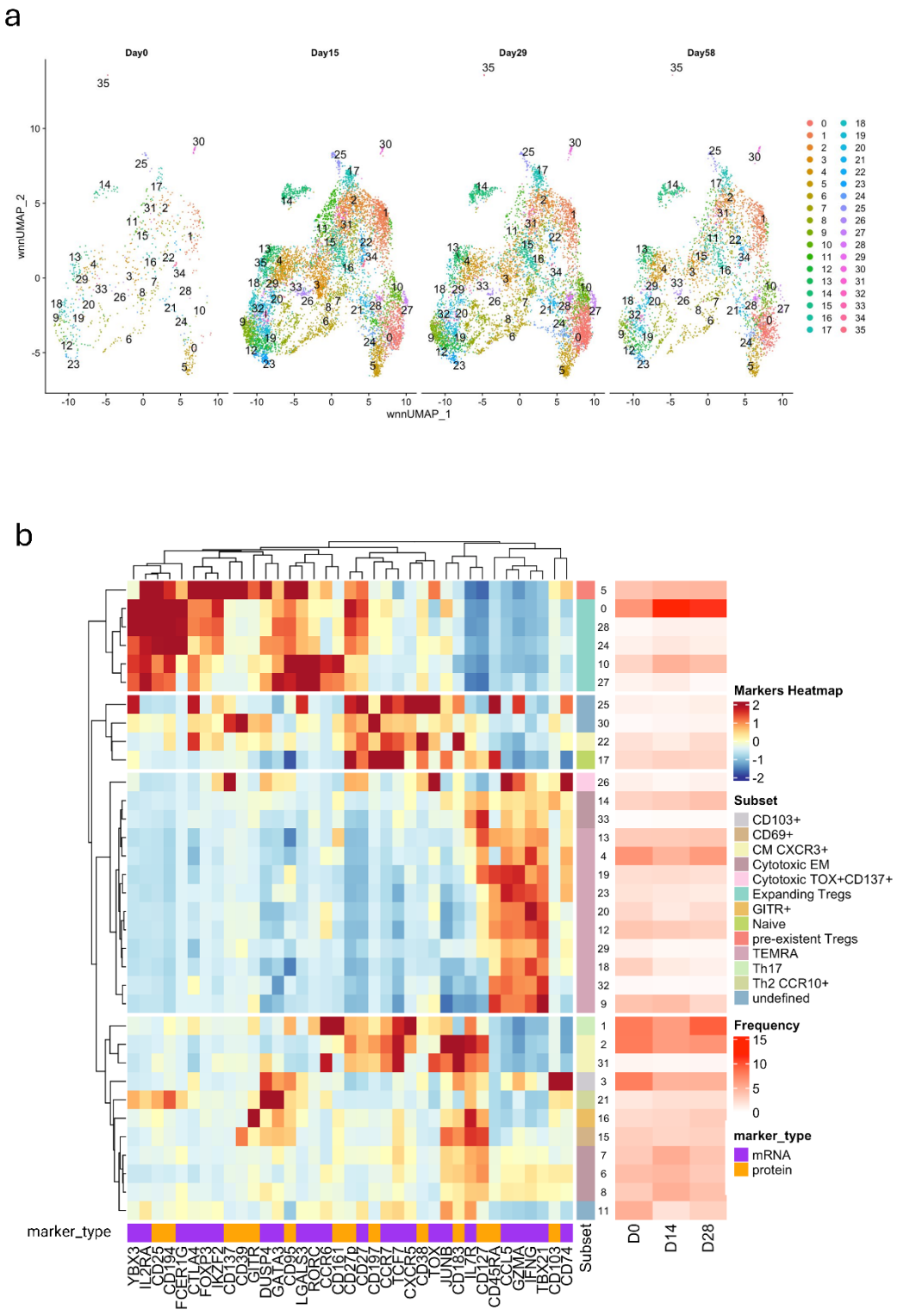

Figure S5: scRNA analysis performed on peripheral blood samples of Patient 2 (a) UMAP plot describing the heterogeneity of CD4^{+} T cells from Patient 2 at different time points as defined by scRNAseq. (b) Combined heatmap showing the expression of selected transcript and protein markers in patient 2 clusters and their frequency at different time points.

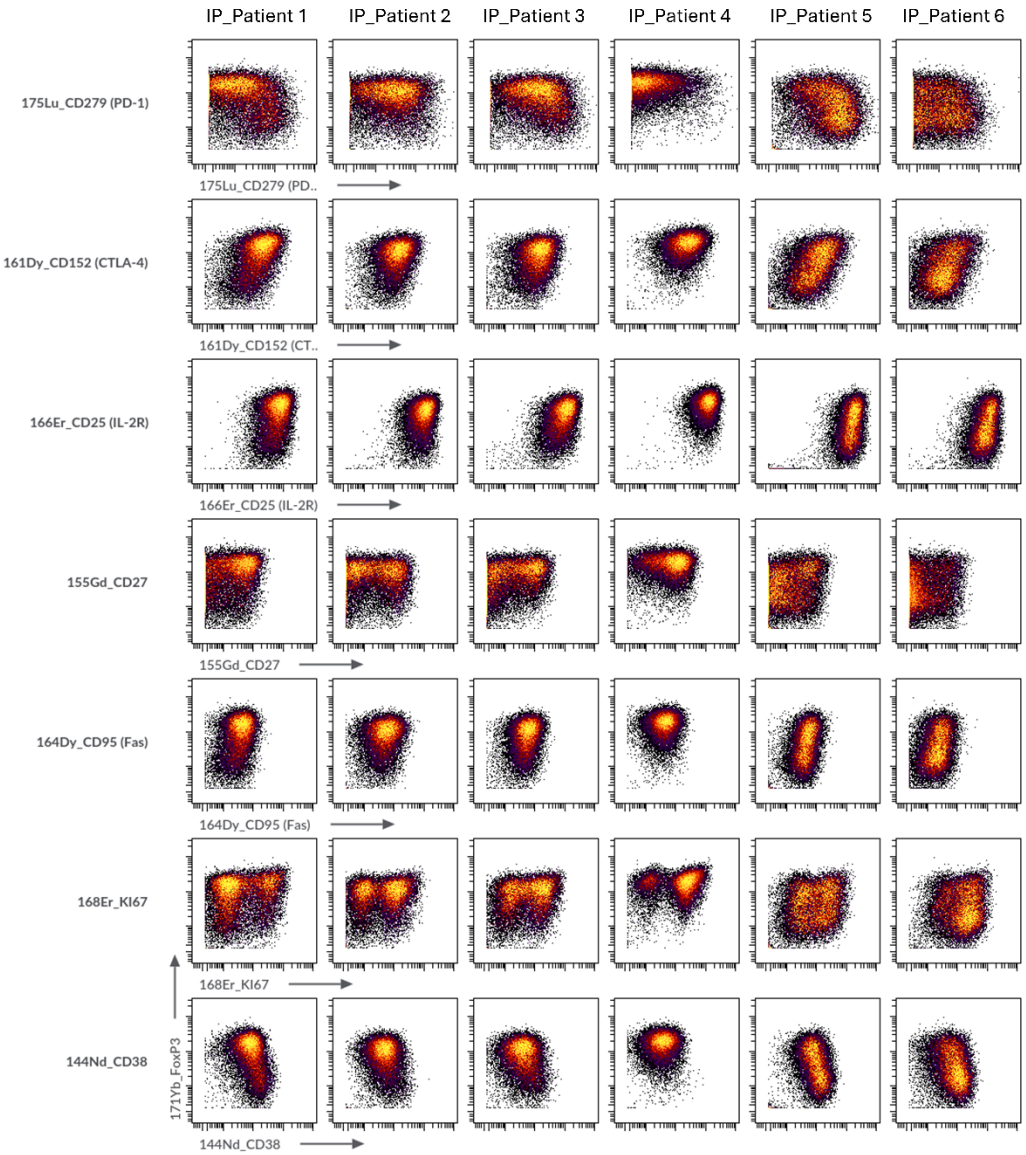

Figure S6: Plots describing the expression of different markers in the infusion products of patients 1 to 6