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Non-transplantable cord blood units as a source for adoptive immunotherapy of leukaemia and a paradigm of circular economy in medicine

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Summary

Advances in immunotherapy with T cells armed with chimeric antigen receptors (CAR-Ts), opened up new horizons for the treatment of B-cell lymphoid malignancies. However, the lack of appropriate targetable antigens on the malignant myeloid cell deprives patients with refractory acute myeloid leukaemia of effective CAR-T therapies. Although non-engineered T cells targeting multiple leukaemia-associated antigens [i.e. leukaemia-specific T cells (Leuk-STs)] represent an alternative approach, the prerequisite challenge to obtain high numbers of dendritic cells (DCs) for large-scale Leuk-ST generation, limits their clinical implementation. We explored the feasibility of generating bivalent-Leuk-STs directed against Wilms tumour 1 (WT1) and preferentially expressed antigen in melanoma (PRAME) from umbilical cord blood units (UCBUs) disqualified for allogeneic haematopoietic stem cell transplantation. By repurposing non-transplantable UCBUs and optimising culture conditions, we consistently produced at clinical scale, both cluster of differentiation (CD)34$^+$ cell-derived myeloid DCs and subsequently polyclonal bivalent-Leuk-STs. Those bivalent-Leuk-STs contained CD8$^+$ and CD4$^+$ T cell subsets predominantly of effector memory phenotype and presented high specificity and cytotoxicity against both WT1 and PRAME. In the present study, we provide a paradigm of circular economy by repurposing unusable UCBUs and a platform for future banking of Leuk-STs, as a 'third-party', 'off-the-shelf' T-cell product for the treatment of acute leukaemias.

Keywords: acute leukaemia, cord blood, dendritic cells, Wilms tumour 1, preferentially expressed antigen in melanoma, leukaemia-specific T cells.

Introduction

The therapeutic effect of allogeneic haematopoietic stem cell transplantation (allo-HSCT) in leukaemias lies with the ability of donor-derived T cells to eradicate the malignant clone mainly through recognition of recipient's minor human leucocyte antigen (HLA) or leukaemia-associated antigens (LAAs), known as the graft-*versus*-leukaemia (GvL)

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effect.[1,2] However, the beneficial donor T-cell-mediated GvL effect is often mitigated by the appearance of the undesirable graft-*versus*-host disease (GvHD) as the allograft's non-specific T cells can also recognise peptides on recipient's normal non-haematopoietic tissues; GvHD and disease recurrence represent the major causes of treatment failure after allo-HCT.[3] Hence, harnessing the power of T cell anti-leukaemic activity, without triggering GvHD, has become a challenging puzzle over recent decades.[4]

Genetically modified T cells to express chimeric antigen receptors (CAR-Ts) targeting antigens specifically expressed on the B-cell surface, have shown remarkable efficacy in the treatment of refractory B-cell malignancies.[5]

However, CAR-Ts design for acute myeloid leukaemia (AML) treatment encounters major challenges, mainly due to the lack of unique surface antigens on myeloid blasts, which would allow selective targeting of malignant cells, while sparing normal myeloid or progenitor cells. In another T-cell immunotherapeutic approach for leukaemia, T-cell receptor (TCR)-engineered T cells (TCR-Ts) express a HLA-matched TCR targeting an antigen of interest. In addition, the mispairing of the transferred with the endogenous TCR and the major histocompatibility complex (MHC) restriction barrier[6] pose significant limitations to wider clinical implementation. Despite efficacy, CAR-Ts and TCR-Ts have been associated with severe, even fatal, either 'on-target, on-tumour' or 'on-target, off-tumour', toxicities.[7,8] Moreover, genetic engineering for both products is costly and labour-intensive, while their dubious long-term survival[9,10] raises concerns about prolonged efficacy.

Non-engineered T cells generated by 'educating' naive T cells *ex vivo* to recognise LAAs and expand, represent an attractive alternative to gene-modified T cells with a low probability of inducing severe off-target effects. Indeed, *ex vivo* expanded antigen-specific T cells have shown a good safety profile with only few treatment-related adverse events.[11,12] Nonetheless, a major hurdle for clinical implementation is the very high numbers of dendritic cells (DCs) required for manufacturing.[13] The low frequency of DCs in blood[14] and the suboptimal effectiveness of current protocols to generate DCs from monocytes, challenge the up-scaling of DC production for clinical use. Given that DCs are naturally originated from HSCs and that 50–60% of the donated umbilical cord blood units (UCBUs) are disqualified for allo-HSCT,[15] the non-transplantable, albeit enriched in cluster of differentiation (CD)34$^+$ cells UCBUs, could be exploited as a source of CD34$^+$ cells for DC generation and as an autologous source for T cells to produce antigen-specific T cells.

In the present study, we provide a unique paradigm of 'circular economy' in medicine, by repurposing non-transplantable UCBUs to generate billions of DCs and subsequently clinically relevant numbers of third-party, bivalent-leukaemia-specific T cells (Leuk-STs) targeting two common LAAs [Wilms tumour 1 (WT1) and preferentially expressed antigen in melanoma (PRAME)].

Methods

The study was approved by the Institutional Review Board of the George Papanikolaou Hospital. Non-transplantable, rejected due to small volume, UCBUs were obtained from the hospital's Public Cord Blood bank, under maternal signed informed consent.

Generation of UCBU-DCs

Immuno-magnetically selected CD34$^+$ cells (Miltenyi Biotec, Bergisch Gladbach, Germany) were cultured in DC medium [Iscove's Modified Dulbecco's Medium (IMDM), 100 u/ml penicillin, 0.1 mg/ml streptomycin, 2 mmol/l Glutamax, 10% human AB serum] supplemented with 100 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF) and 50 ng/ml stem cell factor (SCF) for 4 weeks, followed by a week with GM-CSF and 50 ng/ml interleukin-4 (IL-4) (Fig 1A), as described previously.[16] The CD34$^-$ fraction of the processed UCBU was kept frozen to be later used as a source of autologous T cells. When >2 × 10^6 cells developed in culture, they were either transferred in G-Rex bioreactors (Wilson Wolf Manufacturing, Saint Paul, MN, USA) or continued in conventional plates replenishing the medium twice weekly. On day 33, DCs were induced to maturation with a Toll-like receptor ligand (TLR-L) cocktail of TLR-L3 (20 μg/ml polyI:C) and TLR-L7/8 (4 μg/ml R848) (InvivoGen, San Diego, CA, USA) for 24–48 h.

Bivalent-Leuk-ST generation

The UCBU-derived CD34$^-$ fraction, was stimulated after thawing, with autologous DCs, pulsed with peptide pools of WT1 and PRAME (1 μg/ml; JPT Peptide Technology, Berlin, Germany), at a 1:1 stimulator:responder ratio. Cells were cultured in T-cell medium [Advanced Roswell Park Memorial Institute (RPMI) containing 45% Click's medium, 2 mmol/l Glutamax and 5% human AB serum) supplemented with 10 ng/ml IL-7, 5 ng/ml IL-15, 10 ng/ml IL-12 and 100 ng/ml IL-6, as described previously.[17] On days 10, 17 and 24, cells were re-stimulated with peptide-pulsed DCs, at the same stimulator:responder ratio, and cultured with IL-7/IL-15, and subsequently IL-15 (days 13–31).

Cytotoxicity assay

Cytotoxicity against antigen-pulsed and unpulsed, autologous or partially/fully mismatched phytohaemagglutinin (PHA) blasts was performed as previously described[18] with some modifications. Autologous, non-pulsed PHA blasts and 2 h-antigen-pulsed PHA blasts were labelled with low (0·625 μmol/l) and high (5 μmol/l) concentration of CFSE (CellTrace™ CFSE Cell Proliferation Kit; Invitrogen, Waltham, MA, USA), respectively and mixed (1:1) and co-

cultured with effector cells at various effector (E) to target (T) ratios. The PHA blasts were maintained at 2×10^4 cells, in triplicates for each condition. After 20-h incubation, the cells were stained with 7-aminoactinomycin D (7AAD) to exclude dead cells. Using the ratio of $CFSE^{high}$ and $CFSE^{low}$ alive target cells without the presence of effector cells as baseline, specific cytotoxicity was calculated based on the following equation: Cell lysis (%) = 100 − [100 × Sample ($CFSE^{high}/CFSE^{low}$)/Baseline ($CFSE^{high}/CFSE^{low}$)]. Analyses were performed in a fluorescence-activated cell sorting (FACS) Calibur device with the CellQuest Pro6 software (Becton Dickinson, Franklin Lakes, NJ, USA).

Results

Log-scale expansion of DCs from UCBU-derived CD34$^+$ cells

Using a cytokine cocktail previously optimised for the expansion of DCs from commercial UCB-derived $CD34^+$ cells[16] and starting from a mean $8{\cdot}5 \pm 1{\cdot}6 \times 10^5$ $CD34^+$ cells obtained from rejected, small-volume UCBUs ($58{\cdot}75 \pm 10{\cdot}29$ ml, $n = 8$), we reached a 7×10^3 fold-expansion (Fig 1B) and clinically relevant DC numbers ($5{\cdot}9 \pm 2{\cdot}5 \times 10^9$) (Fig 1C). Expansion was irrespective of the cell culture container [G-rex devices or plates, P = not statistically significant (ns)].

Characterisation of UCBU-derived DCs

Morphology and phenotype. The UCBU-derived, TLR-L-matured DCs exhibited under microscopy the classical semi-round appearance with long dendrites (Fig 2A). During culture, $CD34^+$-expressing cells were gradually shifted to cells expressing the DC-specific integrin, CD11c, representing on average $91 \pm 7{\cdot}2\%$ of total cells by day 35 (Fig 2B, C). At the end of the culture, double positive for the myeloid-associated markers CD33 and CD11c ($88 \pm 5{\cdot}5\%$, Fig 2B, D) cells predominated, whereas the non-myeloid or lymphoid-associated markers CD19 and CD3 were insignificantly expressed ($6{\cdot}9 \pm 3\%$, $1{\cdot}5 \pm 1{\cdot}1\%$ and $2{\cdot}5 \pm 0{\cdot}3\%$, respectively, Fig 2E). In agreement with previous reports[19,20], the TLR-L-matured DCs expressed surface markers for antigen processing and presentation (Fig 2F, G), implicating adequate T-cell stimulation upon antigen encounter.

Expansion and immunophenotype of bivalent-Leuk-STs

To generate bivalent-Leuk-STs from non-usable UCBUs, the $CD34^+$-UCBU-derived DCs were pulsed with peptide pools of two LAAs (PRAME and WT1) and primed the cryopreserved 'autologous' $CD34^-$ fractions (Fig 1A). After four stimulations and an overall six fold $CD3^+$ cell expansion, the cell products reached a mean of $1{\cdot}47 \pm 0{\cdot}31 \times 10^8$ cells with two (UCB-4 and -5, Fig 3A) yielding considerably higher cell doses ($\times 1{\cdot}9$ and $\times 2{\cdot}7 \times 10^8$) over the remaining products. HLA typing was performed in all six T-cell products in order to detect possible relationships between HLAs and cell expansion (Table SI); however, no direct association could be drawn, conceivably due to the rather small sample size. The final T-cell products consisted predominantly of $CD3^+$ cells ($89 \pm 8\%$; $1{\cdot}31 \pm 0{\cdot}27 \times 10^8$ cells) (Figs 3B and 4A). Bivalent-Leuk-STs were polyclonal, with varying content in $CD4^+$ and $CD8^+$ T cells ($32 \pm 11\%$ and $64 \pm 14\%$ respectively), expressing predominantly effector memory markers, while containing insignificant numbers of myeloid DCs ($CD11c^+/CD33^+$) and regulatory T cells (Tregs) ($CD3^+CD4^+CD25^{high}$) (Fig 4A, B). Importantly also, their content in naïve T cells (1%) ($CD45RA^+/CD62L^+$), implicated in graft-*versus*-host reactivity,[21] was negligible by the end of the culture (Fig 4B). To investigate whether the prolonged antigen exposure impaired the functionality of bivalent-Leuk-STs, T-cell exhaustion markers were measured, namely programmed cell death-1 (PD-1), cytotoxic T-lymphocyte-associated protein 4 (CTLA4), T cell immunoglobulin and mucin-domain containing-3 (TIM3) and lymphocyte activation gene-3 (LAG3) after each stimulation. Both the percentage and the mean fluorescence intensity of these markers remained stably low throughout the culture (P = ns, Fig 4C, D), suggesting that the generated cell products will potentially maintain anti-leukaemic responses *in vivo*.

Specificity and cytotoxicity of bivalent-Leuk-STs

To confirm the dual-LAA-specificity, the T-cell products were evaluated after each stimulation for interferon gamma (IFN-γ) secretion in response to individual antigens. Consecutive, up to four, stimulations significantly enriched the bivalent-Leuk-STs population ($P < 0{\cdot}05$, Fig 5A, B), suggesting effective antigen-specific priming of the 'uneducated', naïve T cells by UCBU-$CD34^+$-derived DCs. Notably, at the end of the culture, all UCBU-derived T-cell products were specific against both targeted antigens (Fig 5C), after subtracting the low background generated by the unstimulated cells [15±11 spot forming cells (SFC)/ $2x10^5$ input cells]. In agreement with previous reports,[22] specificity against PRAME was not only observed earlier but it was also higher than WT1 ($P < 0{\cdot}05$, Fig 5A, C), confirming the immunodominance of PRAME over WT1. Irrelevant pulsing with influenza/matrix protein 1 (MP1) peptide did not yield any anti-leukaemic specificity.

To investigate the cytolytic potential of these cells *in vitro*, we co-cultured bivalent-Leuk-STs against a mix of autologous antigen pulsed PHA blasts or partially/fully mismatched unpulsed PHA blasts with unpulsed autologous PHA blasts as control. The LAA-loaded targets were specifically recognised and lysed by the bivalent-Leuk-STs (40:1 E:T $47{\cdot}2 \pm 4{\cdot}2\%$). Irrelevant peptide-loaded and mismatched PHA blasts were barely lysed (40:1 E:T: $2{\cdot}5 \pm 1{\cdot}6\%$/

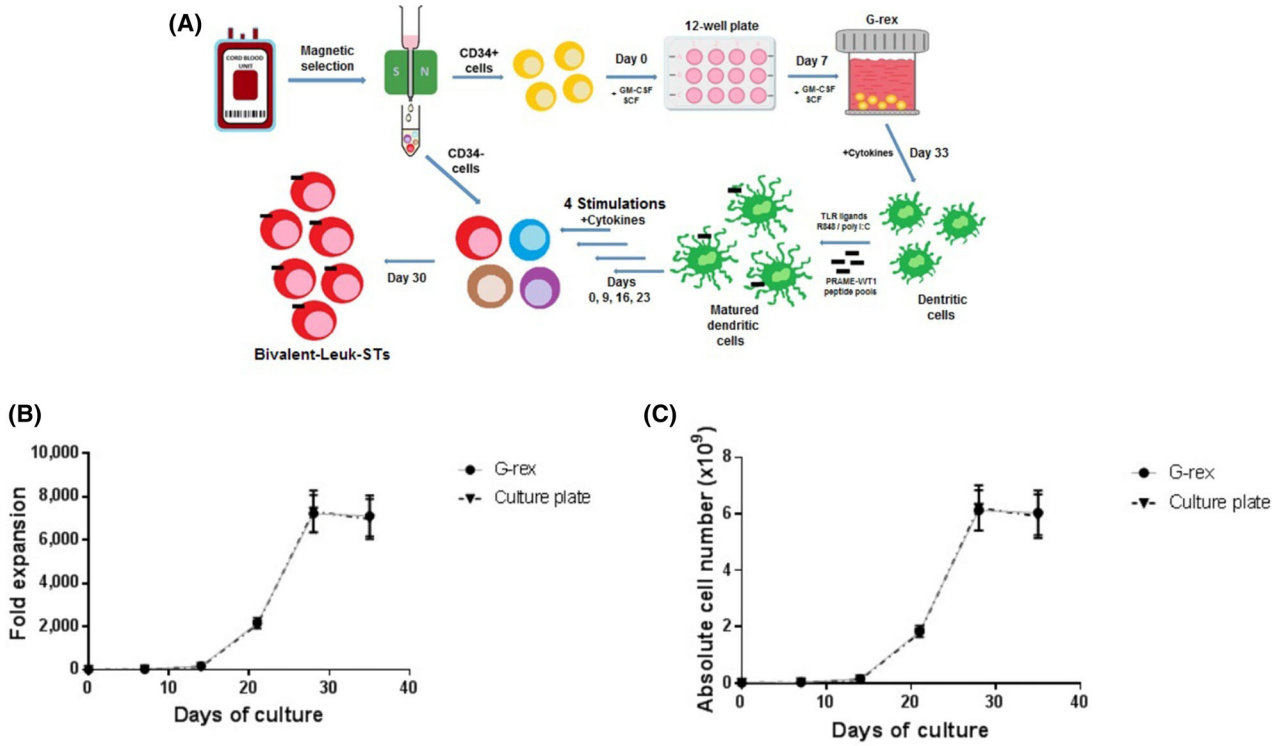

Fig 1. Experimental design and DCs expansion from CD34$^+$ cord blood cells. (A) Immuno-magnetically selected CD34$^+$ cells from non-transplantable UCBUs were cultured in the presence of growth factors in 12 well-plates and later transferred to G-rex devices to generate DCs. DCs were matured, pulsed with the leukaemic antigens and co-cultured with the CD34$^-$fraction in the presence of cytokines to generate, after up to four stimulations, Leuk-STs. (B) Average fold expansion of DCs in G-rex devices (n = 8) and in plates (n = 3) (P = ns) (G-rex:UCBU 1–8, plate:UCBU 2, 3, 5). (C) Average DC numbers obtained after expansion in G-rex devices (n = 8) and in plates (n = 3) (G-rex:UCB 1–8, plate: UCB 2, 3, 5). DCs, dendritic cells; dual-Leuk-STs, T cells targeting two leukaemia-associated antigens; GM-CSF, granulocyte-macrophage colony-stimulating factor; PRAME, preferentially expressed antigen in melanoma; TLR, toll-Like receptor; UCBU, umbilical cord blood unit; WT1, Wilms tumour 1.

2·3 ± 1·5% respectively), thus confirming the specific cytolytic potential and non-alloreactivity as a safety feature of dual-STs (Fig 5D).

Discussion

Currently, T-cell immunotherapy with CAR-Ts has transformed the outcome of refractory/relapsing B-cell malignancies[4] and represents the spearhead of research in malignant haematology. Nevertheless, CAR-Ts are associated with manufacturing complexity and considerable toxicity,[23,24] while their efficacy in myeloid leukaemias lag well behind B-cell malignancies, mainly due to the lack of appropriate targetable antigens.[6,25]

Non-genetically engineered, pathogen-specific T cells, expanded *ex vivo* from the memory T cell compartment[12,26-28] have provided impressive clinical results in controlling opportunistic infections in the allo-HSCT setting. Unfortunately, aside from the Epstein–Barr virus (EBV)-associated lymphoma or post-transplant lymphoproliferative disease (PTLD), the highly immunogenic viral antigens are rarely found in other haematological malignancies. Moreover, the administration of HLA-A*0201-restricted WT1-specific donor-derived CD8 cytotoxic T-cell clones for the treatment of refractory myeloid leukaemia has only met with limited clinical success.[29] The generation of specific T cells for certain LAAs, has been challenged by (i) the requirement of very high numbers of antigen-presenting cells (APCs), (ii) the weak stimulation of T-cell immunity by the 'self-reactive' LAA-STs, (iii) the lack of circulating memory LAA-STs, (iv) the narrow specificity of single-epitope LAA-STs providing an increased risk of tumour escape and (v) the lack of practicality of single HLA class I-restricted antigen-specific T-cell products.[30]

In the present study, we propose the 'recycling' of non-usable UCBUs to upscale the generation of professional DCs and facilitate the production of clinically meaningful doses of specific T cells targeting two of the most common transcription molecules overexpressed in leukaemias[31] and multiple

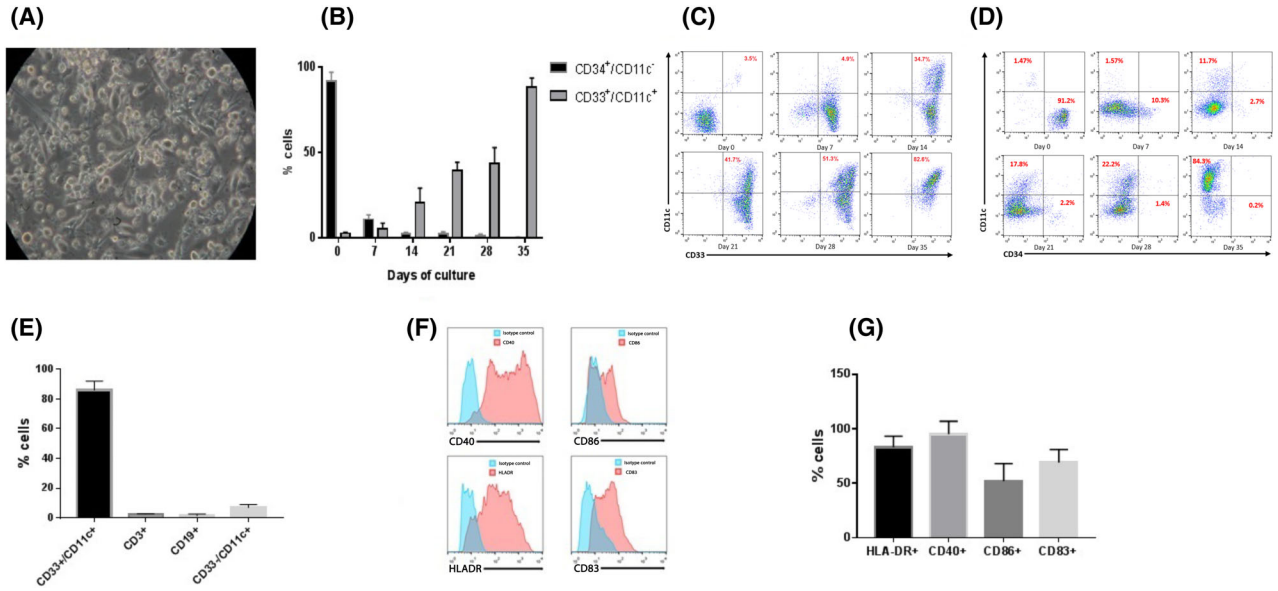

Fig 2. Characterisation of DCs. Morphology, immunophenotype and cytokine expression profile of matured DCs. (A) Reverse phase-contrast microscopy (×200). Matured DCs showing the characteristic semi-round appearance with long dendrites. (B) During culture, cells gradually lose their primitive $CD34^+$ phenotype starting to express the CD33 myeloid marker and later the CD11c dendritic cell marker. At the end of the culture (day 35) the majority of the cells are myeloid dendritic cells ($CD33^+/CD11c^+$) ($n = 8$). (C) Representative flow cytometry analysis of CD11c and CD34 during culture. (D) Representative flow cytometry analysis of CD11c and CD33 during culture. (E) On day 35, the majority of the cells were myeloid DCs ($CD33^+/CD11c^+$) with only minimal expression of lymphoid-associated markers (CD19, CD3) as well as non-myeloid markers ($CD33^-/CD11c^+$) ($n = 8$). (F) Mean fluorescence intensity of expression of markers for antigen processing and presentation from matured DCs. (G) Percentage of expression of markers for antigen processing and presentation from matured DCs ($n = 8$). DCs, dendritic cells; UCBU, umbilical cord blood unit.

antigenic epitopes, via stimulation with overlapping peptides spanning the whole proteins.

Effective adoptive immunotherapy requires multiple stimulations with an at least 1:4 APCs:T cells ratio.[32,33] The low number of circulating DCs hampers their isolation in clinically relevant doses, whereas obtaining high numbers of monocyte-derived DCs requires leucaphaeresis from the donor and generating artificial APCs[34,35] adds greatly to the complexity of the procedure. Alternatively, UCBU-derived $CD34^+$ cells after 3–5 weeks of culture with different growth factor cocktails provided doses of myeloid DCs between $2{\cdot}7 \times 10^7$ [36] to 5×10^9 [16] respectively. By adapting the Harada *et al.* protocol, in the present study we exploited rejected UCBUs to ultimately obtain DC doses at a $\times 10^9$ scale and enable a 1:1 ratio of DCs:T cells and more efficient T-cell priming. G-rex bioreactors did not further improve the ultimate cell output over conventional culture plates, although their use significantly minimised labour intensity and contamination risk.

WT1 and PRAME are known for their antigenicity and associated in some instances with the induction of immune responses and sustained disease remission.[30] The emergence of WT1-specific $CD8^+$ T cells after allo-HSCT has been associated with a decrease in leukaemia load, suggesting a WT1-driven GvL effect.[37] Adoptive immunotherapy with non-genetically engineered donor-derived T cells recognising LAAs through their endogenous TCR has been evaluated in the clinic as prophylaxis or treatment of leukaemia. However, in those early trials, $CD8^+$ LAA-specific T cells were activated by single HLA-restricted epitopes from breakpoint cluster region-Abelson (BCR-ABL) or/and pathogenesis-related protein 1 (PR1) or/and WT1 antigens, thus being restricted to certain HLA genotypes and a subset of patients,[38] while they had limited longevity after infusion, lacking essential growth and survival signals from T-helper (Th) cells.

Over recent years, peripheral blood- or UCBU-derived, polyclonal T cells recognising various antigens and multiple epitopes expressed in lymphoid malignancies have been produced using overlapping peptide libraries spanning the chosen antigens,[17,22] thus overcoming the constrains of specific HLA types. Those preclinical studies have shown that LAA-STs, generated after stimulation with monocyte-derived DCs, were cytotoxic against malignant cell lines expressing the targetable antigens; however, thelow cell numbers of the final products in those studies ($0{\cdot}7–1 \times 10^7$), greatly challenged the robust scalability required for clinical use. Our study offers an unlimited source of $CD34^+$-derived Leuk-STs by repurposing otherwise rejected UCBUs to generate clinical scale ($1{\cdot}03 \pm 0{\cdot}12 \times 10^8$), polyclonal, both $CD4^+$ and $CD8^+$ Leuk-STs targeting WT1 and PRAME, and favouring

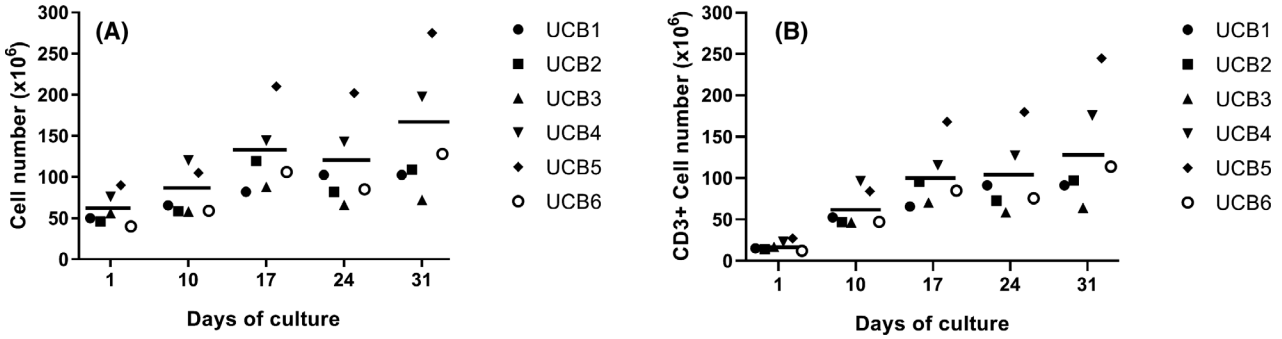

Fig 3. Leuk-STs expansion. (A) Total cell expansion after four stimulations with DCs loaded with WT1 and PRAME peptides ($n = 6$). (B) Dual-Leuk-STs expansion after four stimulations with DCs loaded with WT1 and PRAME peptides ($n = 6$). DC, dendritic cell; dual-Leuk-STs, T cells targeting two leukaemia-associated antigens; PRAME, preferentially expressed antigen in melanoma; UCBU, umbilical cord blood unit; WT1, Wilms tumour 1.

prolonged persistence after infusion due to their memory phenotype.

A major limitation of T-cell immunotherapy with either HLA-epitope restricted LAA-STs or CAR-T cells is the escape risk of the malignant clone and subsequent relapse, mainly due to downregulation or loss of the target epitope/antigen.[39,40] Such a likelihood for immune evasion of the malignant clone could be minimised by multiplexing CAR-Ts to express more than one chimeric receptor,[41] further adding to their manufacturing complexity and design sophistication, or by generating tumour-associated antigen-STs targeting more than one antigen or epitope.[42] Our dual-Leuk-STs by simultaneously targeting two of the best characterised leukaemia-associated (WT1 and PRAME) antigens[43] and also a plethora of antigenic epitopes via stimulation with overlapping peptides spanning the whole proteins, provide the benefits of both minimising the risk of tumour escape and reducing manufacturing times and complexity.

Moreover, T cells functioning through their native receptors are often associated with epitope spreading, a process in which endogenous T cells with new specificities against non-tumour-targeting antigens arise *in vivo*, thus amplifying anti-tumour activity[44] and making the overall probability of antigen loss considerably lower than that observed with CAR-Ts.[45] Recently, two clinical trials tested autologous specific-T cells produced from a large quantity of peripheral blood as starting material, targeting multiple (three to five) solid tumour- or myeloma-expressed tumour-associated antigens (TAAs).[46,47] Despite limitations, both studies provided encouraging results in poor prognosis patients demonstrating longer than expected time to progression, no evidence of antigenic competition and an association of objective responses either with expanded TAA-reactive T-cell clonotypes derived from the infused product or antigen spreading.

The precise phenotype of LAA-STs capable of inducing powerful T-cell leukaemia responses has not yet been determined. Ideally, LAA-STs should consist of both effector and memory $CD8^+$ and $CD4^+$ Th1 cells, to enable effector function and long-term persistence and low Treg frequency to avoid T-cell anergy[48] and disease relapse.[49] Increased levels of exhaustion markers, namely PD-1, CTLA4, TIM3, LAG3 have dampened effector T-cell functions resulting in poor clinical outcome,[50] whereas LAA-STs exhibit exhaustion markers in patients prone to relapse, in contrast to those maintaining long-term remission.[49]

Importantly, the bivalent-Leuk-STs generated in our present study from UCBUs, demonstrated a mixed population of $CD8^+$ and $CD4^+$ cells, highly expressing effector and memory markers [central memory + effector memory: 49 ± 6%, TEMRA (effector memory cells re-expressing CD45RA): 48 ± 8%], but only low levels of Tregs. Peripheral blood-derived LAA/TAA-STs have been shown to exhibit a predominant central over effector memory phenotype[17] or vice versa.[22,47] Our UCB-derived dual-Leuk-STs displayed a predominant effector memory phenotype and consisted of >40% TEMRA cells known to exert potent *ex vivo* cytotoxicity and ability to produce Th1 cytokines upon stimulation.[51] Moreover, in contrast to peripheral blood-derived LAA-STs, the UCB-originated bivalent-Leuk-STs represent naïve-derived effector T cells that have been shown to exert superior anti-tumour effects and better *in vivo* expansion and persistence than effector cells derived from memory subsets.[52,53] Importantly, bivalent-Leuk-STs expressed very low to barely detectable levels of exhaustion markers including LAG3, the increased expression of which in the T-cell product was correlated with non-responsiveness *in vivo*.[46]

T-cell therapies with non-genetically engineered cells, targeting either viruses or TAAs[11,22,32,33] have provided minimal rates of cytokine release syndrome and GvHD,[54] even when administered as third-party products. Banked multi-virus-specific T-cell lines, partially matched (1/8 to 7/8 HLA alleles) to viruses-reactivating allo-transplanted patients but with specificity against target antigens through shared HLA alleles, presented a good safety and efficacy profile and enabled coverage of 97% of the affected recipients.[55] Similarly, and further supported by their UCB-origin allowing for

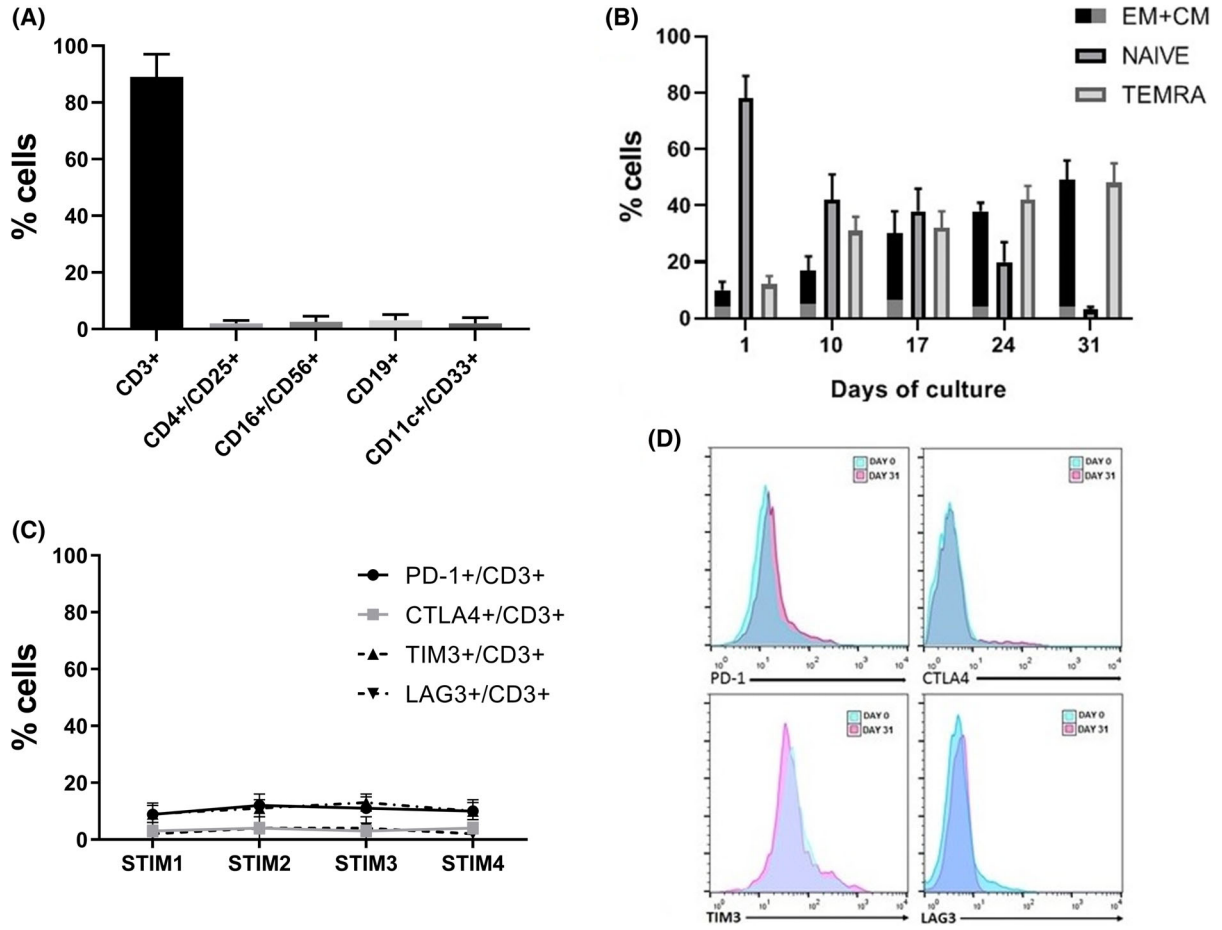

Fig 4. Immunophenotypic characterisation of dual-Leuk-STs. (A) Generated cell products were enriched in $CD3^+$ cells, comprising of both $CD8^+$ cells and $CD4^+$ cells and containing insignificant number of T-regulatory $CD4^+/CD25^{high}$ cells, B-lymphocytes and NK ($CD16^+/CD56^+$) ($n = 6$). (B) The cell products expressed effector memory (EM: $CD45RA^-/CD62L^-$), central memory (CM: $CD45RA^-/CD62L^+$) and EM RA (TEMRA: $CD45RA^+/CD62L^-$) markers while they contained insignificant numbers of naïve T cells ($CD45RA^+/CD62L^+$) by the end of the culture (day 31) ($n = 4$). (C) Low and stable expression levels of PD-1, CTLA4, TIM3 and LAG3 throughout the culture (P = ns) ($n = 4$). (D) Mean fluorescence intensity of PD-1, CTLA4, TIM3 and LAG3 expression at the end of the culture. CTLA4, cytotoxic T-lymphocyte-associated antigen 4; dual-Leuk-STs, T cells targeting two leukaemia-associated antigens; LAG3, lymphocyte activation gene-3; NK, natural killer; PD-1, programmed cell death protein 1; TIM3, T cell immunoglobulin and mucin-domain containing-3; STIM, stimulation; UCBU, umbilical cord blood unit.

low allo-reactivity[56] and no stringent HLA matching, 'recycled' UCBUs-derived LAA-STs, targeting two full-length antigens may be used as third-party, 'off-the shelf' products, although in this setting, criteria for the extent of HLA matching, shared HLA specificity and the number of cell lines required to cover a diverse transplant population might need to be established.

To be amenable to clinical translation and broad implementation, T-cell immunotherapy with LAA-STs needs to deliver scalable quantities, products capable of inducing major clinical responses with low risk of severe toxicity and cost-effectiveness. In the present study, we provide a unique paradigm, where, disqualified UCBUs are 'recycled' to serve as an unlimited source for scalable production of third-party, non-genetically engineered, double targeting AML-specific T cells with a potentially improved safety profile over other T-cell therapies. This platform could form the basis for the establishment of T-cell banks towards global availability of anti-leukaemia T-cell products.

Competing interests

The authors declare that they have no competing interests relevant to the subject matter of this article.

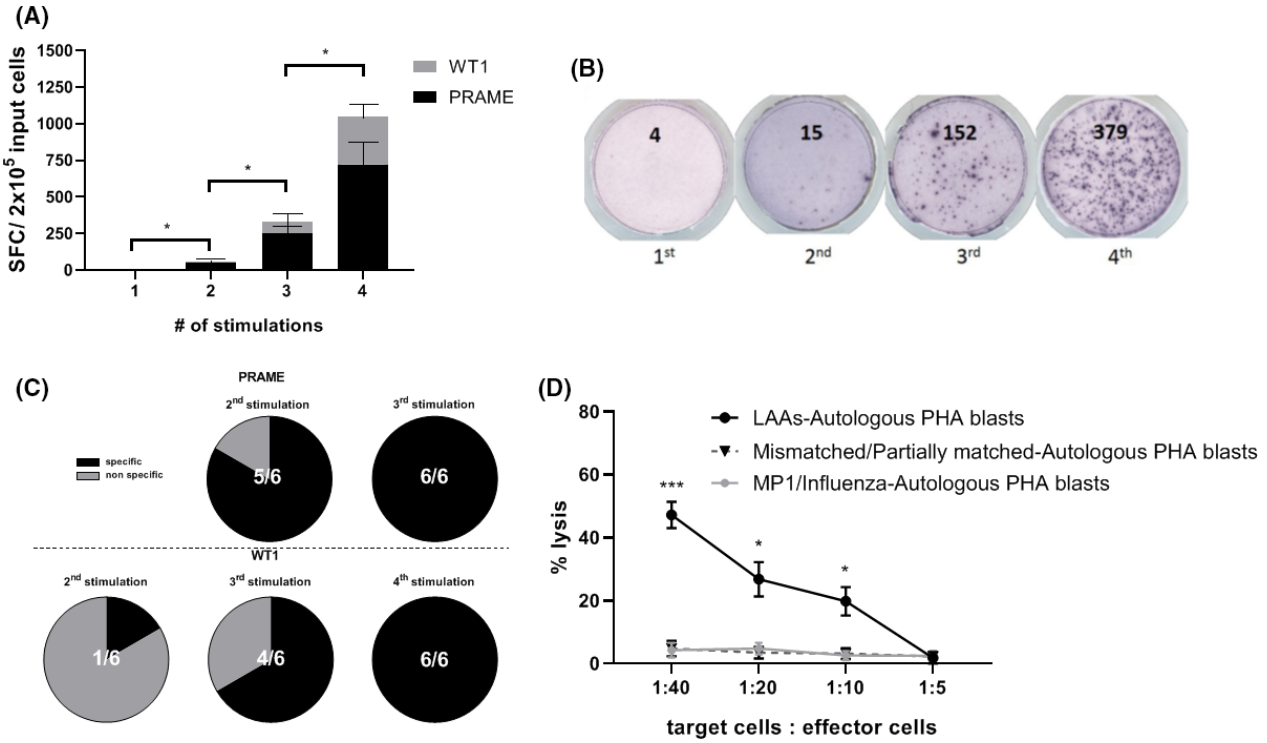

Fig 5. Specificity of dual-Leuk-STs against PRAME and WT1. (A) Specificity of generated cell lines ($n = 6$), by ELISPOT measuring IFN-γ secretion, after four stimulations with DCs presenting PRAME and WT1. Enrichment of IFN-γ secreting T cells after each stimulation [mean SFC/ 2×10^5 cells] ($P < 0{\cdot}05$). (B) A representative ELISPOT of a T-cell product after consecutive stimulations. (C) Three or four stimulations were required to achieve specificity for 100% of the cell products against PRAME and WT1 respectively. (D) Cytotoxic activity of the generated dual-Leuk-STs against autologous peptide-pulsed (LAA: PRAME/WT1, irrelevant antigen:Influenza-MP1) PHA blasts or partially/fully mismatched PHA blasts ($n = 3$). DCs, dendritic cells; dual-Leuk-STs, T cells targeting multiple leukaemia-associated antigens; IFN-γ, interferon gamma; LAA, leukaemia-associated antigens; MP1, matrix protein 1; PHA, phytohaemagglutinin; PRAME, preferentially expressed antigen in melanoma; SFC, spot forming cells; WT1, Wilms tumour PRAME, preferentially expressed antigen in melanoma; WT1, Wilms tumour 1.

Author contributions

Kiriakos Koukoulias: performed the research, designed the research study, analysed the data, wrote the paper. Anastasia Papadopoulou: performed the research, designed the research study, contributed essential reagents or tools, analysed the data, wrote the paper. Anastasios Kouimtzidis: performed research. Penelope-Georgia Papayanni: performed research. Andri Papaloizou: performed research. Damianos Sotiropoulos: contributed essential reagents or tools. Minas Yiangou: contributed essential reagents or tools. Paul Costeas: analysed data and contributed essential reagents or tools. Achilles Anagnostopoulos: contributed essential reagents or tools. Evangelia Yannaki: designed the research study, contributed essential reagents or tools, analysed the data, wrote the paper. Panayotis Kaloyannidis: designed the research study, analysed the data, wrote the paper.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Data S1. Materials and methods.

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