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Targeting the Tumour Antigen 5T4 using CAR-T Cells for the Treatment of Acute Myeloid Leukaemia

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ABSTRACT

Chimeric antigen receptor (CAR) T cells represent a novel targeted approach to overcome deficits in the ability of the host immune system to detect and subsequently eradicate tumours. The identification of antigens expressed specifically on the surface of tumour cells is a critical first step for a targeted therapy that selectively targets cancer cells without affecting normal tissues. 5T4 is a tumour-associated antigen expressed on the cell surface of most solid tumours. However, very little is known about its expression in haematological malignancies. Herein, we assess the expression of 5T4 in different types of leukaemias, specifically Acute Myeloid Leukaemia (AML), and normal haematopoietic stem cells (HSCs). We also provide an *in vitro* assessment of safety and efficacy of 5T4-targeting CAR-T cells against HSCs and AML tumour cell lines. 5T4 expression was seen in about 50% of AML cases; of relevance for therapeutic purposes, leukaemic stem cells (LSCs) are also positive for 5T4 expression. 5T4 CAR-T cells efficiently and specifically killed AML tumour cell lines, including the LSC population. Co-culture of 5T4 CAR-T cells with HSCs from healthy donors showed no impact on subsequent colony formation, thus confirming the safety profile of 5T4. A proof-of-concept study using a murine model for AML demonstrated that CAR-T cells recognise 5T4 expressed on cells and they can kill tumour cells both *in vitro* and *in vivo*. These results highlight 5T4 as a promising target for immune intervention in AML and that CAR-T cells can be considered a powerful personalised therapeutic approach to treat AML.

Keywords: 5T4 tumour antigen; chimeric antigen receptor (CAR); acute myeloid leukaemia (AML); immunotherapy

Declarations:

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Code availability: Not applicable

Ethics approval (include appropriate approvals or waivers): Bone marrows and lymphocytes cells used for this study were purchased from Cellular Technology Ltd (CTL), Stem Cell Technologies, BioIVT/Tissue Solutions and Manchester Biobank or conducted by our Collaborators from University of Sheffield (REC reference number 18/YH/0318) and University of Birmingham (IRAS number 280215/NHS REC reference number 20/NW/0286) using historical and/or leftover clinical samples after approval by the local institution boards.

Animal studies were conducted by Charles River Laboratories (CR) (Morrisville, NC). The animal care and use program at Charles River Discovery Services is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC), which assures compliance with accepted standards for the care and use of laboratory animals.

INTRODUCTION

The number of naturally occurring tumour antigen specific T cells is a key factor limiting the therapeutic potential of the host immune system to eradicate malignant cells in cancer patients. Therapeutic strategies designed to re-direct patients' tumour specific T cells encompass genetic modification of T cells to express chimeric antigen receptors (CARs) with defined antigen specificities. CAR-T cells function independently of both the natural T cell receptor and major histocompatibility complex restriction and can provide therapeutic doses of tumour-specific T cells to the tumour site. CAR-T cell therapies represent a revolution in the treatment of blood cancer with their ability to induce durable clinical responses [1]. Impressive clinical responses have been demonstrated in several haematological cancers [2,3]; for example, response rates of >80% using CAR-T cells targeting the CD19 antigen, which is expressed on several B-cell malignancies [4,5].

One tumour-associated antigen that could be an attractive target for CAR-T cell therapies is 5T4 (also known as TPBG). 5T4 is a 72-kDa transmembrane protein that is expressed on the placenta together with a wide range of human carcinomas but is rarely seen on normal tissues [6, 7]. While its expression is well documented in solid tumours, very little is known about its expression on liquid tumours. Castro *et al.* [8] reported that 5T4 is expressed in pre-B acute lymphoblastic leukaemia patients who are at high risk of relapse and is associated with a more invasive phenotype. In this study we have validated 5T4 as a target in haematological malignancies. We screened blood derived cells obtained from several haematological malignancies for the expression of 5T4. Our attention focused specifically on Acute Myeloid Leukaemia (AML), an aggressive malignancy involving abnormal myeloid stem cell differentiation [9].

AML is a highly heterogeneous cancer which makes a "one size fits all" approach to effective treatment highly challenging. However, significant progress in the understanding of the pathophysiology of AML has led to improvements in therapies available to patients. Indeed, since 2017, nine products have been approved for AML [9]. Despite such advances, AML survival remains poor, especially for older patients which show a 5-year survival rate of about 15% [9].

Targeted therapies in AML are being developed using several approaches, including antibody-drug conjugates (ADC) such as CD33-targeting agent gemtuzumab ozogamicin (GO) [10], CD123xCD3, CD33xCD123 bispecific agents, and CD33xCD3 bispecific T effector cell engagers [11]; immune checkpoint blockade with agents targeting PD-1, CTLA-4, TIM-3 and CD47 [12]; CAR-T and CAR-NK cell therapies targeting CD33, CD123, Flt-3, CD44v6, CLL-1, CD7, GRP78, TIM-3 and, WT1 [13-14]. Unfortunately, some of these markers are also expressed on normal myeloid progenitor cells.

In this report, we have investigated the expression of 5T4 in AML patients with the view to assessing its potential as a candidate target for CAR-T therapy, either as a mono- or combi-therapy for AML patients.

MATERIALS AND METHODS

Assessment of 5T4 Expression in haematological malignancies by Flow Cytometry

Bone marrow (BM) and/or peripheral blood mononuclear cells (PBMCs) were selected from patients with multiple myeloma (MM), chronic myeloid leukaemia (CML), chronic lymphocytic leukaemia (CLL), B-cell acute lymphoblastic leukaemia (B-ALL) and T-cell acute lymphoblastic leukaemia (T-ALL). The samples were obtained from Tissue Solutions Ltd, Histologix Ltd, Blood Service Birmingham University, Sheffield University, and the Manchester Cancer Research Centre (MCRC) biobank.

Antibodies for multi-parameter flow cytometry (MFC) were purchased from BD Bioscience: CD19-APCH7 (RRID:AB_1727437), CD20-PE-Cy7 (RRID:AB_1727450), CD34-APC (RRID:AB_398614), CD45-FITC (RRID:AB_395874), mouse IgG1-FITC (RRID:AB_396090), mouse IgG1-APC (RRID:AB_398613) and mouse IgG1-APC-H7 (RRID:AB_1645642); from Biolegend: CD138-APC (RRID:AB_10896946), CD56-AlexaFluor 700 (RRID:AB_604096), CD38-FITC (RRID:AB_314355), Zombie Aqua™; from Thermo Fisher Scientific: mouse IgG1-PE (RRID:AB_470060). Anti-5T4 (clone H8) was produced in-house and PE- labelled by Bio-Rad.

The cryovials containing PBMCs and BM cells were thawed, and the cells were revived in RPMI-1640 media supplemented with 10% FBS, 5mM L-glutamine, 100 U/ml penicillin and 100UI/ml streptomycin, then spun, washed, and resuspended in PBS. The cells were stained with the Zombie Aqua™ dye as per Manufacturer's protocol, washed in BD FACS Stain Buffer and stained in the dark at 4 °C with the antibody mix between 30 and 60 minutes. Afterwards, the cells were washed in stain buffer and resuspended in BD CytoFix™ buffer as per manufacturer's instructions, spun and resuspended in PBS.

Data acquisition was performed using a FACSVerse™ flow Cytometer (3-laser standard configuration, BD Biosciences, Mountain View, CA). For CLL samples, the acquisition was performed on LSRII flow Cytometer (4-laser bench top cytometer using standardised settings, BD Biosciences, Mountain View, CA) and FACSCanto™ II (3-laser clinical cytometer using standardised setting, BD Biosciences, Mountain View, CA) in two different laboratories (Sheffield University and Birmingham University). For all samples at least 10,000 events were collected in the live cell gate for all samples and analysed using FlowJo v10 software (BD Biosciences, RRID:SCR_008520).

Initial gating strategy was carried out on live cells, then 5T4 expression was evaluated on leukaemic cells using the following markers: CLL on CD20+ and CD34+ cells; MM samples, on CD38+CD56+ cells and CD34+ cells or CD138- cells; CML on the totality of live cells and CD34+ cells; AML samples, on CD34+ CD38- cells ; B-ALL samples, on CD19+ cells and CD34+ cells; T-ALL samples, on the totality of live cells and CD34+ cells; Acute Pro-Myelocytic Leukaemia (APML) samples, on CD34- (hyper-granular APML population) cells.

A sample was reported to be 5T4 positive if the rMFI is ≥1.5. The rMFI is calculated as MFI of test sample/MFI of isotype control. The rMFI was deemed more accurate in giving an index of positivity in frozen samples and in subpopulations with a certain degree of variability from patient to patient [15].

Cell lines and cell culture reagents

Human AML cell lines Kasumi-1 (RRID:CVCL_0589), THP-1 (RRID:CVCL_0006), and AML-193 (RRID:CVCL_1071) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA); media, foetal bovine serum (FBS), GlutaMax, HEPES, used to culture the cells from Gibco, ThermoFisher Scientific; human AB Serum, N-Acetyl-L-Cysteine (NAC), Sodium Pyruvate, DMSO, 2-mercaptoethanol, insulin, transferrin, GM-CSF from Sigma-Aldrich; IL-2, IL-7 and IL-15 from R&D Systems.

Kasumi-1 cells were cultured in RPMI-1640 medium supplemented with 20% FBS; THP-1 cells were cultured in RPMI-1640 supplemented with 0.05 mM 2-mercaptoethanol and 10% FBS; AML-193 cells were cultured in Iscove's modified Dulbecco's medium supplemented with 0.005 mg/mL insulin, 0.005 mg/mL transferrin, 5 ng/mL GM-CSF, and 5% FBS.

Generation of 5T4 CAR-T Cells

Second generation CAR-T cells were generated using scFv sequence from a murine anti-5T4 antibody (H8 clone) subcloned onto HIV-1 lentiviral vector encoding CD8 transmembrane domain, 4-1BB co-stimulatory domain and a CD3ζ intracellular domain (Supplementary Fig. 1). PBMCs from healthy donors were purchased from Cellular Technology Limited (CTL Cleveland, OH, USA). 5T4 CAR-T cells were maintained in TheraPEAK™ X-VIVO™-15 Serum-free Hematopoietic Cell Medium supplemented

with 1% MEM Eagle Vitamin Mix (Lonza) 5% Human AB Serum, 2 mM GlutaMax, 20 mM HEPES, 10 mM NAC, 1 mM Sodium Pyruvate, and 100 IU/mL of IL-2. To activate T cells, anti-CD3/anti-CD28 Dynabeads (Thermo Fisher Scientific) were added to PBMCs at a 3:1 bead to cell ratio. Vector was added at a multiplicity of infection (MOI) of 0.6 by integration titre. After 18-24h, a second dose of lentiviral vector was added at an MOI of 0.6 (total MOI = 1.2). Every 2-3 days, cell concentration was maintained at 5×10^5 viable cells/mL through the addition of fresh media containing 100 IU/mL IL-2. T cell expansion continued for 14 days when 5T4 CAR-T cells were cryopreserved at 2×10^7 viable cells/mL in Cell Freezing Medium containing DMSO.

Non-transduced control T cells were produced in the same manner with no lentiviral vector added.

For the *in vivo* experiments, PBMCs were generated from Leukopaks by BioIVT (Royston, UK) and stored overnight at ambient temperature. T-cells were sorted using anti-CD3/anti-CD28 Dynabeads. T cell transduction and expansion was performed as stated above, except that IL-7 and IL-15 were used in place of IL-2 and the vector was added once on the day of T cell stimulation. Cells were harvested either after 4 days or 7 days after transduction and stored as stated above.

In vitro testing of 5T4 CAR-T cells and Tumour Cell Lines

AML cell lines were labelled using CellTrace™ Far Red (ThermoFisher Scientific) and co-cultured in 96-well round-bottom plates with 5T4 CAR-T cells or non-transduced control T cells at a 1:1 (effector:target) ratio. T cells or tumour cells were cultured alone as negative controls. Supernatant was harvested after 18-24h for assessment of IFN-γ and Granzyme-B production using a cytometric bead array (CBA) kit (BD Biosciences), according to the manufacturer's instructions. After 42-48h, cells were stained using Aqua viability dye (ThermoFisher Scientific), and PE-conjugated anti-human 5T4 or murine IgG1κ-PE isotype control antibody (Clone P3.6.2.8.1, eBioscience, ThermoFisher Scientific). Flow cytometry data were acquired to assess tumour cell killing using a BD FACSVerse™ flow cytometer. Analysis of flow cytometry data was performed using FlowJo v10 software (BD Biosciences, RRID:SCR_008520).

In vitro testing of 5T4 CAR-T cells and human CD34+ bone marrow cells

Co-cultures of 5T4 CAR-T cells or non-transduced cells with normal human BM CD34+ cells (STEMCELL Technologies Inc.) or Kasumi-1 cells were performed by STEMCELL Technologies using their proprietary media (Vancouver). Experiments were performed in StemSpan™ SFEM II media with StemSpan™ CC100. 4×10^3 BM CD34+ cells were added to 4×10^4 5T4 CAR-T cells or non-transduced cells (effector:target cell ratio of 10:1). Co-cultures with Kasumi-1 cells were performed as a positive control (5×10^4 Kasumi-1 cells were added to 5×10^5 5T4 CAR-T cells or non-transduced cells at effector:target cell ratio of 10:1). Target only and T cell only negative controls were included. Each condition was set up in triplicate and cultures were incubated at 37°C, 5% CO_2 for 4 hours.

After 4 hours of co-culture, each BM CD34+ co-culture was added to MethoCult™ H4435 Enriched medium, plated in triplicate into 6-well SmartDish™ at 500 cells/well and incubated for 12-14 days and the total number of erythroid (BFUE), myeloid (CFU-GM) and mixed (CFU-GEMM) colonies were scored based on morphology. A similar co-culture assay was performed to assess the impact of 5T4 CAR-T cells on colony formation of Kasumi-1 cells.

The mean colony numbers (BFU-E, CFU-GM, CFU-GEMM or Total CFC) from 5T4 CAR-T cell and non-transduced T cell co-cultures were compared with target only control cultures. The mean colony numbers from CAR-T co-cultures were compared with mean colony numbers from corresponding non-transduced T cell co-cultures. Standard t-Tests were performed for comparison.

THP-1 xenograft model

Mouse experiment was conducted at Charles River Laboratories (CR) (Morrisville, NC).

Briefly, 5 x 10^6 THP-1-luc cells (RRID:CVCL_C8WH; THP-1 cells were purchased from ATCC and Luc-tagged at CrownBio, Suzhou, Jiangsu, China) were implanted intravenously (i.v.) in seven groups of eight NCG mice (NOD-*Prkdc*^{em26Cd52}*Il2rg*^{em26Cd22}/NjuCrl, RRID:IMSR_CRL:572). Twelve days post-implantation, a single-dose of 5T4 CAR-T therapies were administered to Group 3 (D4 at 5 x 10^5 cells/animal) and Groups 5, 6, and 7 (D7- at 5 x 10^5, 1.5 x 10^6, and 5 x 10^6 cells/animal, respectively). Appropriate controls were set as no treatment Group 1, as well as Groups 2 and 4, treated with their non-transduced counterparts UTC(D4) and UTC(D7) at 5 x 10^5 and 5 x 10^6 cells/animal, respectively. Whole-body BLI measurement started on Day 0, then continued from Day 6 and once per week until

Day 69, with a total of ten imaging timepoints (Days 6, 13, 20, 27, 34, 41, 48, 56, 62, and 69). The data were exported into Living Image software 4.7.3. (Perkin Elmer, MA RRID:SCR_014247) and analysed.

Flow cytometry analysis

Blood (100 µL) was collected via mandibular bleeds without anaesthesia from all animals in all groups at Day 0, Day 7, Day 21 and Day 35; BM samples from the left and right femurs and spleens were collected at termination. Red blood cells lysed with lysis buffer (Quality Biological, Inc.) lymphocytes were then stained using both mouse and human antibodies with the following corresponding parent gates (FSC-A vs Live/Dead): mCD45- vs hCD45+, hCD45+ vs CD3+, CD4- vs CD8+, CD4+ vs CD8-, CD3+ vs anti-idiotype+, CD8+ vs anti-idiotype+, CD4+ vs anti-idiotype+, hCD45+ vs CD3-, and hCD45+ vs 5T4+. Data were acquired on a BD LSRFortessa™ flow cytometer (BD Biosciences) and analysed with FlowJo software (BD Bioscience, RRID:SCR_008520). The analysed flow cytometry data are presented in graphical forms, according to the Live or parent cell population gates.

Increased Life Span (ILS) Assessment and Tolerability

Animals were weighed twice a week and monitored individually to an endpoint of moribundity due to systemic tumour progression. Full hind limb paralysis (unable to reach food and water), severe ocular proptosis, or moribundity, all called for euthanasia, and all deaths due to tumour progression were classified as death on survival study (DSS). The time to endpoint (TTE) was recorded in days for each mouse. Any animal with weight loss exceeding 30% for one measurement or 25% for three measurements was euthanised. Acceptable toxicity was defined as a group mean body weight loss of less than 20% during the study and no more than 10% deaths related to treatment (TR deaths). Death may also be classified as TR if due to unknown causes during the dosing period or within 14 days of the last dose. Death was classified as non-treatment related (NTR) if there was no evidence that the death was related to treatment side effects. If an animal exited a group by sudden death or was euthanised due to moribundity, blood and organs such as BM, spleen, liver, and lungs were harvested for further efficacy and toxicology analyses; necropsy examination, where possible, was also performed.

Statistical Analysis and Graphical Presentation

GraphPad Prism 9.3.1 for Windows 10 (RRID:SCR_002798) was used for all statistical analyses and graphical presentations. For survival analysis, the logrank test and Kaplan-Meier test were employed. The Group mean body weight changes over the study time course were plotted as percentages from Day 1. Error bars, when present, represent one standard error of the mean (SEM).

RESULTS

5T4 expression in patient samples from different haematological malignancies

Table 1 shows 5T4 positivity on PBMCs and BMs from patients affected by CLL, AML, CML, MM, APML, T-ALL and B-ALL. Expression of 5T4 was negligible in CLL (23 samples screened for CLL); low in T-ALL and CML samples, respectively 18% and 29% (11 samples screened for T-ALL and 7 samples for CML); moderate to high in B-ALL, AML, MM and APML samples, respectively 52%, 53%, 71% and 78% (21 samples screened for B-ALL, 36 samples for AML, 31 samples for MM and 9 samples for APML). APML samples resulted positive according to the rMFI criteria applied; however, they were very faintly positive for 5T4, and the number of samples was very limited too.

Functional activity of 5T4 CAR-T cells in vitro

A range of AML cell lines were screened for 5T4 target antigen expression by multiparameter flow cytometry (MFC, Supplementary Table 1). Kasumi-1 (Supplementary Fig. 2) and THP-1 were both positive for 5T4, while AML-193 showed no notable expression (Figure 1A).

The functional activity of 5T4 CAR-T cells was then assessed against these cell lines (Figure 1B-D). Figure 1 shows that there was significant production of IFN-γ (1B) and Granzyme-B (1C) by 5T4 CAR-T cells in response to co-culture with THP-1 or Kasumi-1 cell lines. Moreover, 5T4 CAR-T cells and non-transduced T-cells showed low IFN-γ and Granzyme-B levels when co-cultured with a 5T4-negative AML cell line (AML-193). 5T4 CAR-T cells induced significant killing of THP-1 and Kasumi-1 cell lines compared to non-transduced T-cells, but no killing of the 5T4 negative AML-193 cell line as assessed by MFC (Figure 1D). Of note, the percentage of dead cells in the killing assay for both Kasumi-1 and THP-1 cells was higher than the percentage of cells estimated to be positive for 5T4 by MFC (Figure 1A and D). To assess whether 5T4 CAR-T cells were preferentially killing cells with high surface expression of 5T4, the MFI of 5T4 positive cells was divided in three arbitrary ranges: cells with an MFI value of >120 were considered positive, “borderline negative” with MFI values between 70-120, and “negative” with an MFI <70. As expected, very few cells with an MFI > 120 remained alive following co-culture with 5T4 CAR-T cells; interestingly, there was a significant reduction in the percentage of cells considered to be “borderline negative” (MFI between 70-120), while there was no or negligible reduction

in the percentage of cells considered to be “negative” (MFI <70) (Figures 2C and 2D). This data suggests that 5T4 CAR-T cells were able to induce cell death for cells with low surface expression of 5T4, which highlights the therapeutic potential of 5T4 CAR-T cells.

5T4 CAR-T cells kill LSCs in vitro

Kasumi-1 cells are known to contain a putative stem cell compartment (LSC) defined as CD34+CD38- [16]. The presence of this component of Kasumi-1 cells was confirmed by MFC, with around 40% of Kasumi-1 cells found to be CD34+CD38- (Supplementary Figure 2. To note, we did not differentiate between dim and bright CD34+ cells). After co-culture with 5T4 CAR-T cells, there was an almost complete eradication of the CD34+CD38- Kasumi-1 compartment only in cells co-cultured with 5T4 CAR-T cells (Figure 3). A significant reduction in colony forming units was also detected following co-culture of 5T4 CAR-T cells with Kasumi-1 target cells (P=0.001 compared to target cells only and P=0.004 compared to non-transduced control cells), but not with non-transduced control cells (Table 2). Collectively, these results strongly suggest that 5T4 CAR-T cells kill the colony-forming stem sub-population within Kasumi-1, which broadly translates to the ability of 5T4 CAR-T cells to target the persistent subset of cells within a leukaemic population.

Target antigen expression on HSCs and impact of 5T4 CAR-T cells on colony formation

Given the expression of 5T4 in AML and on putative LSCs in Kasumi-1 cells, it was important to determine whether 5T4 is expressed on haematopoietic stem cells (HSCs). CD34+ bone marrow (BM) cells recovered from 10 healthy (non-AML) donors were screened for expression of 5T4 (Figure 4). 5T4 expression was detected on all samples, albeit at different levels, ranging from 10% to 60% with an average of 30% expression on CD34+ cells. Subsequently, the potential impact of 5T4 CAR-T cells on colony formation was determined using the same 10 donors. There was no statistically significant decrease in CFC colony output or negative impact on colony morphology observed after a 4-hour co-culture of BM CD34+ cells from these 10 healthy donors with 5T4 CAR-T cells when compared to non-transduced controls (Figure 4). Donor 5 did show a statistically significant decrease in total CFC output after co-culture with 5T4 CAR-T cells when compared with target only control cells, but a significant decrease was also seen with non-transduced control cells (Figure 4B). No significant differences in

BFU-E (Figure 4C), CFU-GM (Figure 4D) and CFU-GEMM (Figure 4E) colonies were detected for any of the 10 donors.

We also tested the expression of 5T4 on healthy tissue using an IgG harbouring the same scFv expressed on 5T4 CAR-T cells. We confirmed that the only tissue highly positive for 5T4 was the placenta, as expected as 5T4 is highly expressed in the trophoblast. In addition, localised weak staining was visible in the epithelia of the fallopian tube, urothelium of the bladder, endometrium, mesothelium and epithelium of the ovary, and ureter. Isolated positive cells were visible in the pituitary gland, the muscularis mucosa of the duodenum and the oesophagus (Supplementary Figure 3A-C). To note, as highlighted in Supplementary Figure 3C, five tissues showing moderate positivity for 5T4 were obtained from patients whose cause of death was described as cancer.

5T4 CAR-T cells are effective in reducing the tumour burden in an in vivo model of AML

To demonstrate the efficacy *in vivo* of 5T4 CAR-T cells a preclinical study was conducted in a murine model of AML. Firefly luciferase expressing THP-1 cells (5×10^6 cells/animal) were injected into female NCG mice at CRL, Morrisville (NC). Ten days after receiving THP-1 cells, mice were distributed into experimental groups equally according to measured BLI. The subsequent day, mice were left untreated, or treated with non-transduced cells or 5T4 CAR-T cells. Analysis of whole blood taken from mice at Day 7, Day 21 and Day 35 showed a progressive increase of human CD3+ cells (Fig. 5A) and, even more importantly, a steady increase of 5T4 CAR-T cells (Fig. 5B) in the groups of animals treated with 5T4 CAR-T cells in a concentration-dependent manner, for 5T4 CAR-T expanded for 7 days. Non-transduced control cells showed no presence of CAR-T cells, only an increase of CD3+ cells, most likely cytokine-induced Killer cells, as these cells were generated in media containing IL-7 and IL-15. Average BLI for the experimental groups was not different at the point of treatment; however, from Day 21 the group treated with the highest dose of 5T4 CAR-T cells showed a marked decrease in BLI compared to the group receiving the same dose of non-transduced cells, and the untreated control group; however, a decrease in the BLI signal can be also observed for Day 7 low and medium doses (Fig. 5C and Fig. 5D-J). Despite the lowest BLI recorded and the expansion of 5T4 CAR-T cells, the Group 7 animal, treated with the highest dose of 5T4 CAR-T cells, showed a marked decrease in body

weight and had to be sacrificed (Fig. 5K). The necropsy did not show any sign of tumours, but shrunken and pale livers. As no tissue immunohistochemistry was conducted at the time of the necropsy, we can only speculate that this might be a case of tumour lysis syndrome or GvHD; interestingly, the non-transduced control group did not show the same marked decrease in body weight and, upon inspection post-mortem, large tumour masses were found mostly on liver and ovaries.

DISCUSSION

5T4 is an oncofoetal protein that has been identified by our and other teams around the world as a promising target to treat solid malignancies [6, 7, 17, 18, 19]. Indeed, previous studies using different therapeutic approaches (a vaccine targeting 5T4, a 5T4-targeted antibody super-antigen and a 5T4 targeted antibody-drug conjugate) have all shown a good safety profile in pre-clinical and clinical testing with no reported autoimmune reaction [7]. Its safety profile would make 5T4 an ideal candidate for Chimeric Antigen Receptor (CAR) T cell therapies, if an extensive characterisation was available for haematological malignancies, in addition to the one publication by Castro and colleagues [8] demonstrating 5T4 expression in B-ALL.

As an initial step, we started with target validation in liquid malignancies by screening a number of leukaemic samples for the expression of 5T4. While 5T4 expression was negligible or low in CLL, T-ALL and CML, moderate to high in B-ALL, AML, APML and MM expressed 5T4, evaluated as ratio between the MFI of the sample stained with anti-5T4 antibody (H8 clone) and the isotype control. The caveat associated with this analysis is that it is a binary assessment. In particular for the APML samples, were the number of cases was limited and the samples just passed the acceptance criteria, the screening of a bigger data set would be advised.

Overall, the 5-year survival rate for MM is more than 50%, while for AML it decreases according to the age of the patients, and it is on average 15% for patients diagnosed between 60-69 years of age [9]. Leukaemic stem cells (LSCs), particularly those sequestered in the bone marrow (BM), may play a pivotal role in the resistance of AML to chemotherapy [20, 21]. We decided to focus our attention on AML because of its dismal survival, and also because CAR-T cells can access the BM compartment, providing an alternative treatment in chemotherapy-resistant patients suffering from AML. From our 5T4 expression study, CAR-T cells targeting 5T4 could be beneficial in AML either alone, but possibly combined with another AML-associated antigen target [22, 23].

The subsequent step was to prove that CAR-T cells could target and kill *in vitro* specifically those leukaemic cell lines expressing 5T4. We showed that THP-1 and Kasumi-1 cell highly express 5T4, while AML-193 is virtually negative. Co-culture of 5T4-CAR-T cells with these cell lines induced significant killing of THP-1 and Kasumi-1 cell lines compared to non-transduced T-cells, but no killing

of the 5T4 negative AML-193 cell line. Mirroring this data, but on a more functional level, there was a significant production of IFN-γ and Granzyme-B by 5T4-CAR-T cells in response to co-culture with THP-1 or Kasumi-1 cell lines. In contrast, non-transduced T-cells showed no significant production of IFN-γ and Granzyme-B in control co-cultures. Our *in vitro* analysis not only demonstrated the ability of 5T4 CAR-T cell to re-direct cell killing of cell lines expressing 5T4, but that 5T4 CAR-T cell killing was not totally dependent on the level of expression of the receptor on leukaemia cells. This suggests that this propensity to kill 5T4-low expressor cells could translate into a greater than predicted therapeutic efficacy. Consequently, this observation has direct impact for safety testing (e.g. on-target off-tumour toxicity), for the selection of patients who might benefit from treatment with CAR-T cells as well as eradication of LSCs. Our *in vitro* data are in line with reports suggesting that treatment with CD19 CAR-T *in vitro* results in elimination of CD19-positive myeloma cells, including those with <100 CD19 molecules per cell [24].

To assess the risk associated with off target effect of 5T4, immunohistochemistry assessment was conducted on several tissue biopsies which confirmed its limited expression in normal tissues. Like many targets in AML, expression of 5T4 was evaluate on haematopoietic stem cells. 5T4 expression was detected on CD34+ BM cells from healthy donors as for CD33 and CD123 on CD34+CD38- and CD34+CD38+ BM cells [25]. The data reported in this study suggest that both the percentage of 5T4 expression was lower than that published for CD33, CD123 and CD200 [25]. The effects of 5T4 CAR-T cells or non-transduced T cells on human hematopoietic progenitor cells from ten healthy donors were evaluated using *in vitro* CFC assays. There was no statistically significant decrease in CFC colony output or negative impact on colony morphology following co-culture with 5T4 CAR-T cells. In contrast, co-culture of 5T4 CAR-T cells with the human AML cell line, Kasumi-1, resulted in a statistically significant decrease in CFC colony numbers. From the literature, our data appear to exhibit a much safer profile when compared with CAR-T cells targeting CD123 which showed a significant impact on myeloid colony formation and total cell number following short-term exposure to human CD34+ cells [26].

Our next and final step was to show that 5T4 CAR-T cells could expand *in vivo*, retain their therapeutic ability to recognise and kill 5T4 expressing cells, and that high expression level of 5T4 on cancer cells does not give therapeutic advantage, as suggested by our *in vitro* data that even cells expressing low number of molecules per cells will be targeted by 5T4 CAR-T cells.

Cell-line derived xenograft into immunodeficient mice is a relatively simple and easy way to develop leukaemia mouse models [27-30]. Injection of a cell line stably expressing firefly luciferase allows tracking of cancer development by monitoring the bioluminescent intensity (BLI) from each animal over time to provide a non-invasive method to monitor the change in tumour burden over the running of the in-life experiment [31]. For the *in vivo* study, THP-1 firefly luciferase -expressing cells were injected into NCG mice; 5T4 CAR-T cells or non-transduced control cells were generated using a manufacturing protocol optimised using IL-7/IL-15 instead of IL-2 to generate a larger number of cells of a less differentiated T cell phenotype with the aim that the generation of "stem-like" CAR-T cells would lead to greater *in vivo* persistence at day 4 and day 7 post-transduction [32-34]. Day 4 manufacturing was chosen to shorten the manufacturing process, reduce time and cost while maintaining stem like cells phenotype within the CAR-T population. The endpoints for the pilot murine experiment were designed to prove the expansion of 5T4 CAR-T cells and their ability to control tumour growth by monitoring BLI over-time.

Data reported in this *in vivo* study show that transduced cells were successfully identified in the blood of treated mice injected with different doses and preparations of 5T4-CAR-T cells, starting at day 7 and peaking at day 35 post treatment. The mice treated with the highest dose of 5T4-CAR-T cells 7 days post transduction were able to keep under control the tumour growth as demonstrated by the lower BLI detected in this group from day 14 post treatment and for the subsequently three weeks.

Interestingly but not surprisingly, day 7 non-transduced T Cells (control group day 7) were comparable to the lower doses of 5T4-CAR-T cells in terms of BLI output, thus suggesting that using IL-7 and IL-15 combination during manufacturing generated not only generate more stem-like cells [33] but likely contribute to produce Cytokine-Induced Killer (CIK) cells. CIK cells have been developed as a therapeutic treatment [35], whether CIK cells in the CAR-T cells preparation might add a synergistic effect to CAR-T treatment, it should be considered carefully to understand the dose regimen in patients. Most of the animals treated with the highest dose of 5T4 CAR-T cells lost weight and reached the humane endpoint point and were sacrificed. Post-mortem examination showed no tumour growth in livers (the organ mostly affected by THP-1 tumour cells [36]). However, post-mortem examination revealed a shrivelled and pale liver, which suggested sign of toxicity due to tumour lysis syndrome (TLS) or GvHD, but no histology was performed on these animals.

If the reason of the weight loss in the animal injected with the high concentration of 5T4-CAR-T cells was truly TLS, the time of treatment after implant is crucial: while CAR-T cells are expanding, THP-1 cells are expanding too and they do express 5T4, therefore the killing may have been so extensive that it induced toxicity.

Future experiments to prove efficacy and dose-response of 5T4 CAR-T cells should consider injection of CAR-T cells much earlier after tumour cell implant, as reported by other studies. We injected the CAR-T cells at day 11 post THP-1 tumour injection, whereas most, if not all, publications that we have reviewed report experiments when the CAR-T cells are injected earlier (between day 2 and 4) post tumour cell line injection, albeit using different cell and/or route of administration [37-38].

This experiment demonstrates that if the tumour cells do express 5T4, they will be targeted and killed. This would be important for patients who express a low level of 5T4, as this means that this treatment can be combined with other therapeutics to eradicate blasts that might otherwise escape treatment.

In summary, the data presented here are validate 5T4 as a target to treat AML using CAR-T cells and potentially other types of immunotherapies targeting this antigen, such as bi-specific antibodies. In particular we also demonstrated that 5T4 CAR-T cells could also target the LSC compartment. 5T4 showed a low expression in healthy tissues and in BM cells from healthy donors, which will likely translate into a good safety profile in humans when targeted in patients. 5T4-CAR-T cells can kill *in vitro* AML cell lines. 5T4-CAR-T cells can expand *in vivo* in a THP-1 model and are able to kill 5T4 positive cancer cells. These results demonstrate that 5T4 is a promising target for immune intervention in AML and CAR-T cells should be considered for personalised cancer immune therapy to treat AML.

Authors' Contributions

R. Harrop, D. Blount, B. Souberbielle, K. Mitrophanous, A. Ettorre: Conceptualization, visualisation, resources, supervision, formal analysis, methodology, writing-original draft, writing–review and editing. M. Soyombo, L. Moyce, J. Down: Formal analysis, investigation, visualization, writing–review and editing. M. Lawson, N. Khan, M. Drayson, D. O'Connor, R. Nimmo, Y. Lad: Investigation, resources, supervision, project administration, review and editing.

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Table 1. Summary of Screening of leukocytes obtained from patients diagnosed with different types of blood malignancies.

Type of Leukaemia	Total number of Samples	Total number of samples positive for 5T4+	Percentage of 5T4 positive samples
Chronic Lymphocytic Leukaemia (CLL)	9 + 14 (23)	0	0%
Multiple Myeloma (MM)	31	22	71%
Acute Myeloid Leukaemia (AML)	36	19	53%
B-Cell Acute Lymphoblastic Leukaemia (B-ALL)	21	11	52%
T-Cell Acute Lymphoblastic Leukaemia (T-ALL)	11	2	18%
Chronic Myeloid Leukaemia (CML)	7	2	29%
Acute Promyelocytic Myeloid Leukaemia (APML)	9	7	78%

Table 1 summarises the results of the screening of leukocytes obtained from patients diagnosed with different types of blood malignancies stained and analysed by flow cytometry as described in the related Materials and Methods section. A sample was reported to be 5T4 positive if the rMFI is ≥1.5. The rMFI is calculated as MFI of test sample/MFI of isotype control.

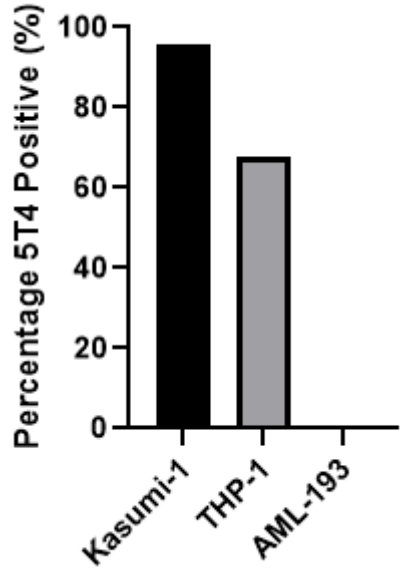
Table 2. Effect of 5T4 CAR-T cells on colony formation of Kasumi-1 cells.

E:T Ratio and Replicate Number		Mean Colonies ± SD	P-Value cf Kasumi-1 only	P-Value cf non-transduced control cells
Kasumi-1 Cells Only	1	113 ± 7	N/A	N/A
	2	111 ± 5		
	3	111 ± 2		
Kasumi-1 + 5T4 CAR-T Cells				
10:1	1	56 ± 10	0.001	0.004
	2	45 ± 7		
	3	67 ± 6		
Kasumi-1 + Non-transduced T Cells				
10:1	1	100 ± 9	N.S.	N/A
	2	98 ± 4		
	3	112 ± 21		

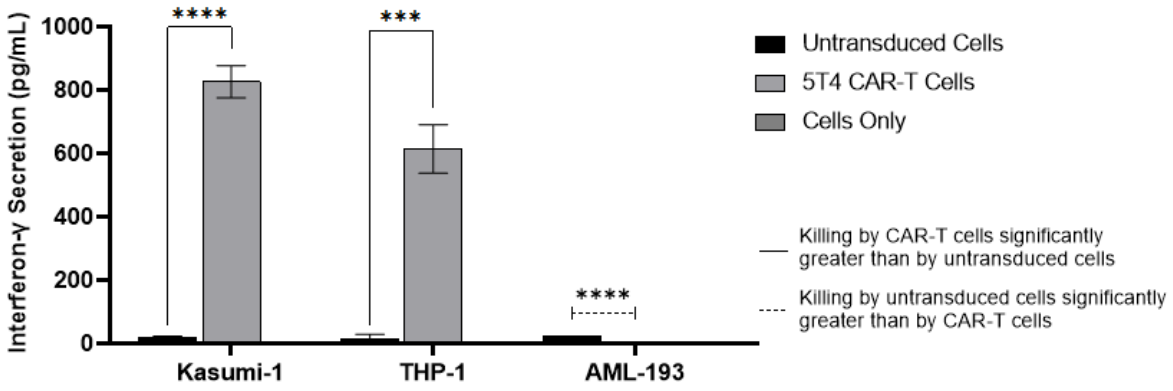
Kasumi-1 cells were co-cultured for 4 hours with 5T4 CAR-T cells or non-transduced control cells. Average colony counts were assessed after 13-day culture in MethoCult. Statistical significance compares each test condition to the corresponding target (Kasumi-1) only control or comparison of +5T4 CAR-T cells to +non-transduced control cells. Key: N/A = not applicable; N.S. = Non-significant.

Figure 1. Functional activity of 5T4 CAR-T cells. Functional activity of 5T4 CAR-T cells following co-culture with 5T4-positive (Kasumi-1 and THP-1) and 5T4-negative (AML-193) tumour cell lines was assessed at an effector to target (E:T) ratio of 1:1. Percentage 5T4 expression was assessed by flow cytometry prior to functional activity assays (A), experimental read-outs of efficacy included quantification of IFNɣ (B), Granzyme-B (C) and cell killing (D). Statistical significance is indicated (p value < 0.0001 is denoted by ' **** ', p value between 0.0001 to 0.001 is denoted by ' *** ', p value between 0.001 to 0.01 is denoted by '**' and a p value ≥ 0.05 is denoted by 'ns'; not significant).

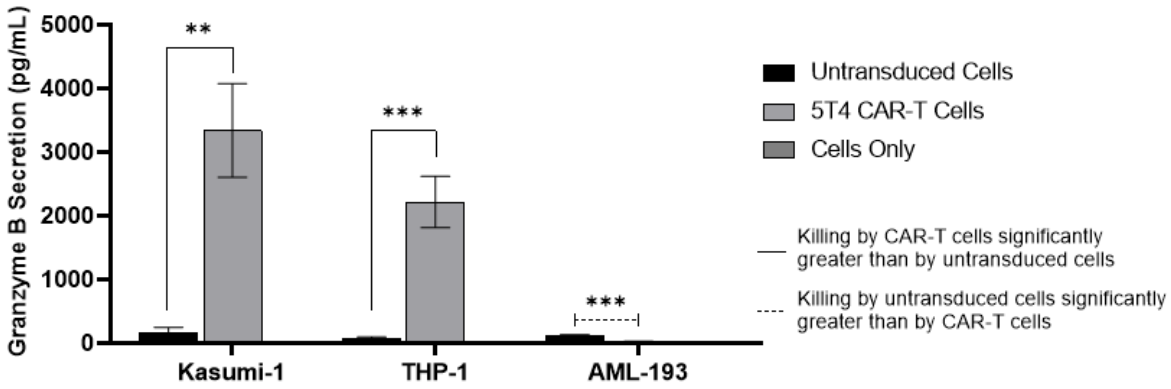
A. Percentage 5T4 Expression



B. IFNɣ



C. Granzyme-B



D. Cell Killing

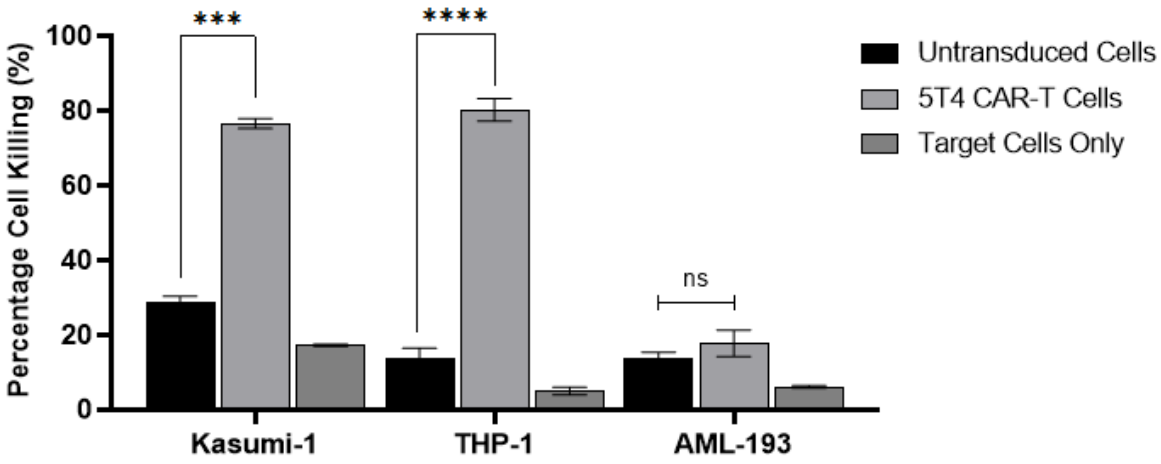
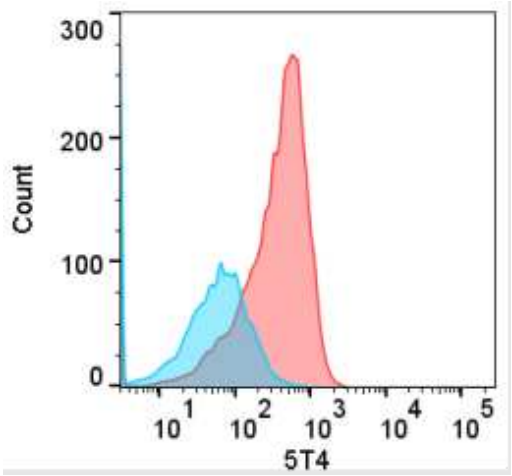
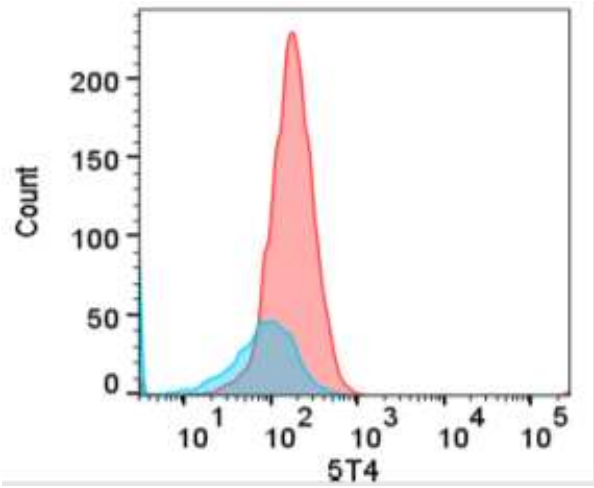


Figure 2. 5T4 CAR-T cells kill target Kasumi-1 and THP-1 cells that are categorised as 5T4 negative by FACS. Figure 2A and 2B shows overlaid histogram plots of the 5T4 staining of Kasumi-1 (A) or THP-1 (B) cells left alive following co-culture with 5T4 CAR-T cells (blue) or non-transduced control cells (red). Figures 2C and 2D show the percentage of Kasumi-1 (C) or THP-1 (D) cells left alive following co-culture with 5T4 CAR-T cells relative to non-transduced control cells. Cells have been stratified into 3 arbitrary MFI ranges for 5T4 expression: positive by flow (MFI>200), weakly negative (MFI 70-200) or strongly negative (MFI <70).

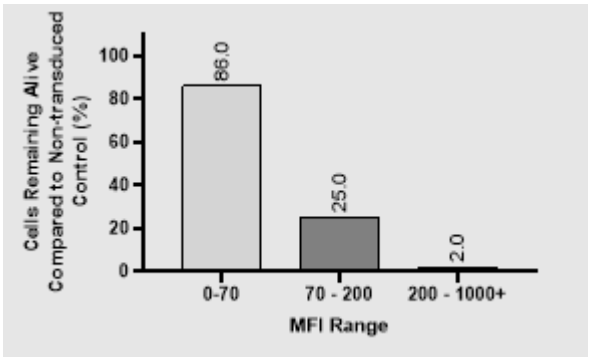
A: Kasumi-1 Cells



B: THP-1 Cells



C: Kasumi-1 Cells



D: THP-1 Cells

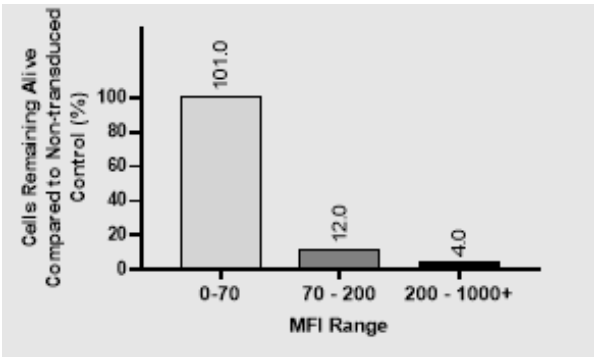


Figure 3. 5T4 CAR-T cells kill Kasumi-1 leukaemic stem cells. The number of Kasumi-1 cells remaining alive following co-culture with 5T4-CAR T cells or non-transduced control cells is plotted stratified by cell phenotype (CD34+CD38- cells = leukaemic stem cells; LSCs). CAR-T cells derived from 2 different donors were tested. Statistical significance is indicated (p value < 0.0001 is denoted by ' **** ' and a p value between 0.0001 to 0.001 is denoted by ' *** ').

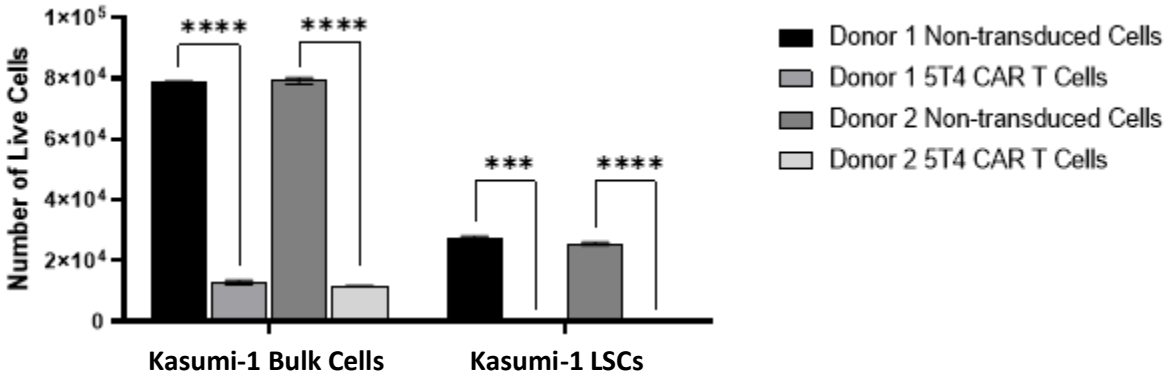
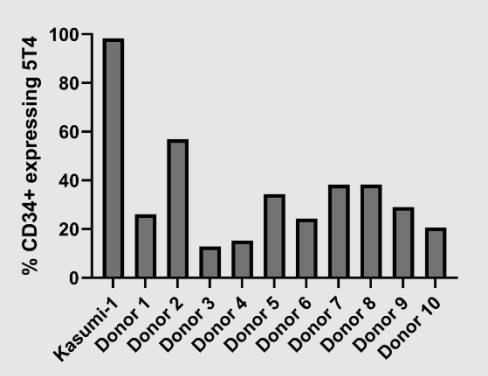
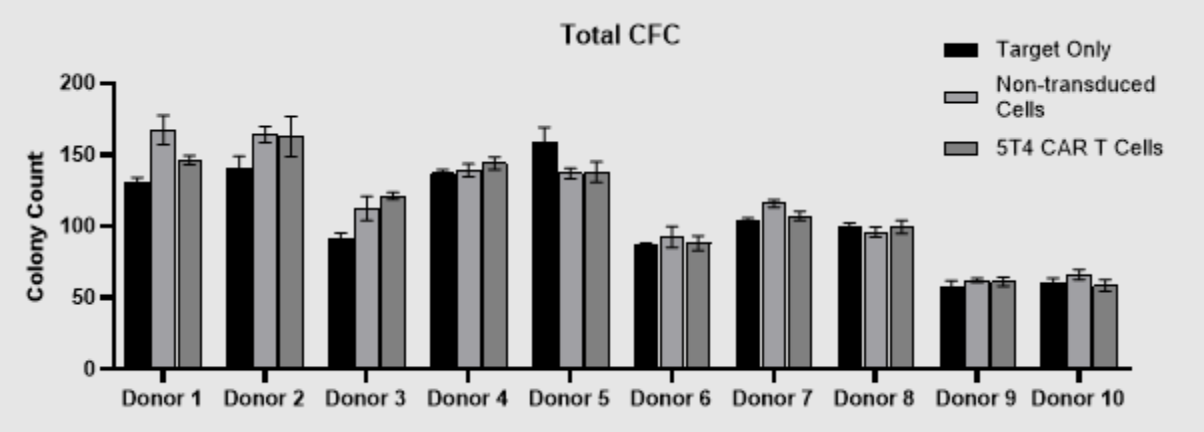


Figure 4: Impact of 5T4 CAR-T cells on colony formation.
Expression of 5T4 on the 10 donors tested in this study (A); the number of total colonies (B), BFU-E (C), CFU-GM (D) and CFU-GEMM (E) colonies following co-culture with 5T4 CAR-T cells, non-transduced T cells or cells alone are illustrated.

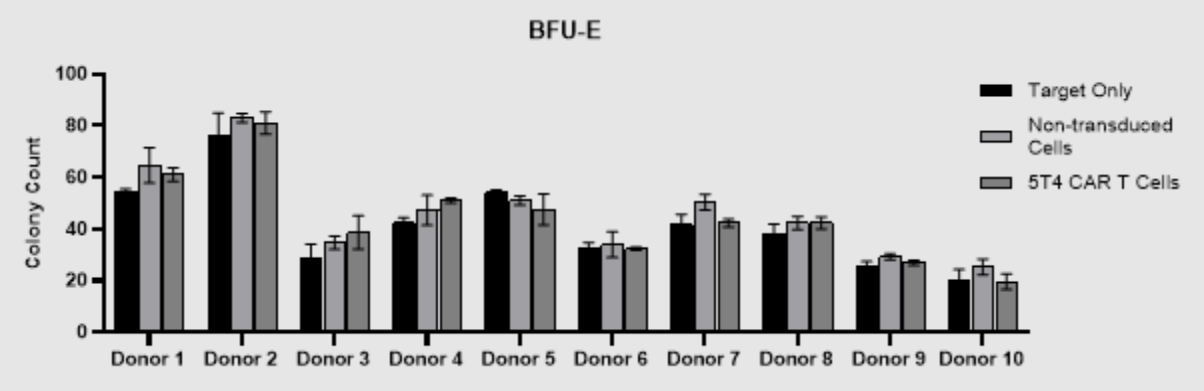
A: Expression of 5T4 as percentage



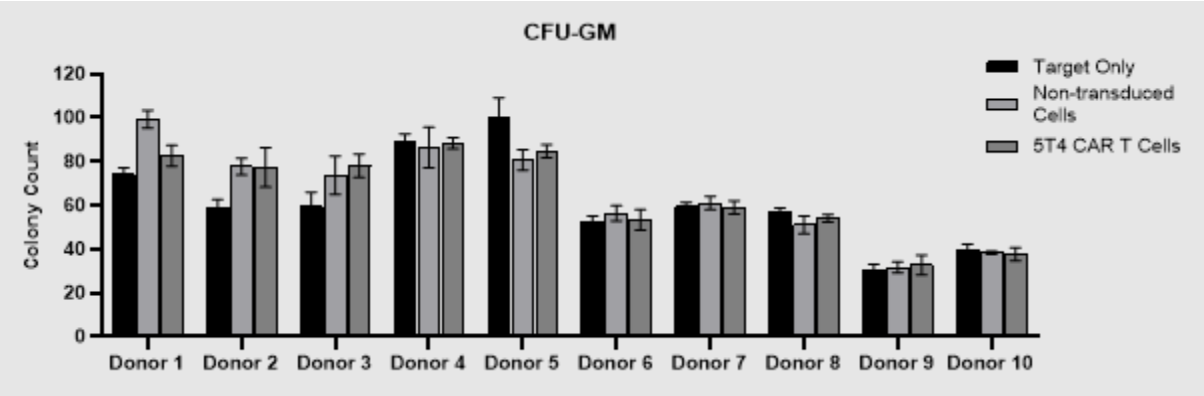
B: Total Colonies



C: BFU-E



D: CFU-GM



E: CFU-GEMM

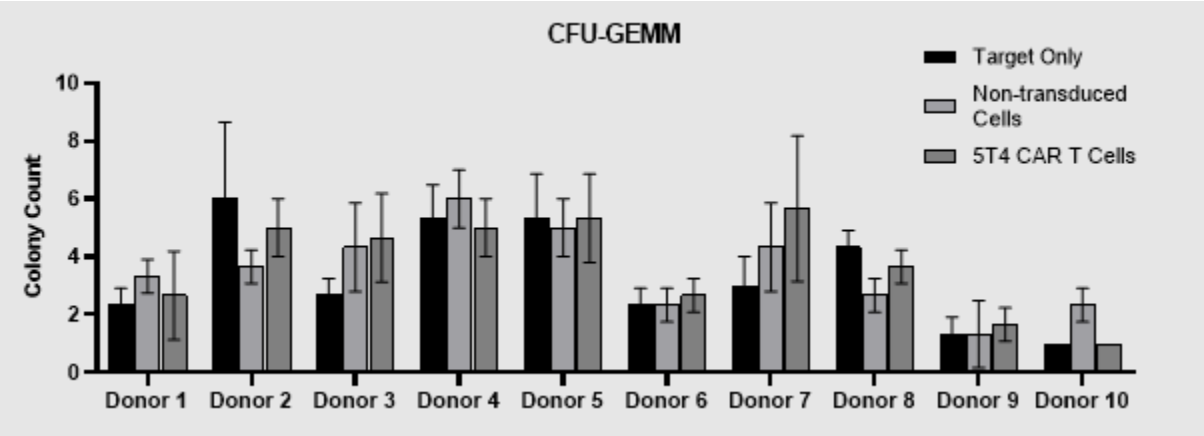
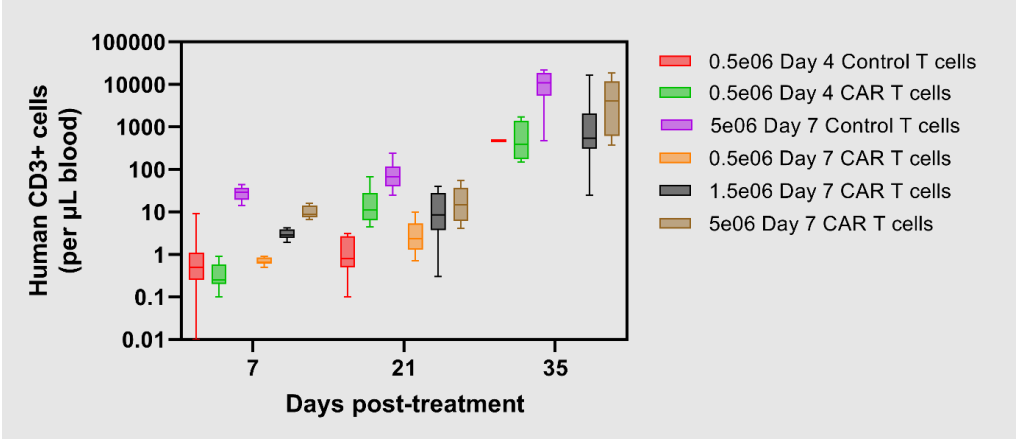
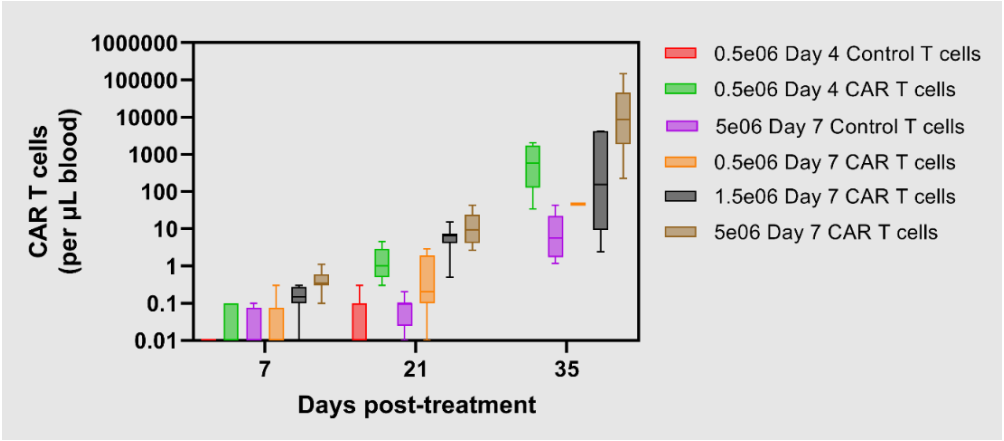


Figure 5: 5T4 CAR-T cell activity in an NCG mouse model of AML.
NCG mice were dosed with firefly luciferase-labelled THP-1 cells and either not treated or treated in a single dose model using Day 4 or Day 7 manufactured CAR-T cells at increasing doses. Circulating CD3+ T cells (A) or 5T4 CAR T-cells (B) were measured by MFC, 7-, 21-, and 35-days post-treatment. Tumour progression was estimated by BLI imaging (C). Example images taken at Day 28 are shown for mice in experimental groups - No Treatment (D), 0.5e06 Day 4 non-transduced cells (E), 0.5e06 Day 4 5T4 CAR-T cells (F), 5.0e06 Day 7 non-transduced cells (G), 0.5e06 Day 7 5T4 CAR-T cells (H), 1.5e06 Day 7 5T4 CAR-T cells (I), and 5.0e06 Day 7 5T4 CAR-T cells (J). Survival was monitored until study termination (K).

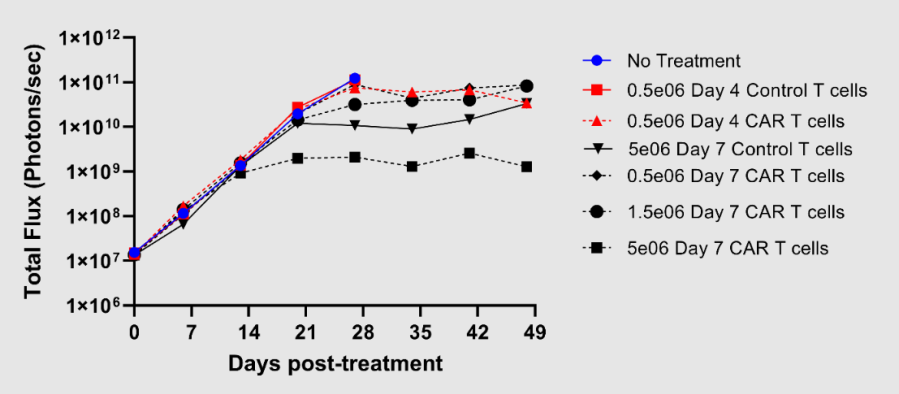
A



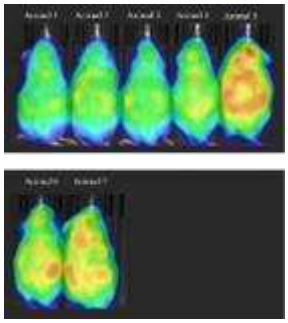
B

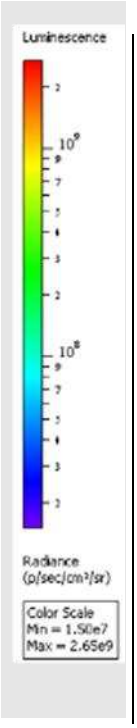


C

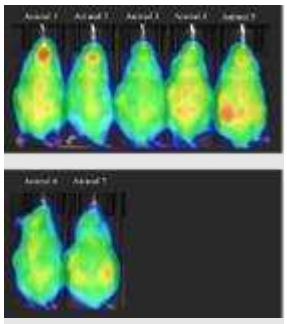

Total Flux (Photons/sec)
1×10^12
1×10^11
1×10^10
1×10^9
1×10^8
1×10^7
1×10^6
0
7
14
21
28
35
42
49
Days post-treatment
No Treatment
0.5e06 Day 4 Control T cells
0.5e06 Day 4 CAR T cells
5e06 Day 7 Control T cells
0.5e06 Day 7 CAR T cells
1.5e06 Day 7 CAR T cells
5e06 Day 7 CAR T cells

D

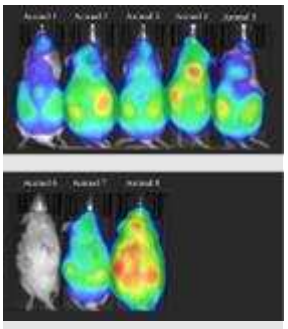



Luminescence
Radiance (p/sec/cm²/sr)
Color Scale
Min = 1.50e7
Max = 2.65e9

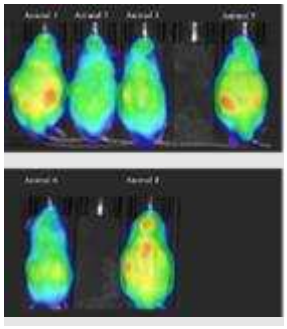
E



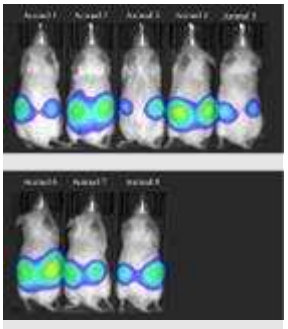
F



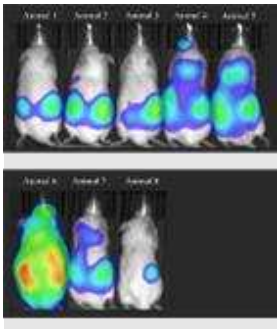
G



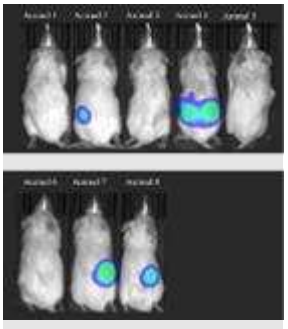
H



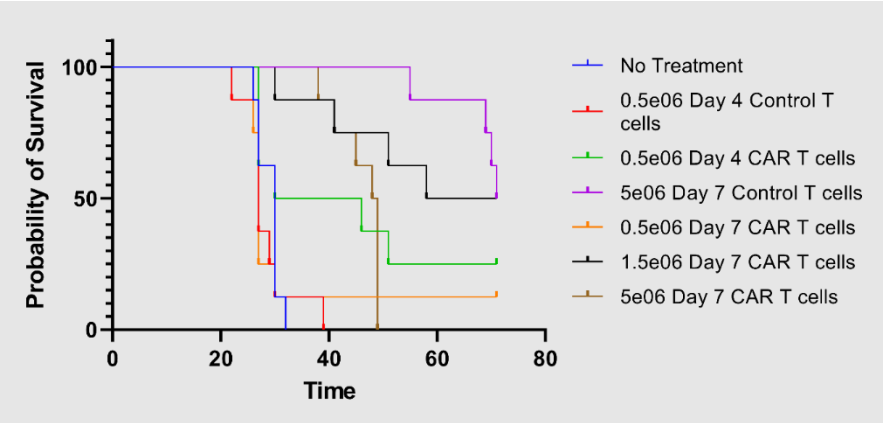
I



J



K

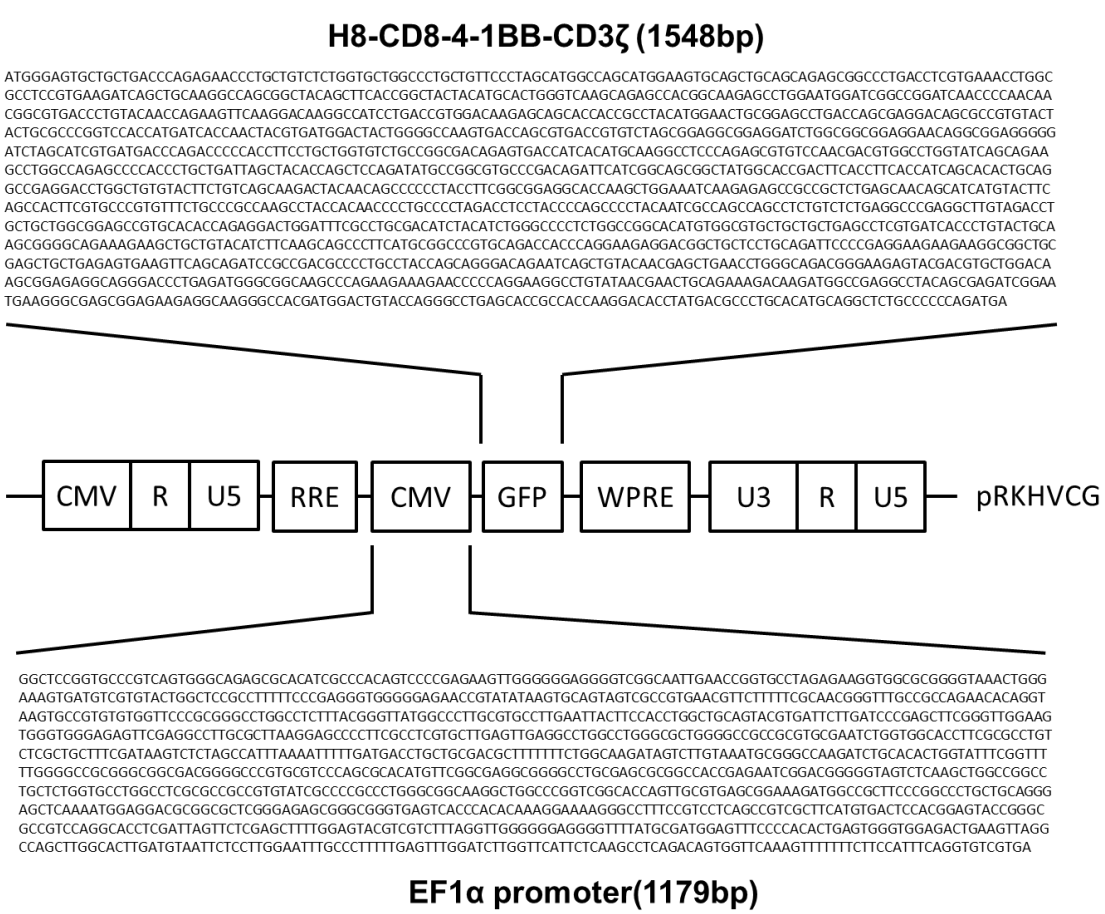

Probability of Survival
100
50
0
0
20
40
60
80
Time
No Treatment
0.5e06 Day 4 Control T cells
0.5e06 Day 4 CAR T cells
5e06 Day 7 Control T cells
0.5e06 Day 7 CAR T cells
1.5e06 Day 7 CAR T cells
5e06 Day 7 CAR T cells

Supplementary Table 1: 5T4 Expression in AML Cell Lines.

Cell Line	FAB	Protein Quantification				
		% 5T4+	MFI	rMFI	ABC	5T4 Status (FACS)
AML-193	M5	0.1	15.8	0.88	0	Negative
Kasumi-1	M2	87.1	262	17.47	4954	Positive
Kasumi-3	M0	0.23	256	0.81	N/A	Negative
KG-1a	M1	0.02	10.5	0.77	0	Negative
MV4-11	M5	0.45	19.5	0.96	0	Negative
NB-4	M3	0.52	396	0.88	N/A	Negative
NOMO-1	M5a	15.2	75.7	N/A	187	Positive
OCI-AML-2	M4	93.8	447	N/A	1332	Positive
TF-1	M6	99.9	12572	N/A	53571	Positive
THP-1	M5	40.5	171	5.1	2225	Positive

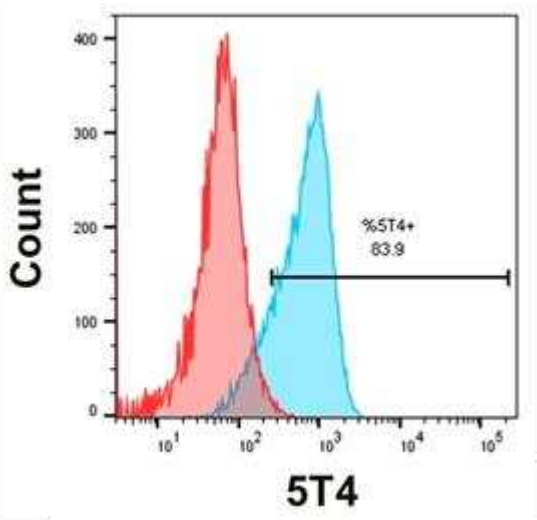
Expression of 5T4 was evaluated by means of flow cytometry staining and positivity established as described in the related section of Materials and Methods.
The percentage of cells positive for 5T4 was calculated by subtracting the isotype control from the total percentage of positive cells, to correct for non-specific binding.
The MFI is calculated on the live cell population. The rMFI is calculated as MFI of test sample/MFI of isotype control.
The ABC value is calculated as per manufacturer's protocol (Quantum™ Simply Cellular, Bangs Laboratories).
Human AML cell lines Kasumi-1 (RRID:CVCL_0589), Kasumi-3 (RRID:CVCL_0612), THP-1 (RRID:CVCL_0006), and AML-193 (RRID:CVCL_1071) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA); KG-1a (RRID:CVCL_1824), MV4-11 (RRID:CVCL_0064), NB-4 (RRID:CVCL_0005) were purchased from CLS Cell Lines Service GmbH (now Cytion, Germany); NOMO-1 (RRID:CVCL_1609) was purchased from Leibniz Institute DSMZ (Germany), OCI-AML-2 (RRID:CVCL_1619) and TF-1 (RRID:CVCL_0559) were screened by CrownBio (Suzhou, Jiangsu, China).
All cell lines mentioned above were cultured accordingly to the Suppliers' instructions.

Supplementary Figure 1. Schematic representation of 5T4 construct.

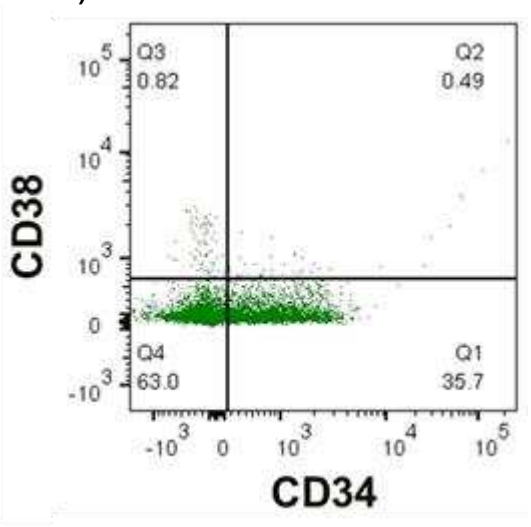


Supplementary Figure 2. A) 5T4 is highly expressed in the AML cell line Kasumi-1. Red: Isotype Antibody; Blue: anti-5T4 Antibody. B) Flow cytometry was used to identify the putative leukaemic stem cell population based on the expression of CD34 and absence of CD38 (CD34+CD38-; Q1 quadrant). Approximately 36% of Kasumi-1 cells were identified as the putative leukaemic stem cell subset.

A) Kasumi-1 5T4 expression



B) Leukaemic stem cell subset



Supplementary Figure 3: 5T4 expression in normal tissues.

Immunohistochemistry studies were conducted by Covance, UK (now Labcorp, UK). Tissue sections were fixed in Acetone for 10min and then let them air-dry before blocking then with a solution of H_2O_2 in methanol (1ml:50ml) for additional 10 min. afterwards the sections were washed three time for 5 minutes each time with Washing Buffer (PBS/0.02% Tween20). The sections were then blocked with rabbit serum and then with Avidin/Biotin blocking kit as per manufacturer's instructions (RRID:AB_2336231, Vector Laboratories Inc., Newark, US). Sections were then rinsed with PBS and stained for 1h with anti-5T4 antibody (H8 clone) (dilution 1/1,000 in rabbit serum). After the incubation, the slides were washed three times for 5minutes each time with Washing Buffer (PBS/0.02% Tween20). Sections were then incubated with the secondary antibodies for 30 to 45 minutes at room temperature with biotinylated rabbit anti-mouse according to the ABC kit's instructions. After the incubation, the slides were washed three times for 5 minutes each time with Washing Buffer (PBS/0.02% Tween20). Prepare and add the tertiary reagent to the sections for 30 minutes at room temperature, according to the manufacturer's instructions (RRID:AB_2336819, ELITE anti-mouse ABC detection kit, Vector Laboratories, Newark, US). After the incubation, the slides were washed three times for 5 minutes each time with Washing Buffer (PBS/0.02% Tween20). Apply DAB solution, made up as per manufacturer's instructions (Vector Laboratories, Newark, US), for up to 10 minutes. Repeat the washing step, three times for 5 minutes each time in distilled water. Counterstain with Haematoxylin & Eosin for approximately 20 seconds, then was again the slides in water. The slides were then dehydrated through graded alcohols (70%, 90% and 100% Ethanol), cleared in Xylene and finally coverslips were added with DPX.

Sections prepared from placenta were used as positive control, as 5T4 is highly expressed in the trophoblast. An example of the staining pattern can be seen in Supplementary Fig. 3B.

Staining with H8 clone localised in the epithelia of the fallopian tube, urothelium of the bladder, endometrium and ureter. Staining was also visible in the mesothelium and epithelium of the ovary. Isolated positive cells were visible in the *muscolaris mucosa* of the duodenum and of the oesophagus, and the pituitary gland (Supplementary Fig. 3A and B). To note, as highlighted in Supplementary Fig. 3B, five tissues showing moderate positivity for 5T4 were obtained from patients whose cause of death was described as cancer.

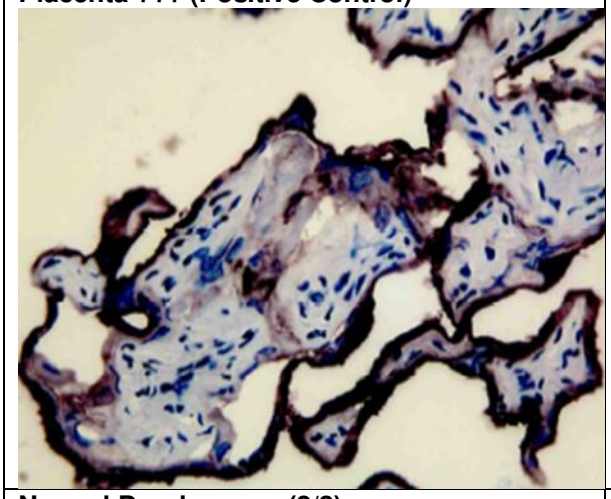
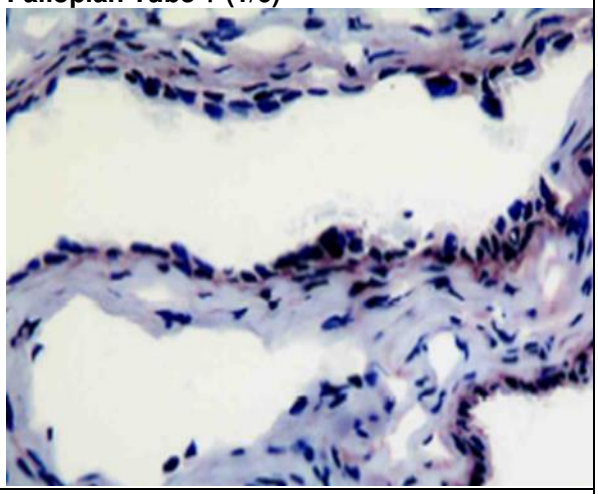
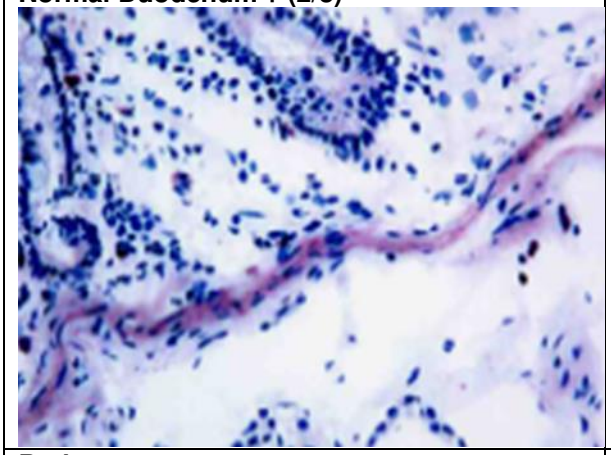
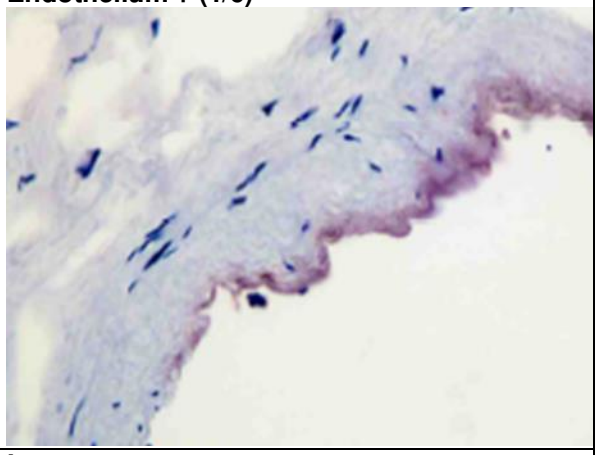
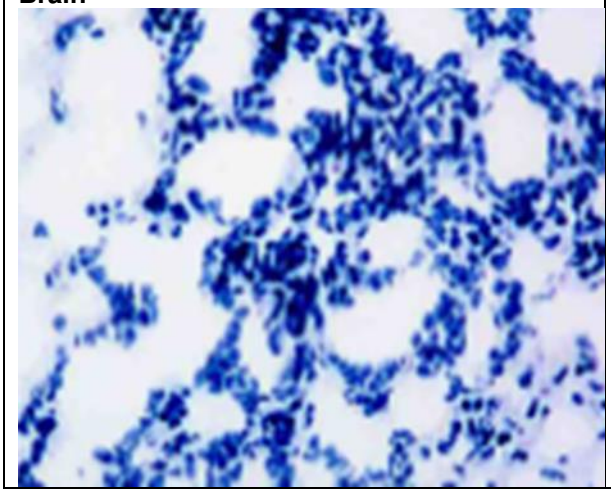
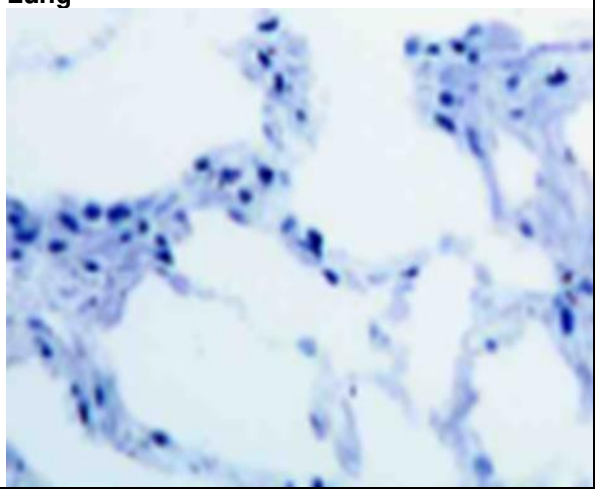
The data presented in Supplementary Fig. 3A and 3B suggest that anti-5T4 antibody (clone H8) showed minimal cross-reactivity with normal tissues. Our data confirm previous reports suggesting the limited expression of 5T4 in normal tissues, which makes it an ideal cancer target for onco-therapies.

Supplementary Figure 3A. 5T4 positivity in different normal tissues.

Tissue	Anti-5T4 ANTIBODY H8 clone	Level of Staining
Spinal Cord	0/3	neg
Brain Cerebellum	0/3	+
Brain Cortex	0/3	neg
Breast	0/3	neg
Colon	0/3	neg
Duodenum	2/3	+
Ileum	0/3	neg
Eye	0/3	neg
Fallopian Tube	1/3	+
Ovary	1/6	+
Uterus (Cervix)	0/3	neg
Uterus (Endometrium)	1/3	+
Placenta	6/6	+++
Heart	0/3	neg
Kidney	0/3	neg
Ureter	3/5	+
Bladder	4/6	+
Liver	0/3	neg
Pancreas	0/3	neg
Spleen	0/3	neg
Lung (Bronchus)	0/3	neg
Parathyroid	0/3	neg
Adrenal Gland	0/3	neg
Thyroid Gland	0/3	neg
Pituitary	2/5	+
Lymph-node	0/3	neg
Prostate	0/3	neg
Testis	0/3	neg
Skin	0/3	neg
Skeletal Muscle	0/3	neg

Tissues were analysed and staining score: negative (neg); positivity: weak staining (+), low/medium staining (++), high staining (+++)

Supplementary Figure 3B: Example of tissue staining.

Placenta +++ (Positive Control)	**Fallopian Tube + (1/6)**
	
Normal Duodenum + (2/3)	**Endothelium + (1/6)**
	
Brain –	**Lung –**
	

Supplementary Figure 3C. Scoring system used for the tissue presented in Supplementary Figure 3A.