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Allo-defensive, multiplex base-edited, anti-CD38 CAR T cells for 'off-the-shelf' immunotherapy

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Disclosures

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Author contributions

W.Q., R.P., and C.G., designed the project. R.P., C.G., O.G., R.K., A.J., E.C., and D.K., performed experiments and analysed data; R.P., and W.Q. wrote the manuscript. All authors have reviewed and approved the manuscript.

Data availability

Raw data presented and analysed in this manuscript can be requested from the corresponding author.

Abstract

Chimeric antigen receptor (CAR) T cell therapies are being widely investigated in both autologous and allogeneic settings, with gene editing providing new strategies to address barriers to mismatched cell therapies. Currently 'universal' donor derived T cell therapies require intensive lymphodepletion and are still prone to host-mediated rejection. CD38, a transmembrane glycoprotein involved in cell activation and bioenergetics, is a promising immunotherapy target for haematological malignancies. Disruption of CD38 expression using base editing prevented fratricide between T cells expressing anti-CD38 CAR (CAR38). Additional base editing enabled generation of 'universal' donor CAR38-T cells, devoid of endogenous TCRαβ and Human Leukocyte Antigen (HLA) molecules after disruption of *T Cell Receptor Beta Constant* (*TRBC*), *Beta-2 microglobulin* (*B2M*), and *Regulatory Factor X5* (*RFX5*). Removal of cell surface HLA expression enabled evasion of anti-HLA antibodies in sera from sensitised donors and reduced allo-stimulation in mixed lymphocyte cultures (MLCs), while TCRαβ disruption prevented allo-reactivity. In MLCs, CAR38 expression enabled potent 'allo-defense' activity against $CD38^+$ allo-reactive cells. Multiplex-base-edited CAR38-T cells exhibited antigen-specific anti-leukemic activity against human B, T, and myeloid malignancies and inhibited disease progression in humanised murine xenograft models. CAR38-T cells offer a potent 'off-the-shelf' strategy against $CD38^+$ haematological malignancies and long-lived plasma cells which can be associated with autoantibody production.

Introduction

Chimeric antigen receptor (CAR) T cells offer new avenues for B cell malignancies including acute lymphoblastic leukaemia (ALL), with products targeting CD19 or B-cell maturation antigen (BCMA) commercially available (1, 2). Therapeutic applications are also under investigation for auto-immune disorders where CAR-mediated B cell elimination has induced remissions of Systemic Lupus Erythematosus (SLE) and other conditions (3-6). Limitations and challenges of autologous approaches include disease and host immune cell heterogeneity (7-9), unwanted toxicities from shared antigens (10), and risks of antigen-masking following accidental transduction of blasts (11). Genome-edited allogenic CAR T cells manufactured from healthy donors offer 'off-the-shelf' alternatives that can be pre-manufactured and used for a variety of indications (12-14). We have previously manufactured allogeneic anti-CD19 CAR T cells using TALEN or CRISPR/Cas9 editing to remove the T-cell receptor-αβ (TCRαβ) preventing graft-versus-host disease (GVHD) and CD52 to promote survival in the presence of the lymphodepleting antibody Alemtuzumab (12, 13, 15). We have also investigated base edited anti-CD7 CAR T cells (BE-CAR7) (10, 14), and BE-CAR33 T cells (16) in human studies against T-ALL and acute myeloid leukaemia (AML), respectively. For all these settings, healthy donor derived allogeneic CAR (allo-CAR) T cells mediated potent anti-leukemic effects but relied on intense lymphodepletion with augmented doses of fludarabine and cyclophosphamide as well as alemtuzumab. Here we report T cells armed with an anti-CD38 CAR, generated using cytidine base editing to first remove CD38 expression, (17) can mediate potent anti-leukemic effects and acquire allo-defensive properties.

CD38 is an extracellular type-II glycoprotein with multiple immune regulatory functions and has been exploited as an immunotherapy target using anti-CD38 monoclonal antibodies daratumumab or isatuximab, which have been approved for indications including multiple myeloma (MM) (18), acute lymphoblastic leukaemia (ALL) and AML (19, 20). Phase-I clinical trials have also reported autologous anti-CD38 CAR (CAR38) T cells with encouraging safety and efficacy profiles (21-25). Genome editing now offers opportunities to improve CAR38 products by disrupting CD38 expression to prevent fratricide and address barriers to allow mismatched allogenic T cells to be used without Human Leukocyte Antigen (HLA) matching.

We combined lentiviral vector delivery of CAR38 with cytosine deaminase mediated base editing to knockout TCRαβ and CD38 alone or in combination with HLA disruption, for universal configurations (***Figure 1A***) (17). Cytidine to thymidine (C>T) conversions introduced premature stop codons or disrupted splice sites of one or more genes at high efficiency and without DNA breaks, allowing BE-CAR38 T cell products to be generated efficiently for investigations *in vitro* and in humanised murine models.

Methods

CAR T cell manufacture

Mononuclear cells (MNCs) from healthy blood donations (UCL, REC: 25257.001 or REC: 19/LO/0447) were activated with TransACT (Miltenyi Biotec, Bergisch Gladbach, Germany) and cultured in TexMACS media (Miltenyi Biotec) supplemented with 3% heat inactivated human serum (Seralab, Sussex, UK) and 20ng/mL human recombinant IL-2 (Miltenyi Biotec) as described previously (10). Genome editing used codon-optimised cytidine base editor-3 (BE3) mRNA (TriLink BioTechnologies, California, USA). Delivery and molecular assessments are described in Supplementary methods. Where indicated, residual TCRαβ- and HLA-expressing cells were depleted using biotin-conjugated anti-TCRαβ (clone: BW242/412, Miltenyi Biotec), anti-HLA-ABC (clone: REA230, Miltenyi Biotec), and anti-HLA-DR,DP,DQ (clone: REA332, Miltenyi Biotec) antibodies. Primary anti-human antibodies used for immunophenotyping are provided in Supplementary Table S1.

CAR lentiviral vectors

CARs were expressed from 3rd-generation lentiviral vector configurations and transductions performed at multiplicity of infection (MOI) 5 (10, 14). Anti-CD38 scFv was derived from Daratumumab (heavy-variable-light-variable orientation with a $GGGGS_3$ linker). We previously described configurations for CAR19 (clone: 4g7) (12, 13), CAR7 (clone: 3A1e) (10, 14), and CAR33 (clone: My96) (26). All CAR configurations contained a CD8α hinge/transmembrane region, 4-1BB co-stimulatory domain, and CD3ζ intracellular signalling domain (scFv-CD8α-4-1BB-CD3ζ).

Flow-based *in vitro* cytotoxicity assay

EGFP antigen-positive and antigen-negative tumour cells were co-cultured at a 1:1 ratio for 4 hours with CAR^+ T cells across effector-to-target (E:T) ratios. Co-cultures were then stained for antigen expression and viability, with the ratio of antigen positive to antigen negative tumour cells used to calculate specific lysis.

Mixed lymphocyte cultures

Irradiated BE-CAR T cells (30Gy) were co-cultured in a 96-well plate (1:1 ratio) with allogeneic MNCs. After 5 days, wells were pulsed with 1µCi 3H-thymidine (Revvity, Massachusetts, USA) and incubated for 18-20 hours before transfer of 3H-thymidine labelled DNA to a Filtermat (Revvity) using a cell harvester (TOMTEC Imaging Systems, Unterschleissheimn Germany). Meltilex (Revvity) was applied to the Filtermat, and 3H-thymidine incorporation was read using a MicroBeta counter (PerkinElmer, Massachusetts, USA). Flow cytometry-based readouts were setup as above before staining and acquisition on day 5.

Flow cytometric crossmatch

BE-CAR T cells were incubated with control or test sera (containing blood donor derived anti-HLA antibodies) for 30 minutes. Samples were stained with anti-human IgG, CD3, and CD19 for 25 minutes before acquisition on a FACSLyric Flow Cytometer (BD).

***In vivo* CAR T cell studies**

Animal studies approved by the UCL Biological Services Ethical Review Committee and licensed under the Animals (Scientific Procedures PP5675666) Act 1986 (Home Office, London, United Kingdom). NOD/SCID/γc–/– (NSG) mice (Charles River, The Jackson Laboratory), were inoculated by intravenous (IV) injection with $EGFP^+LUC^+$ Daudi ($0.5x10^6$), Jurkat ($1x10^7$), or MOLM14 ($1x10^5$) on day 0. Engraftment was confirmed on day 5 by bioluminescence imaging (BLI) using an IVIS Lumina III (PerkinElmer, live image version 4.5.18147). On day 6 mice received either $2.5x10^6$ unmodified, or BE-CAR^+ T cells. Tumour inhibition was assessed by serial BLI and bone marrow at necroscopy.

Statistical analysis

Graphs show mean ± SEM. One-way ANOVA (paired or unpaired) with Tukey's post-hoc test and log-rank survival analysis were performed in GraphPad Prism Version 10.4.1.

Results

Base editing prevents fratricide and enables efficient anti-CD38 CAR T cell production

After activation with anti-CD3/CD28 transact reagent T cells upregulated cell surface expression of CD38 from around 11% to 85% (n=3) compared to around 20% (n=3) in T cells with disrupted *CD38* through the introduction of a premature stop codon by base editing (***Supplementary figure 1A&B***). Sanger sequencing confirmed appropriate C>T conversions in the anticipated window of deamination with 77% conversions at position C6 (protospacer position six) and 68% at position C7 (n=3) (***Supplementary figure 1C***). The effects of CD38 knockout were investigated in CAR19 and in CAR38 T cells after transduction with the respective lentiviral vector with simultaneous genome editing of CD38 and TCRαβ/CD3 to create $TCR\alpha\beta^{-}CD38^{-}CAR^{+}$ effectors. All BE-CAR groups exhibited high levels of TCRαβ knockout, resulting in ~1% residual expression after bead mediated depletion. In CAR19 T cells, expression of CD38 reduced from 50% to 13% (n=5) after *CD38* base-editing, and for BE-CAR38 T cells, there was near complete absence of CD38 expression reflecting 'self-enrichment' and/or antigen masking mediated by CAR38 (n=5, ***Figure 1B***). Molecular analysis confirmed flow evidence of editing at *T cell receptor beta constant 1/2* (*TRBC1/2*) and *CD38* loci with C>T deamination within the anticipated 5bp base-editing window (n=4, ***Figure C***).

Control BE-CAR19 T cells, exhibited similar levels of CAR19 transduction and cell yields irrespective of CD38 base-editing (***Figure 1D & E***) and the phenotype of BE-CAR19 T cells was unaffected by CD38 knockout (***Figure 2A & B***). In contrast, CAR38 T cell yields were significantly greater for $TCR^{-}CD38^{-}$ groups compared to $TCR^{-}CD38^{+}$ T cells. Although high initial transduction efficiency was documented in the $CD38^{+}$ group, these cells were highly activated (high CD25) and released cytokines (***Figure 2B i***) in the absence of target cells and had an increased bioenergetic profile on Seahorse analysis (***Figure 2C***). These differences in phenotype, activation, and bioenergetic profiles were likely due to CAR38 T cell activation during fratricidal effects against CD38. In contrast $TCR^{-}CD38^{-}$ CAR38 T cells released cytokines only in the presence of $CD19^{+}CD38^{+}$ Daudi target cells and profiles were comparable to CAR19 controls (n=4) (***Figure 2B ii***). Favourable metabolic profiles have been reported after CD38 disruption in T cells and NK cells

(27, 28), however immediately after CAR T cell engineering no significant difference in oxidative phosphorylation (measured by oxygen consumption rate, n=3, ***Figure 2C i***) or glycolysis (measured by extra-cellular acidification rate, n=3, ***Figure 2C ii***) were apparent in BE-CAR19 with or without CD38 knockout. CAR19 and CAR38 effector groups all showed similar lysis of $CD19^{+}CD38^{+}$ Daudi target cells across a range of effector: target (E:T) ratios in cytotoxicity assays (***Figure 2D***).

HLA base edited BE-CAR38 T cells evade humoral and cellular allo-responses

Overcoming HLA-mismatches to allow allogeneic T cells to be used without matching requires TCRαβ disruption to prevent GvHD, and additional editing such as conferring resistance to serotherapy or removal of HLA class I and II to tackle host mediated rejection. The latter was achieved by editing *B2M* for disruption of HLA class I expression, and editing of *RFX5*, a major transcriptional factor for HLA class II inhibition. Thus, fully 'universal' (TCR^{-} $CD38^{-}HLAI^{-}HLAII^{-}$) BE-CAR38 and BE-CAR19 T cells were generated by multiplexed disruption of *TRBC1/2, B2M,* and *RFX5 as well as CD38,* followed by bead mediated triple depletion of residual TCRαβ, HLA class I (HLA-I), and class II (HLA-II) expressing T cells (***Figure 3A***). Flow cytometry confirmed knockout and enrichment of highly homogenous $TCR^{-}HLAI^{-}HLAII^{-}$ CAR T cell products (***Figure 3B i & ii***). Preservation of function of 'universal' BE-CAR19 and BE-CAR38 T cell products was confirmed *in vitro* after combined TCRαβ and HLA knockouts, with no significant difference in cytotoxicity or cytokine release against $CD19^{+}CD38^{+}$ Daudi cells (***Figure 3C & D***). Antigen negative $CD19^{-}CD38^{-}$ Daudi cells were spared in cytotoxicity assays, confirming CAR specificity even after undergoing multiplexed base editing (***Supplementary figure 2***).

To investigate cognate recognition and binding of BE-CAR T cells by anti-HLA antibodies we used a flow cross match assay and sera from multiple donors with known HLA sensitisation against the complete repertoire of HLA class I and II molecules expressed by the relevant cell donor (***Figure 4A***). Both BE-CAR19 and BE-CAR38 T cell products with intact HLA expression ($BE\text{-}CAR19^{+}TCR^{-}HLAI^{+}HLAII^{+}$ and $BE\text{-}CAR38^{+}TCR^{-}HLAI^{+}HLAII^{+}$) exhibited high levels of anti-HLA antibody binding, but in contrast cells with HLA-I and HLA-II knockout exhibited minimal binding that was below limits of quantification. This suggests disruption of both HLA-I and -II offers a route to 'universal' BE-CAR products even in subjects with pre-existing anti-HLA antibodies.

Mixed lymphocyte proliferation assays quantified cell proliferation by 3H-thymidine incorporation as a quantifiable response by host T cell mediated TCRαβ recognition of mismatched HLA on BE-CAR T cells. Thus, these co-culture assays modelled allo-recognition of irradiated donor BE-CAR T cells by mismatched host T cells and quantified the impact of HLA removal on the allo-stimulation potential of BE-CAR T cells (***Figure 4B***). In the case of BE-CAR19 T cells, disruption of HLA-I and HLA-II significantly reduced responses by non-matched allogeneic T cells. Co-cultures investigating responses elicited against BE-CAR38 T cells revealed more complex interactions involving CAR38 mediated responses against CD38, which was notably upregulated on alloreactive responder T cells. This resulted in significantly reduced thymidine incorporation in proliferation assessments and flow cytometry confirmed that BE-CAR38 T cells eliminated $CD38^+$ allogeneic cells in these co-cultures (***Supplementary figure 3***).

Experiments also investigated possible NK cells mediated 'missing-self' activity against BE-CAR T cells after HLA-I removal. While there was evidence of NK degranulation against HLA-I^- CAR19 T cells, as the majority of NK cells expressed CD38, BE-CAR38 T cells recognised and eliminated these cells in co-cultures (***Figure 4C***). Overall, these data were consistent with CAR38 mediated 'allo-defense' phenomena and suggest that 'universal' BE-CAR38 T cells may have notable advantages in overcoming host mediated allogeneic responses.

In vivo anti-leukaemia activity of 'universal' $CD38^-TCR^-HLA\text{-}I^-HLAII^-$ CAR38 T cells

To determine *in vivo* antileukemic performance of multiplex edited fully 'universal' BE-CAR19 and BE-CAR38 T cells, NSG mice were inoculated with $CD19^+CD38^+$ Daudi cells expressing EGFP and Luciferase six days prior to BE-CAR T cell injections (***Figure 5A*)**. Disease progression was subsequently tracked weekly by IVIS imaging (***Figure 5B***). All groups receiving BE-CAR T cells exhibited significantly reduced disease progression and longer survival compared to mice receiving unmodified T cell controls. Interestingly, survival was longer in CAR38 T cell groups compared to CAR19 groups despite high level expression of both antigens on targets (*P<0.01*, ***Figure 5C & D***). Base editing of CD38 in BE-CAR19 T cells did not appear to influence responses and nor was there significant impact of HLA disruption on function for either 'universal' BE-CAR product. Flow cytometry of bone marrow at

necroscopy detected CAR T cells and quantified residual $CD19^+CD38^+$ Daudi cells (***Figure 5E***), and consistent with BLI, reductions in leukaemia burden were observed to be greatest in the BE-CAR38 T cell group (***Figure 5F***).

BE-CAR38 T cells were also evaluated against $CD7^+CD38^+$ Jurkat T cell malignant lines and $CD33^+CD38^+$ MOLM14-AML lines, and compared against anti-CD7 CAR T cells (BE-CAR7) (10, 14) and anti-CD33 CAR T cells (BE-CAR33) (26) respectively. Additional editing of *CD7* was incorporated in BE-CAR38 T cells for comparisons to BE-CAR7, as previously described (10). *In vitro*, co-cultures with Jurkat (***Supplementary figure 4A***) or MOLM14 (***Supplementary figure 4B***) cells demonstrated antigen specific BE-CAR38 T-cell cytotoxicity and cytokine release against $CD38^+$ leukemic lines across E:T ratios. Responses were comparable to BE-CAR7 and BE-CAR33 with no evidence that CD38 editing influenced responses (***Supplementary figure 4A & 4B***). Comparisons *in vivo* used NSG mice engrafted with either Jurkat or MOLM14 lines and again responses were comparable to BE-CAR7 (***Supplementary figure 5A***) or BE-CAR33 respectively (***Supplementary figure 5B***). These findings suggest that BE-CAR38 T cells have potential applicability against a wide variety of $CD38^+$ haematological malignancies.

Discussion

CD38 is a promising candidate for immunotherapy with robust expression in multiple haematological malignancies (19, 20, 29, 30) but limited expression on healthy tissue beyond the hematopoietic system (31). Anti-CD38 monoclonal antibodies exemplified by daratumumab and isatuximab, received FDA approval for treatment of MM due to their efficacy and safety profiles (32-36), and have also produced encouraging outcomes against ALL (37-39), although resistance and limited clinical responses have also been documented (40). CD38 is also expressed on long lived plasma cells and there is also interest in targeting these populations for certain autoimmune conditions (41).

Alternative CAR T cell based approaches have also been explored with early clinical trials reporting efficacy and safety data in B-ALL (21), AML (22), chronic myelogenous leukaemia (CML) (24), and MM (23, 25). Cytopenia was commonly observed, likely due to CD38 expression across the haemopoietic system, which may require time-limited applications or bridge-to-transplant strategies for donor

derived reconstitution in some settings. Autologous CAR38 T cell products were reported to have negligible residual $CD38^+$ cells detectable by flow cytometry at the end of manufacture, suggesting possible epitope masking by the CAR and/or enrichment of the CD38 negative T cell populations during manufacture (21).

We found that CD38 disruption by base editing improved CAR38 T cell yields significantly by protecting against cell loss through fratricide and reducing metabolic activation, cytokine release and terminal differentiation of effector cells. CD38 enzymatic activity is known to deplete NAD^+ availability while producing adenosine, which in turn has been associated with T cell immunosuppression (42, 43). Some reports have suggested that inhibition or knockout of CD38 favours oxidative metabolism and offers improved function of CAR T cells (27, 28). We found no evidence that multiplexed CD38 and TCRαβ knockout, affected *in vitro* or *in vivo* activity of BE-CAR38 or other edited CAR T cells targeting CD19, CD7 or CD33. Other strategies for CAR38 T cell or NK-cell approaches have investigated restriction of CD38 with blocking antibody (44), CRISPR/Cas9 genome editing (45-47), affinity-optimised scFvs (48) and adaptor-based CARs (29). We investigated 'universal' allogenic CAR38 T cell approach as an 'off-the-shelf' alternative that could be applied across a variety of indications. Previously, for 'universal' CAR T cells we combined TCRαβ knockout to avoid GVHD, CD7 to prevent fratricide, and CD52 knockout to confer resistance to the anti-CD52 monoclonal antibody alemtuzumab, which is used to lymphodeplete recipients and reduce the risk of host mediated CAR T cell rejection. In the context of CD38 knockout T cells it may be feasible to use daratumumab or isatuximab instead of alemtuzumab to create a similar advantage for allo-CAR T cells. Moreover, removal of both HLA class I and II was incorporated to extend immunological stealth, allowing evasion of pre-existing anti-HLA antibodies and reducing allo-stimulation, likely to trigger host immune cell mediated rejection.

Multiplexed based editing of *CD38*, *TRBC1/2*, *B2M* and *RFX5* enabled 'universal' BE-CAR T cells to evade binding by anti-HLA antibodies and ameliorated cell mediated responses in mixed lymphocyte cultures where alloreactive T-lymphocyte recognition of de-nuded BE-CAR19 iterations was blunted. In the context of BE-CAR38 T cells these cells exhibited responses against activated $CD38^+$ allogeneic T cells and NK cells, a phenomenon akin to 'allo-defense'. The 'allo-defense' concept has been previously described, for example by targeting upregulated 4-1BB on

activated lymphocytes, or through B2M - CD3ζ fusion receptor for depleting allo-reactive T cells upon HLA-I recognition (49, 50). In combination, expression of CAR38 and quadruple base-editing has the potential to both arm T cells against and shelter T cells from host immunity. Therapeutic development against a variety of haematological malignancies and autoimmune conditions using ‘universal’ ‘off-the-shelf’ BE-CAR38 T cells are warranted, either alone or in combination with other CD38 edited ‘off-the-shelf’ CAR T products.

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Figure legends

Figure 1 Production of BE-CAR T cells: A) Timeline of base-edited CAR T cell manufacture. B) Quantification of TCRαβ and CD38 disruption at the end of manufacture by flow cytometry (n=5) and C) Sanger sequencing (n=4). D) CAR positive cells post magnetic bead mediated depletion of residual $TCR\alpha\beta^+$ cells across multiple donors (n=5). * $p \leq 0.05$, paired t test. E) End of manufacture cell yields relative to parallel productions of control BE-CAR19 T cells (n=9 donors). ** $p \leq 0.01$, paired one-way ANOVA with a Tukey's multiple comparison test. Mean ± standard error of the mean (SEM) shown.

Figure 2 CD38 knockout mitigates against CAR mediated activation and fratricidal effects: A) Immunophenotyping at the end of BE-CAR manufacture (n=4) measuring both activation profile (i), CD25 mean fluorescence intensity; (MFI) and memory phenotype (ii) (CD45RA and CD62L expression). Naïve ($CD62L^+CD45RA^+$), central memory T cells (T_{CM}, $CD62L^+$, $CD45RA^-$), effector memory T cells re-expressing CD45RA (T_{EMRA}, $CD62L^-$, $CD45RA^+$), and effector memory T cells (T_{EM}, $CD62L^-$, $CD45RA^-$). **** $p \leq 0.0001$ paired one-way ANOVA with a Tukey's multiple comparison test. Red * indicates analysis between the T_{EM} populations. B) Cytokine bead array quantifying cytokine release in absence (i) and presence (ii) of leukaemia target cells (n=3 technical replicates, dotted line indicates the limit of quantification (50pg/ml). C) Metabolic activation measured using Seahorse-XF analyser (n=3 donors, where each point represents the mean of 4-8 technical replicates, measuring oxygen consumption rate (OCR) as a surrogate for oxidative phosphorylation (i) and extracellular acidification rate (ECAR) as a surrogate for glycolysis. ** $p \leq 0.01$, paired one-way ANOVA with a Tukey's multiple comparison test. D) Lysis of Daudi cells after 4-hour *in vitro* co-culture with effector T cells across a range of Effector: Target (E:T) ratios (n=8 donors for all groups except CAR38 TCR^-CD38^+ which has n=5 donors). Mean ± SEM.

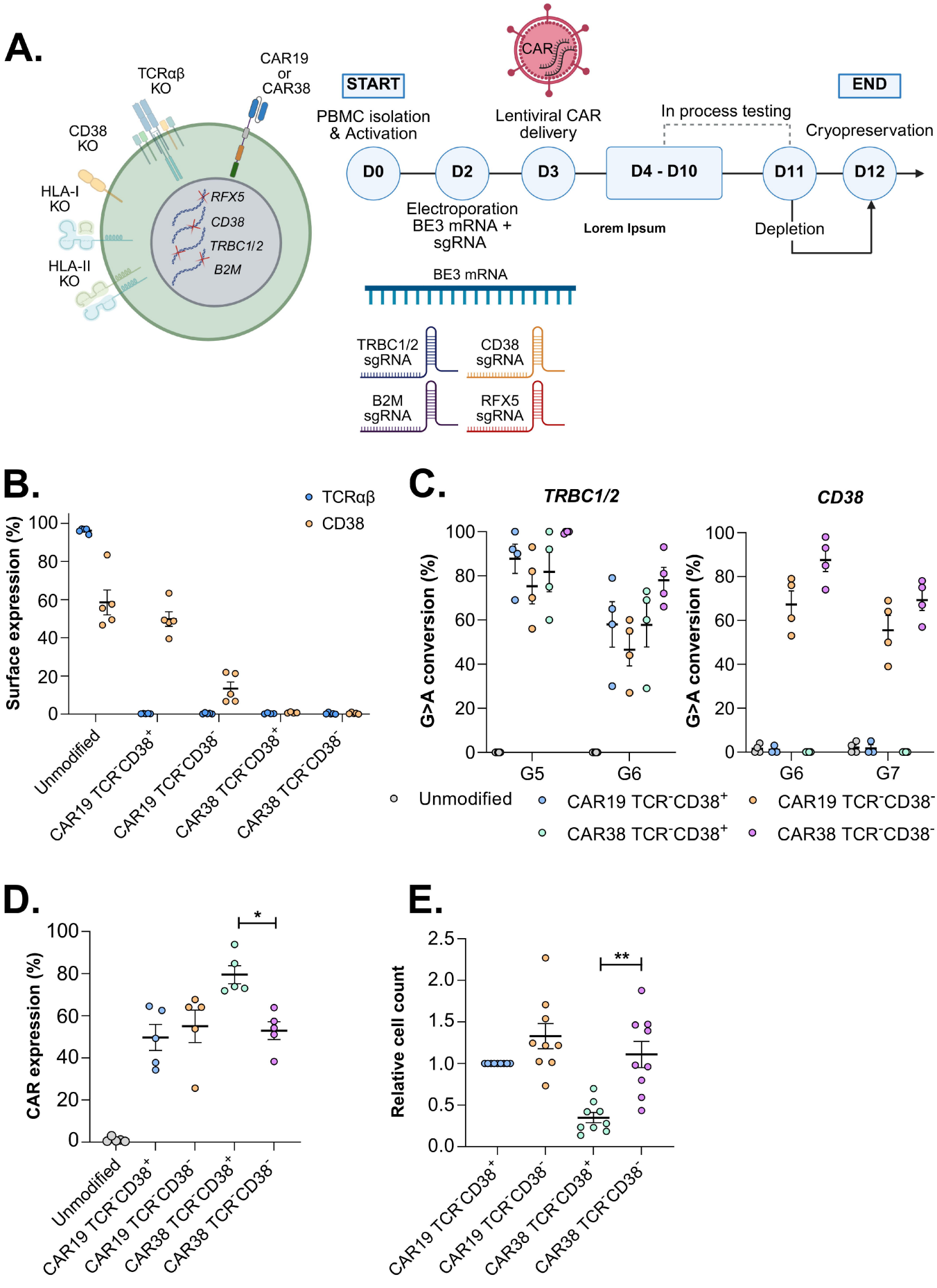
Figure 3 Manufacture of 'universal' CAR T cells with additional HLA knockouts (TCR^-CD38^-HLA-I/II$^-$) retain *in vitro* function: A) Schematic of base-edited loci (*TRBC*, *CD38*, *B2M*, & *RFX5*). The red arrow indicates the position of the desired edit, protospacer sequence is shown below, with numbers indicating protospacer

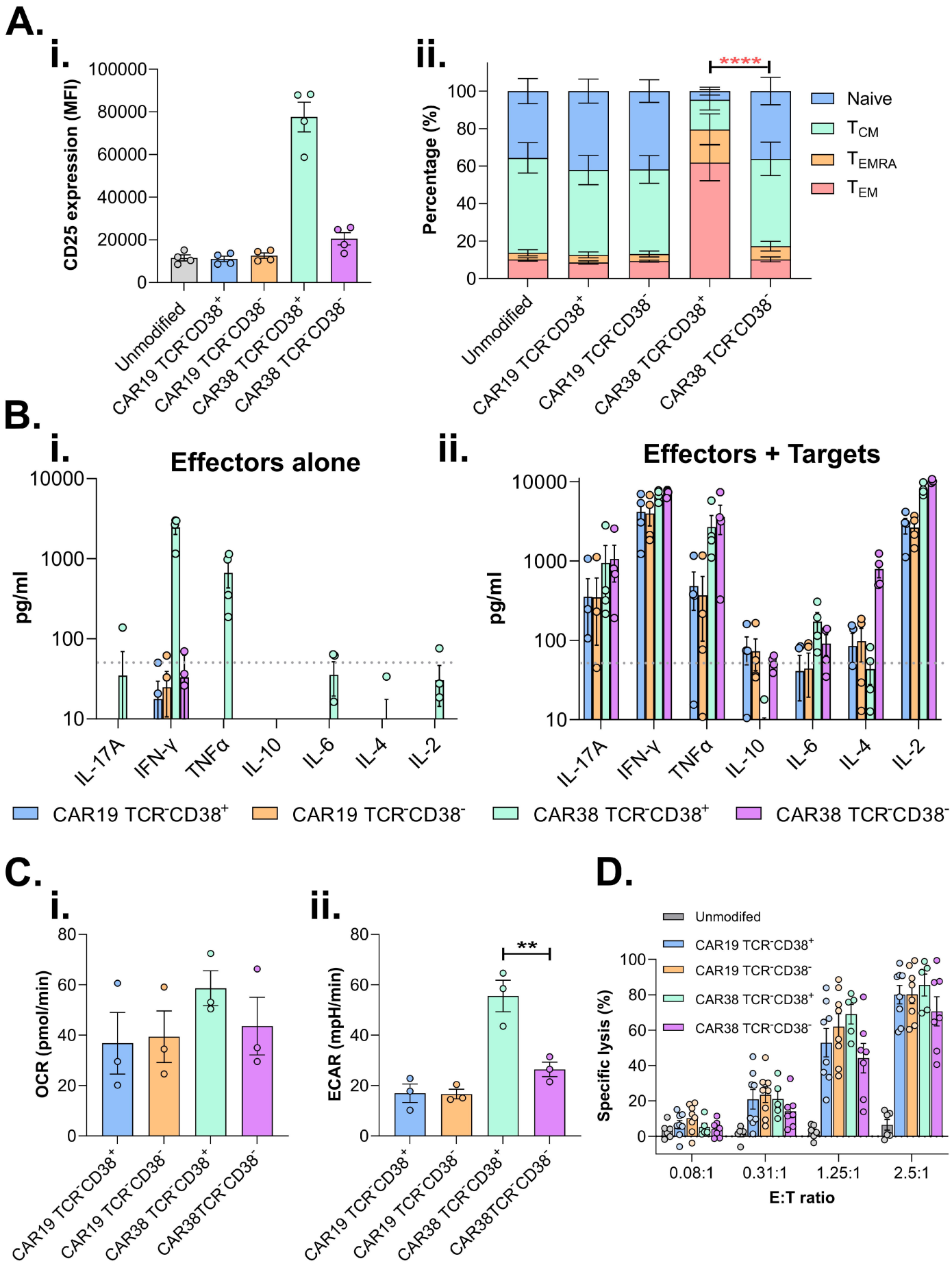
position distal to the protospacer adjacent motif (PAM). Line between positions 4 and 8 shows the optimal editing window for the third-generation cytosine base editor (BE3), with targeted bases in dark green. Representative Sanger sequencing analysed by EDITR to quantify base conversion in CAR38 T cells edited at all loci and depleted for residual TCRαβ and HLA expressing cells. B) Flow-cytometry of unmodified, BE-CAR, and universal CAR T cell groups at the end of manufacture. Representative plots showing CAR, TCRαβ, and HLA expression (i) as well as a summary histogram of n=4 donors (ii). C) *In vitro* cytotoxicity of CAR T cell products against a Daudi line in a 4-hour co-culture across a range of Effector: Target (E:T) ratios (n=4 donors, where each point represents the mean of a technical triplicate). D) Cytokine release of effector T cells after 16-hour co-culture with Daudi target cell (n=3 donors). Each point shows the mean of n=3 technical replicates, with the limit of detection indicated by a dotted line (50pg/ml). Mean ± SEM.

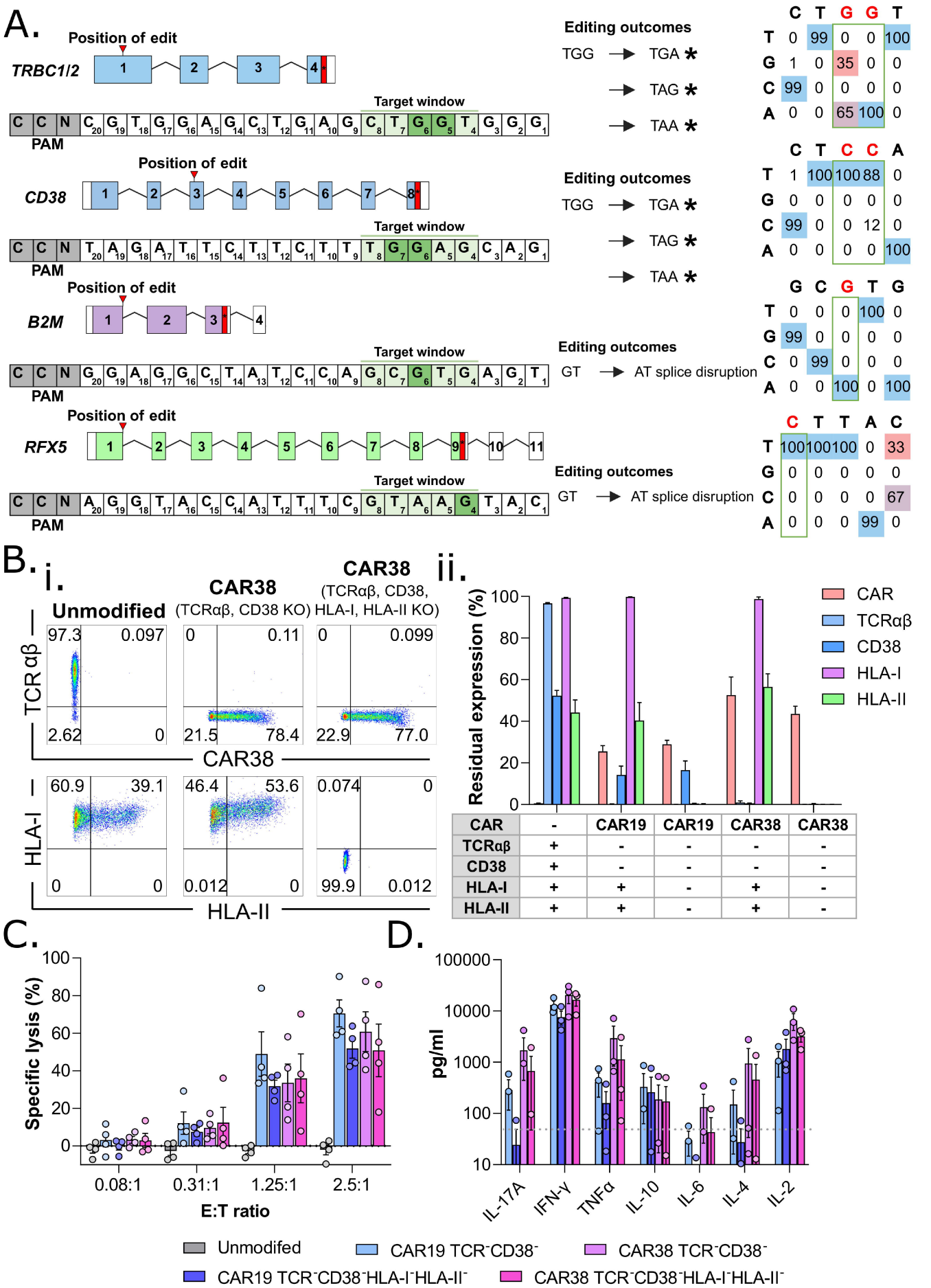
Figure 4 Base-edited $TCR^{-}CD38^{-}HLA\text{-}I/II^{-}$ CAR T cells evade alloreactive humoral and cellular immunity: A) A flow-crossmatch assay was established using human sera with defined anti-HLA antibody profiles. Quantification of anti-HLA antibody binding to BE-CAR T cells from 5 sera measured by mean fluorescence intensity (MFI). B) Mixed lymphocyte reactions measure thymidine uptake by mismatched allo-MNCs against irradiated (30Gy) BE-CAR T cells relative to responses against unmodified T cells (n=6 donors). Each point represents the mean of n=3 technical replicates. One-way ANOVA with a Tukey's multiple comparison test (* $P \leq 0.05$). C) Natural killer (NK) cell degranulation after co-culture with $BE\text{-}CAR^{+}$ $TCR^{-}HLA\text{-}I^{-}HLAII^{-}$ T cells or control K562 cells (n=3 donors, with each point representing the mean of a technical triplicate). NK cells are gated on live $CellTrace^{-}$ $CD2^{+}CD4^{-}CD8^{-}$ $CD3^{-}C56^{+}$. Expression of CD107a (degranulation marker), CD38 (CAR target), and NK events (measured by counting beads) are plotted for each group. Mean ± SEM shown

Figure 5 *In vivo* function of $TCR^{-}CD38^{-}HLA\text{-}I/II^{-}$ CAR T cells after multiplexed base editing: A) Timeline of an *in vivo* experiment with NSG mice engrafted with Daudi cells expressing GFP and Luciferase. B) IVIS images confirming tumour engraftment on day 5, prior to CAR T cell treatment on day 6, and tumour progression over the course of the experiment. Five mice received unmodified, base-edited CAR19 $TCR^{-}CD38^{+}$, CAR19 $TCR^{-}CD38^{-}$, and 'universal' CAR19 $TCR^{-}HLA\text{-}I^{-}II^{-}$

T cells. Six mice received CAR38 $TCR^{-}CD38^{-}$, and four received ‘universal’ CAR38 $TCR^{-}CD38^{-}HLA\text{-}I^{-}II^{-}$ T cells. C) Leukaemia progression measured by average radiance ($p/s/cm^2/sr$) over the course of the experiment. Median of each group is indicated by a solid line, with individual replicates shown as dotted lines. D) Kaplan-Meier curves to day 53, with comparisons between groups performed by log-rank tests. E) Confirmation of both leukaemia burden and residual T cells in the bone marrow at necroscopy. F) Detection of target antigen on remaining GFP^{+} Daudi cells in the bone marrow. Mean ± SEM


A.
TCRαβ KO
CAR19 or CAR38
CD38 KO
HLA-I KO
HLA-II KO
RFX5
CD38
TRBC1/2
B2M
CAR
START
END
PBMC isolation & Activation
Lentiviral CAR delivery
In process testing
Cryopreservation
D0
D2
D3
D4 - D10
D11
D12
Electroporation BE3 mRNA + sgRNA
Lorem Ipsum
Depletion
BE3 mRNA
TRBC1/2 sgRNA
CD38 sgRNA
B2M sgRNA
RFX5 sgRNA
B.
TCRαβ
CD38
Surface expression (%)
Unmodified
CAR19 TCR-CD38+
CAR19 TCR-CD38-
CAR38 TCR-CD38+
CAR38 TCR-CD38-
C.
TRBC1/2
CD38
G>A conversion (%)
G5
G6
G7
Unmodified
CAR19 TCR-CD38+
CAR19 TCR-CD38-
CAR38 TCR-CD38+
CAR38 TCR-CD38-
D.
CAR expression (%)
*
E.
Relative cell count
**

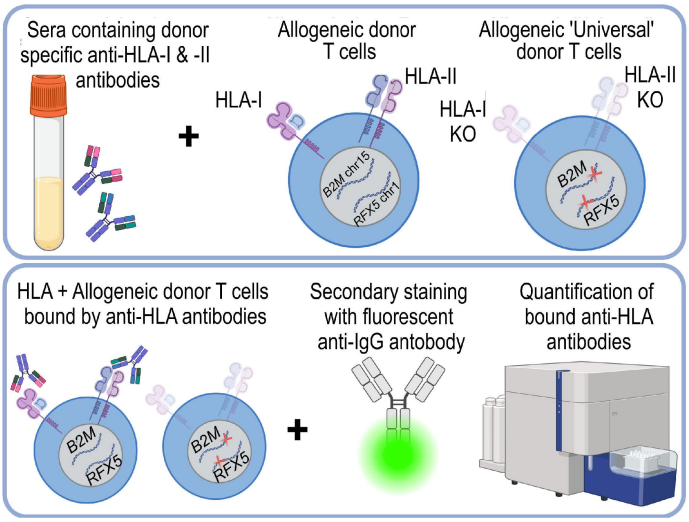

A.
i.
CD25 expression (MFI)
100000
80000
60000
40000
20000
0
Unmodified
CAR19 TCR-CD38+
CAR19 TCR-CD38-
CAR38 TCR-CD38+
CAR38 TCR-CD38-
ii.
Percentage (%)
****
Naive
TCM
TEMRA
TEM
B.
i.
Effectors alone
ii.
Effectors + Targets
pg/ml
10000
1000
100
10
IL-17A
IFN-γ
TNFα
IL-10
IL-6
IL-4
IL-2
CAR19 TCR-CD38+
CAR19 TCR-CD38-
CAR38 TCR-CD38+
CAR38 TCR-CD38-
C.
i.
OCR (pmol/min)
ii.
ECAR (mpH/min)
**
D.
Unmodifed
Specific lysis (%)
E:T ratio
0.08:1
0.31:1
1.25:1
2.5:1

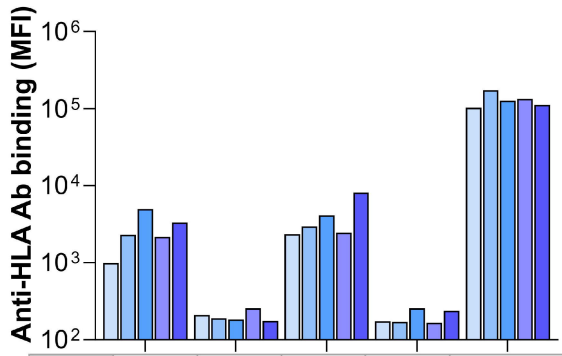

A.
Position of edit
TRBC1/2
Target window
PAM
Editing outcomes
TGG → TGA *
→ TAG *
→ TAA *
CD38
B2M
Editing outcomes
GT → AT splice disruption
RFX5
B.
i.
Unmodified
CAR38 (TCRαβ, CD38 KO)
CAR38 (TCRαβ, CD38, HLA-I, HLA-II KO)
TCR αβ
CAR38
HLA-I
HLA-II
ii.
Residual expression (%)
CAR
TCRαβ
CD38
HLA-I
HLA-II
CAR | - | CAR19 | CAR19 | CAR38 | CAR38
TCRαβ | + | - | - | - | -
CD38 | + | - | - | - | -
HLA-I | + | + | - | + | -
HLA-II | + | + | - | + | -
C.
Specific lysis (%)
E:T ratio
0.08:1
0.31:1
1.25:1
2.5:1
D.
pg/ml
IL-17A
IFN-γ
TNFα
IL-10
IL-6
IL-4
IL-2
Unmodifed
CAR19 TCR⁻CD38⁻
CAR38 TCR⁻CD38⁻
CAR19 TCR⁻CD38⁻HLA-I⁻HLA-II⁻
CAR38 TCR⁻CD38⁻HLA-I⁻HLA-II⁻

A.

HLA type of CAR batches:

A*01:01:01, A*03:01:01, B*07:02:01, B*57:01:01, C*06:02:01, C*07:02:01, DRB1*07:01:01, DRB1*15:01:01, DRB4*01:03:01:02N, DRB5*01:01:01, DQB1*03:03:02, DQB1*06:02:01, DPA1*01:03:01, DPB1*04:01:01, DQA1*01:02:01, DQA1*02:01:01





CAR	CAR19	CAR19	CAR38	CAR38	Cumulative
TCRαβ	-	-	-	-	n/a
CD38	-	-	-	-	n/a
HLA-I	+	-	+	-	n/a
HLA-II	+	-	+	-	n/a

A*01:01, A*03:01, B*07:02, C*06:02, C*07:02, DQB1*03:03, DQA1*02

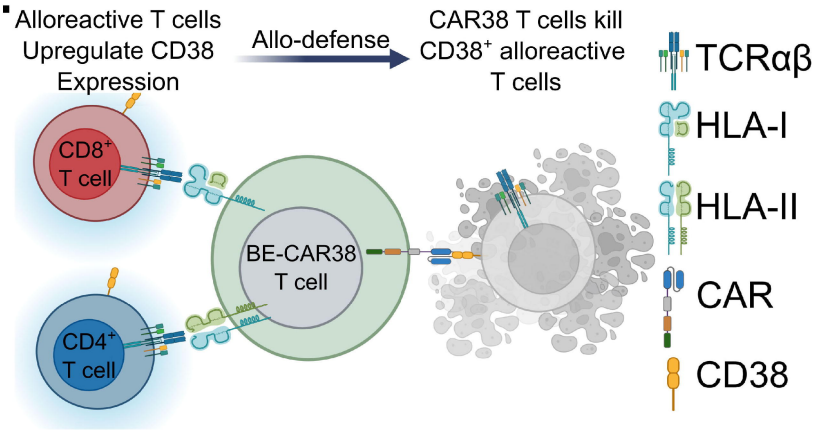
A*01:01, A*03:01, B*57:01, DRB1*07:01, DRB1*15:01, DRB5*01:01, DRB1*06:02, DQB1*03:03, DQA1*02, DPB1*04:01

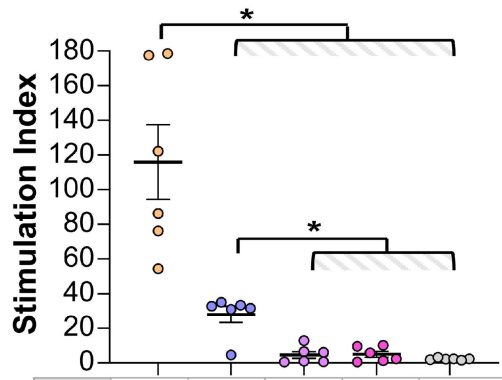
A*01:01, A*03:01, B*07:02, B*57:01, DRB1*07:01, DRB1*15:01, DRB5*01:01

A*01:01, A*03:01, B*57:01, DRB1*07:01, DRB1*15:01, DRB5*01:01, DRB1*06:02, DQB1*03:03, DQA1*02

A*01:01, A*03:01, B*07:02, B*57:01, C*06:02, DRB1*07:01, DRB1*15:01, DRB5*01:01, DQB1*03:03, DPB1*04:01, DPA1*01:03

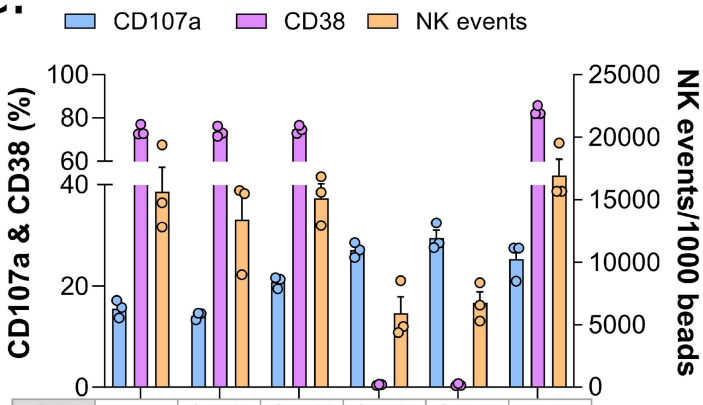
B.



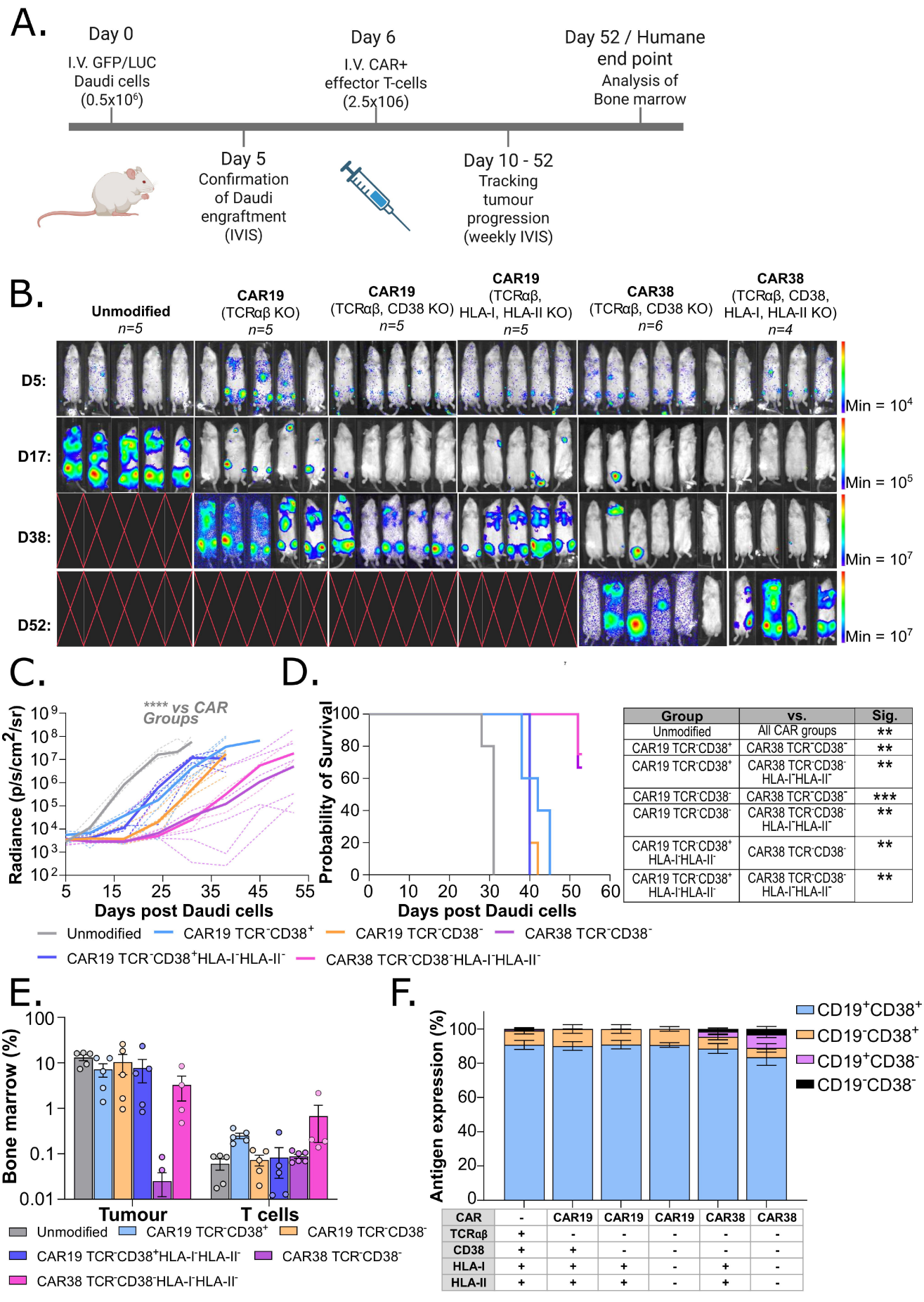


CAR	CAR19	CAR19	CAR38	CAR38	Autologous
TCRαβ	-	-	-	-	+
CD38	-	-	-	-	+
HLA-I	+	-	+	-	+
HLA-II	+	-	+	-	+

C.



CAR	-	CAR19	CAR19	CAR38	CAR38	K562
TCRαβ	+	-	-	-	-	-
CD38	+	-	-	-	-	-
HLA-I	+	+	-	+	-	-
HLA-II	+	+	-	+	-	-


A.
Day 0
I.V. GFP/LUC Daudi cells (0.5x10⁶)
Day 5
Confirmation of Daudi engraftment (IVIS)
Day 6
I.V. CAR+ effector T-cells (2.5x106)
Day 10 - 52
Tracking tumour progression (weekly IVIS)
Day 52 / Humane end point
Analysis of Bone marrow
B.
Unmodified n=5
CAR19 (TCRαβ KO) n=5
CAR19 (TCRαβ, CD38 KO) n=5
CAR19 (TCRαβ, HLA-I, HLA-II KO) n=5
CAR38 (TCRαβ, CD38 KO) n=6
CAR38 (TCRαβ, CD38, HLA-I, HLA-II KO) n=4
D5:
D17:
D38:
D52:
Min = 10⁴
Min = 10⁵
Min = 10⁷
Min = 10⁷
C.
**** vs CAR Groups
Radiance (p/s/cm²/sr)
Days post Daudi cells
D.
Probability of Survival
Days post Daudi cells
Group | vs. | Sig.
Unmodified | All CAR groups | **
CAR19 TCR⁻CD38⁺ | CAR38 TCR⁻CD38⁻ | **
CAR19 TCR⁻CD38⁺ | CAR38 TCR⁻CD38⁻ HLA-I⁻HLA-II⁻ | **
CAR19 TCR⁻CD38⁻ | CAR38 TCR⁻CD38⁻ | ***
CAR19 TCR⁻CD38⁻ | CAR38 TCR⁻CD38⁻ HLA-I⁻HLA-II⁻ | **
CAR19 TCR⁻CD38⁺ HLA-I⁻HLA-II⁻ | CAR38 TCR⁻CD38⁻ | **
CAR19 TCR⁻CD38⁺ HLA-I⁻HLA-II⁻ | CAR38 TCR⁻CD38⁻ HLA-I⁻HLA-II⁻ | **
Unmodified
CAR19 TCR⁻CD38⁺
CAR19 TCR⁻CD38⁻
CAR38 TCR⁻CD38⁻
CAR19 TCR⁻CD38⁺HLA-I⁻HLA-II⁻
CAR38 TCR⁻CD38⁻HLA-I⁻HLA-II⁻
E.
Bone marrow (%)
Tumour
T cells
Unmodified
CAR19 TCR⁻CD38⁺
CAR19 TCR⁻CD38⁻
CAR19 TCR⁻CD38⁺HLA-I⁻HLA-II⁻
CAR38 TCR⁻CD38⁻
CAR38 TCR⁻CD38⁻HLA-I⁻HLA-II⁻
F.
Antigen expression (%)
CD19⁺CD38⁺
CD19⁻CD38⁺
CD19⁺CD38⁻
CD19⁻CD38⁻
CAR | - | CAR19 | CAR19 | CAR19 | CAR38 | CAR38
TCRαβ | + | - | - | - | - | -
CD38 | + | + | - | - | - | -
HLA-I | + | + | + | - | + | -
HLA-II | + | + | + | - | + | -

Supplement

Allo-defensive, multiplex base-edited, anti-CD38 CAR T cells for 'off-the-shelf' Immunotherapy

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Supplementary Methods

Cell lines

Cell lines (293T, K562, Daudi, Jurkat, and MOLM14 cell lines) were obtained from ATCC (Virginia, USA). Cell lines were maintained in either RPMI-1640 (Thermo Fisher Scientific, Massachusetts, USA), or DMEM (Thermo Fisher Scientific), supplemented with 10% FBS (Sigma-Aldrich, Missouri, USA). Transduction with a lentiviral vector expressing enhanced green fluorescent protein (EGFP) and luciferase (LUC) allowed detection via flow-cytometry and *in vivo* tumour tracking.

Target antigen negative populations were generated using *Sp*Cas9 mRNA (TriLink BioTechnologies, California, USA) with single-guide RNAs (sgRNA, Synthego, California, USA), delivered using a Lonza 4D-Nucleofector X unit (Lonza Group AG, Basel, Switzerland). Residual antigen positive populations were removed by sorting on a MoFlo XDP cell sorter (Beckman Coulter, California, USA).

Base editing & Molecular Assessments

Codon optimised cytidine base editor-3 (BE3) mRNA (TriLink BioTechnologies, California, USA) was used at 50µg/ml and combined with 10µg/ml sgRNAs (Synthego, California, USA) to create premature stop codons or disrupt splice-donor site as detailed in Supplementary Table S2. Electroporation was performed in a Lonza 4D-Nucleofector X unit (Lonza Group AG), using program EW138 in buffer P3.

Genomic DNA was extracted from 1-2x10^6 cells using the DNeasy blood and tissue kit (QIAGEN, Hilden, Germany). Primers were designed using Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) to amplify 500-1000bp across the protospacer site and manufactured by Thermo Fisher Scientific. Sanger sequencing was performed by Eurofins Genomics (Konstanz, Germany) and analysed using EDITR (http://baseeditr.com/). Primers sequences (5'-3') are showed in Supplementary Table S3. We previously interrogated off-target editing at both the DNA and transcriptome level, reporting negligible effects following transient expression of coBE3 mRNA (1, 2). This analysis has not been repeated as part of this manuscript.

Flow cytometry

CAR T cell phenotyping by flow-cytometry was carried out using a BD FACSymphony cell analyzer (BD, New Jersey, USA). Primary anti-human antibodies used for immunophenotyping are provide in Supplementary Table S1. CAR19 transduction was assessed using Biotin-SP AffiniPure F(ab'), Fragment Goat, anti-Mouse IgG, F(ab') Fragment Specific antibody (Jackson Immunoresearch, Cambridge, UK) with secondary staining by Streptavidin (BioLegend, California, USA). His-tagged recombinant CD7, CD33, and CD38 proteins (Sino Biological, Beijing, China) were used to detect CAR7, CAR33, and CAR38 expression, followed by staining with Anti-6X His tag (clone: AD1.1.10, Abcam, Cambridge, UK). All samples were acquired on a BD FACSymphony cell analyzer (BD) unless specified otherwise and analysis of was done using FlowJo version 10.10.0.

Flow-based *in vitro* cytotoxicity assay

EGFP antigen-positive and antigen-negative tumour cells were mixed at a 1:1 ratio and co-cultured for 4 hours with CAR^+ T cells across effector to target (E:T) ratios. Co-cultures were then stained for antigen expression and viability, with the ratio of antigen positive to antigen negative tumour cells used to calculate specific lysis.

Cytometric bead array to quantify cytokine release

Co-cultures at E:T of 1:1 were maintained for 16-hours in 96-well plates after which 150µl supernatant was analysed using a Human Th1/2/17 CBA kit (BD) and BD FCAP Array software v3.

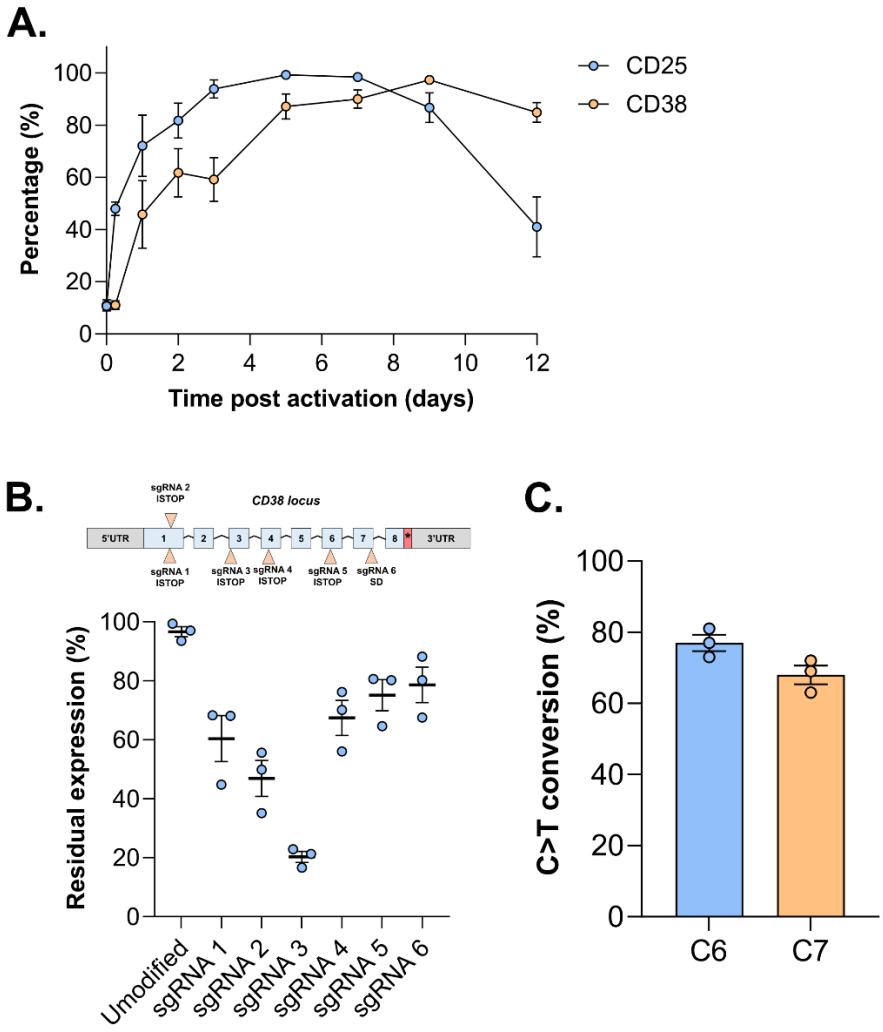
Metabolic analysis by Seahorse XF analyser

An XFe96 Sensor Cartridge (Agilent Technologies, California, USA) hydrated with 200µl of calibration fluid (Agilent Technologies) per well and a XFe96 PDL cell plate (Agilent Technologies) was incubated overnight prior to the assay in a 37^{o}C non-CO_2 incubator. T cells were resuspended in Seahorse XF RPMI medium supplemented with 1% Seahorse XF Glucose (1.0M solution), 1% Seahorse XF Pyruvate (100mM solution), and 1% Seahorse XF L-Glutamine (200mM solution) (Agilent Technologies) and seeded with $1x10^5$ cells per well. A Seahorse XF T Cell Metabolic Profiling Kit (Agilent Technologies) and XFe96 analyser measured both extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) in real time. Data was analysed in the online seahorse analytics portal (https:// seahorseanalytics.agilent.com).

Natural Killer cell degranulation

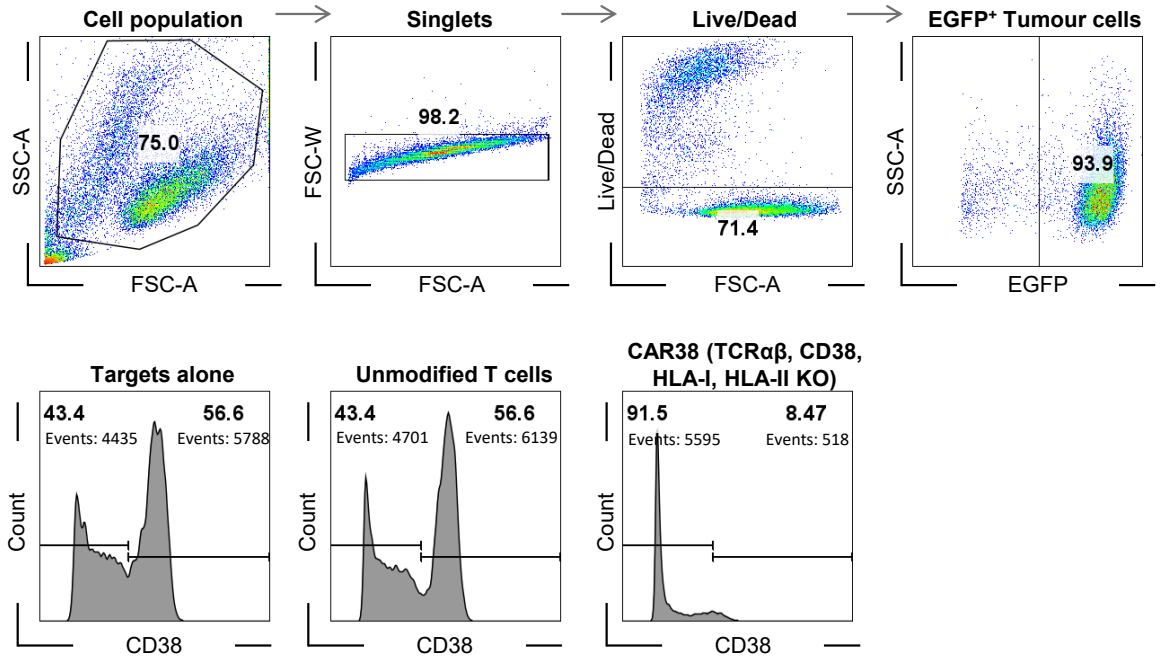
Natural Killer (NK) cells isolated from MNCs using a NK Cell Isolation Kit (Miltenyi Biotec) were co-cultured with BE-CAR T cells in a 96-well plate (1:1 ratio) for 20-hours in TexMACS (Miltenyi Biotec) with 3% heat inactivated human serum (Seralab). Anti-CD107a antibody (clone: H4A3, BioLegend) was added to co-cultures and incubated for 4-hours before analysis.

Supplementary figure 1.



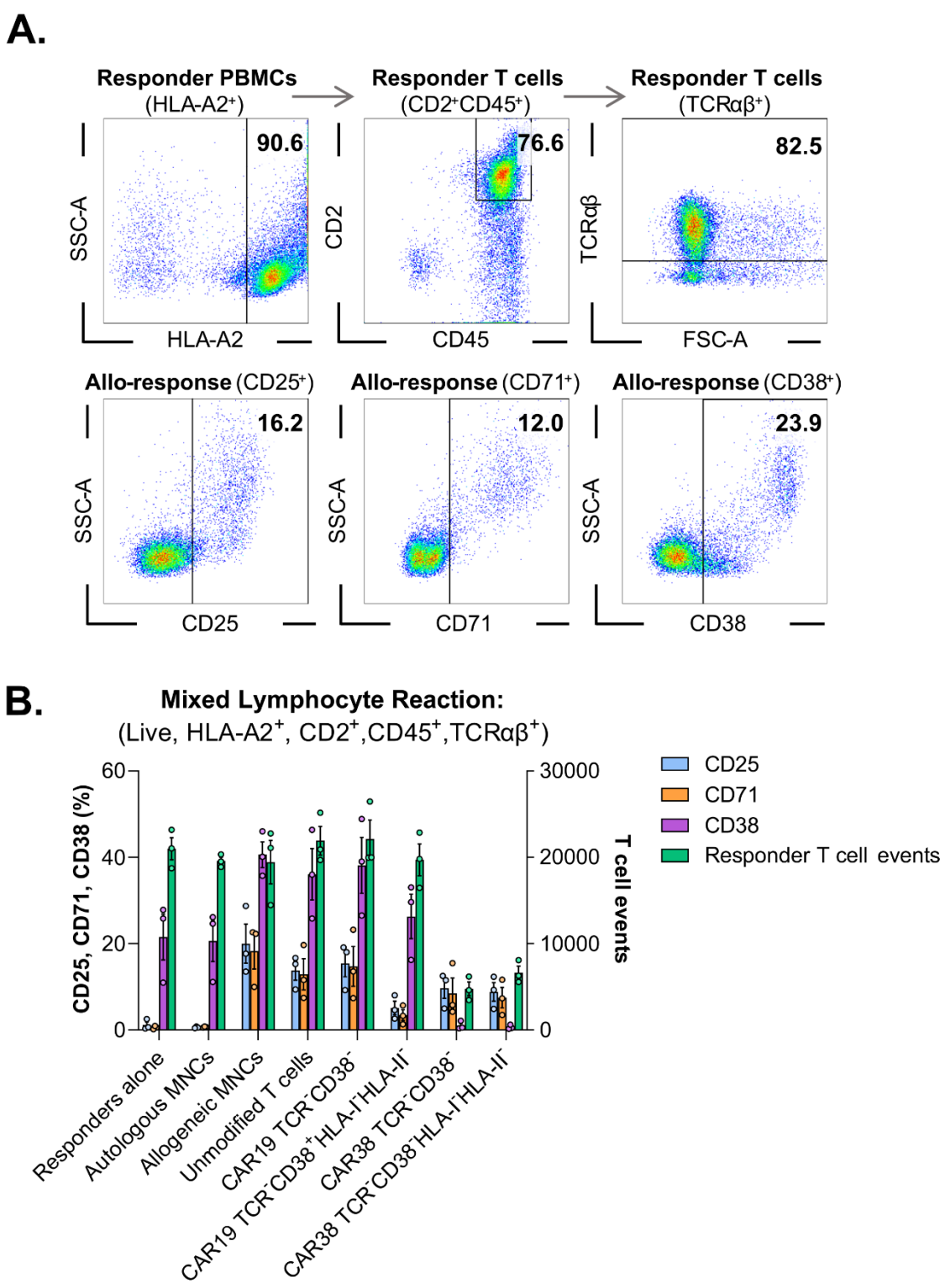
Supplementary figure 1 Kinetics of CD38 expression on primary human T cells after activation and base-editing: A) Flow-cytometry monitoring expression of CD38 and CD25 on T cells ($CD2^{+}CD45^{+}$) from MNC donors pre- and post-activation up to day 12. B) Schematic of CD38 locus. Exons are shown as solid squares, with joining black lines indicating intronic sequence. Triangles show sgRNA binding sites and predicted outcome of premature stop codon creation or splice donor site (SD) disruption. Flow cytometry measuring residual CD38 expression after cytidine deaminase base-editing across all tested sgRNA sequences with sgRNA 3 having the lowest residual expression. C) Sanger sequencing across the protospacer of sgRNA 3 confirming C>T conversion at protospacer positions 6 and 7 within the predicted editing window consistent with flow-cytometry. Analysis performed using EDITR. N=3, mean ± standard error of the mean (SEM).

Supplementary figure 2.



Supplementary figure 2 Specific lysis of CD19^{+}CD38^{+} Daudi cells by Universal CAR T cells: Gating strategy for *in vitro* cytotoxicity assessment (Top line, Targets alone). Antigen negative (CD19^{-}CD38^{-}) and antigen positive (CD19^{+}CD38^{+}) Daudi cells were mixed and at 1:1 ratio, before a 4-hour co-culture with either unmodified (negative control) or CAR^{+} T cell groups. Representative histograms show depletion of CD38^{+} targets by Universal BE-CAR38 T cells (Bottom line).

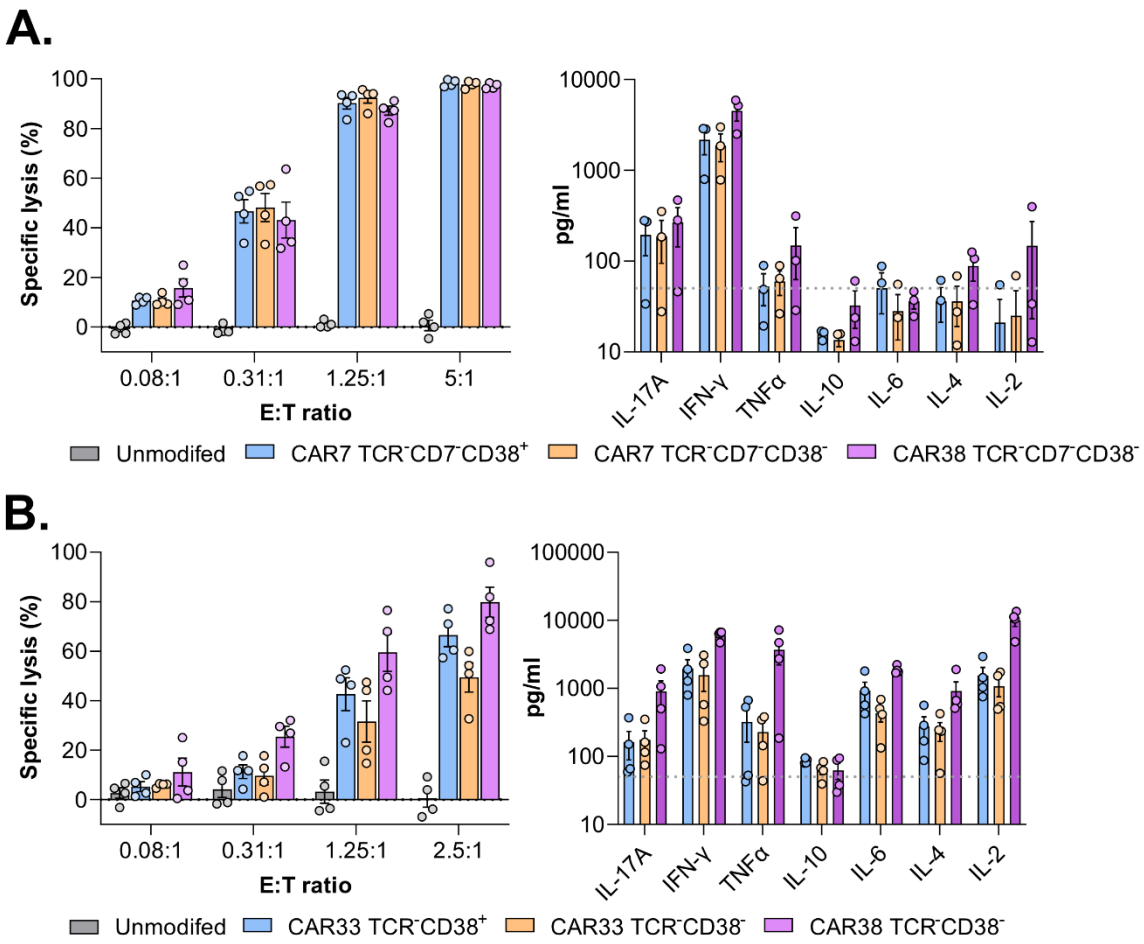
Supplementary figure 3.



Supplementary figure 3 Based-edited CAR38 T cells depleted CD38⁺ allo-responsive T cells in mixed lymphocyte co-cultures: A) Representative flow-cytometry after 5-day mixed lymphocyte co-culture with responder MNCs and irradicated target cells (30Gy, Unmodified T cells). Responder T cells are gated on live

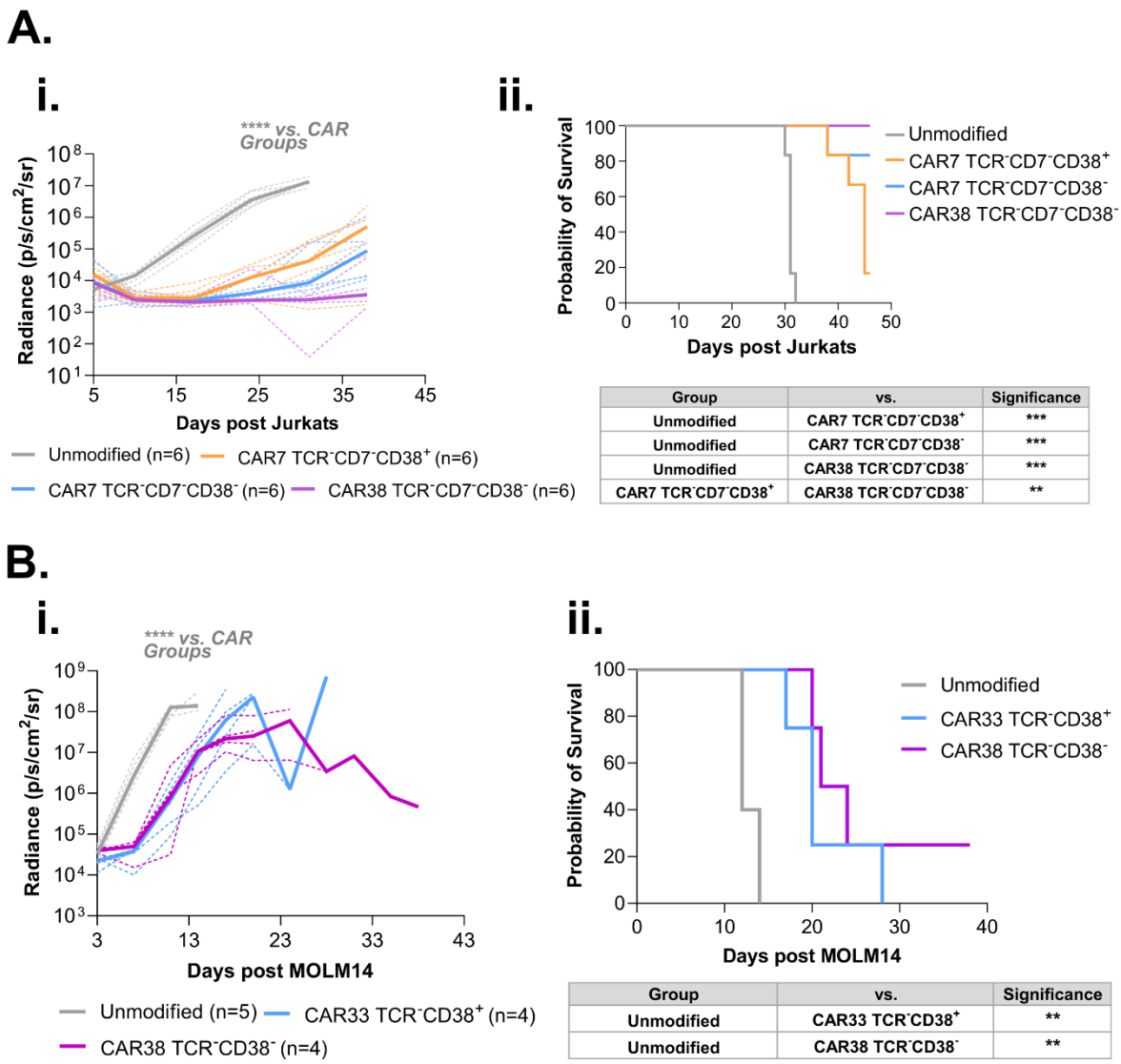
HLA-A2^{+}CD2^{+}CD45^{+}TCRαβ^{+}, with CD25, CD71, and CD38 expression used as measures of activation. These co-cultures were setup in triplicate with responders alone and autologous MNCs as negative controls, allogenic MNCs targets used as positive controls, and BE-CAR groups as test conditions. B) Responder T cell expression of activation markers (left y-axis) and responder T cells events (right y-axis) from N=3 donors against irradiated target groups. Each point represents the mean of a technical triplicate. Data Mean ± SEM.

Supplementary figure 4.



Supplementary figure 4 *In vitro* cytotoxic function of BE-CAR38 T cells against Jurkat and MOLM14 lines: Tumour lysis and cytokine release of unmodified and BE-CAR38 effector T cells against A) Jurkat or B) MOLM14 cell line with BE-CAR7 or BE-CAR33 used as a comparison. N=4, Mean ± SEM

Supplementary figure 5.



Group	vs.	Significance
Unmodified	CAR7 TCR⁻CD7⁻CD38⁺	***
Unmodified	CAR7 TCR⁻CD7⁻CD38⁻	***
Unmodified	CAR38 TCR⁻CD7⁻CD38⁻	***
CAR7 TCR⁻CD7⁻CD38⁺	CAR38 TCR⁻CD7⁻CD38⁻	**

Group	vs.	Significance
Unmodified	CAR33 TCR⁻CD38⁺	**
Unmodified	CAR38 TCR⁻CD38⁻	**

Supplementary figure 5 *In vivo* function of BE-CAR38 T cells in other models of haemopoietic malignancy: NSG mice were engrafted with either (A) Jurkat (N=6 in all groups) or (B) MOLM14 (N=5 Unmodified, and N=4 in both CAR T cell groups) tumour lines ahead of BE-CAR38 effector T cells. Unmodified T cells were given as a negative control, with BE-CAR7 or BE-CAR33 used as a control with known potency. Leukaemia progression measured by average radiance (p/s/cm^2/sr) over the course of the experiment. Solid lines present the median of each group with each replicate shown as dotted lines. Kaplan-Meier survival curves are shown with log-rank statistical analysis.

Supplementary table 1

Anti-human antibody	Clone	Supplier
CD2	LT2	Miltenyi
CD4	OKT4	BioLegend
CD8	SK1	BioLegend
CD7	CD7-6B7	BioLegend
CD16	3G8	BioLegend
CD19	HIB19	BioLegend
CD25	M-A251	BioLegend
CD33	WM53	BioLegend
CD38	HIT2	BioLegend
CD45	REA747	Miltenyi
CD45RA	HI100	BioLegend
CD56	REA196	Miltenyi
CD62L	REA615	Miltenyi
CD71	CY1G4	BioLegend
CD107a	H4A3	BioLegend
HLA-A2	BB7.2	BioLegend
HLA-ABC	W6/32	BioLegend
HLA-DR, DP, DQ	Tü39	BioLegend
TCRαβ	IP26	BioLegend
BD Horizon™ Fixable Viability Stain 700	n/a	BD Biosciences

Supplementary Table 1: Flow cytometry antibodies used for immunophenotyping.

Supplementary table 2

sgRNA ID	Protospacer sequence (5'-3')	Intended effect
TRBC1/2	CCCA**CC**AGCTCAGCTCCACG	Premature stop codon
B2M	ACTCA**C**GCTGGATAGCCTCC	Removes splice donor
RFX5	GTA**C**TTA**C**GAAATGGTACCT	Removes splice donor
CD38 sgRNA 1	GCGC**C**AGCAGTGGAGCGGTC	Premature stop codon
CD38 sgRNA 2	CTC**C**ACTGCTGGCGCCACCT	Premature stop codon
CD38 sgRNA 3	CTGCT**CC**AAAGAAGAATCTA	Premature stop codon
CD38 sgRNA 4	GTTTT**CC**AGAATACTGAAAC	Premature stop codon
CD38 sgRNA 5	AGGTT**C**AGACACTAGAGGCC	Premature stop codon
CD38 sgRNA 6	TTA**C**CTGTAGATATTCTTGC	Removes splice donor
CD7	CACCTGCCAGGCCATCACGG	*Sp*Cas9 indel formation
CD19	GGAACCTCTAGTGGTGAAGG	*Sp*Cas9 indel formation
CD33	TGACAACCAGGAGAAGATCG	*Sp*Cas9 indel formation

Supplementary Table 2: Protospacer sequences used throughout this manuscript compatible with BE3 or SpCas9 genome editing. Sequences are given in 5'-3' orientation, with the target Cytosine highlighted in Red.

Supplementary table 3

Target locus	Forward Primer (5'-3')	Reverse Primer (5'-3')
TRBC1/2	ACACAGAGCCCCTACCAG	GCTACCTGGATCTTTCCA
B2M	CCTCCAGCCTGAAGTCCTAG	GACGAAGTCCACAGCTCTCC
RFX5	GTATGGGGTCAGAGGCAGAA	GGGCTTCTATGCAAGTGCTC
CD7	ATCACCTGCTCCACCAGCGG	GTGTCCTCGCCAGCACACAC
CD33	CTGTAGTCCTTCCCCTCCAC	CAGCGAACTTCACCTGACAG
CD38	GGGAGTTAGCGGAGGGAGTA	GCGGAAACCGCAGAAAAAGT

Supplementary Table 3: Primer sequences used for genomic analysis of base conversion

References

1. Georgiadis C, Rasaiyaah J, Gkazi SA, Preece R, Etuk A, Christi A, Qasim W. Base-edited CAR T cells for combinational therapy against T cell malignancies. Leukemia. 2021;35(12):3466-81.
2. Chiesa R, Georgiadis C, Syed F, Zhan H, Etuk A, Gkazi SA, et al. Base-Edited CAR7 T Cells for Relapsed T-Cell Acute Lymphoblastic Leukemia. N Engl J Med. 2023.