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

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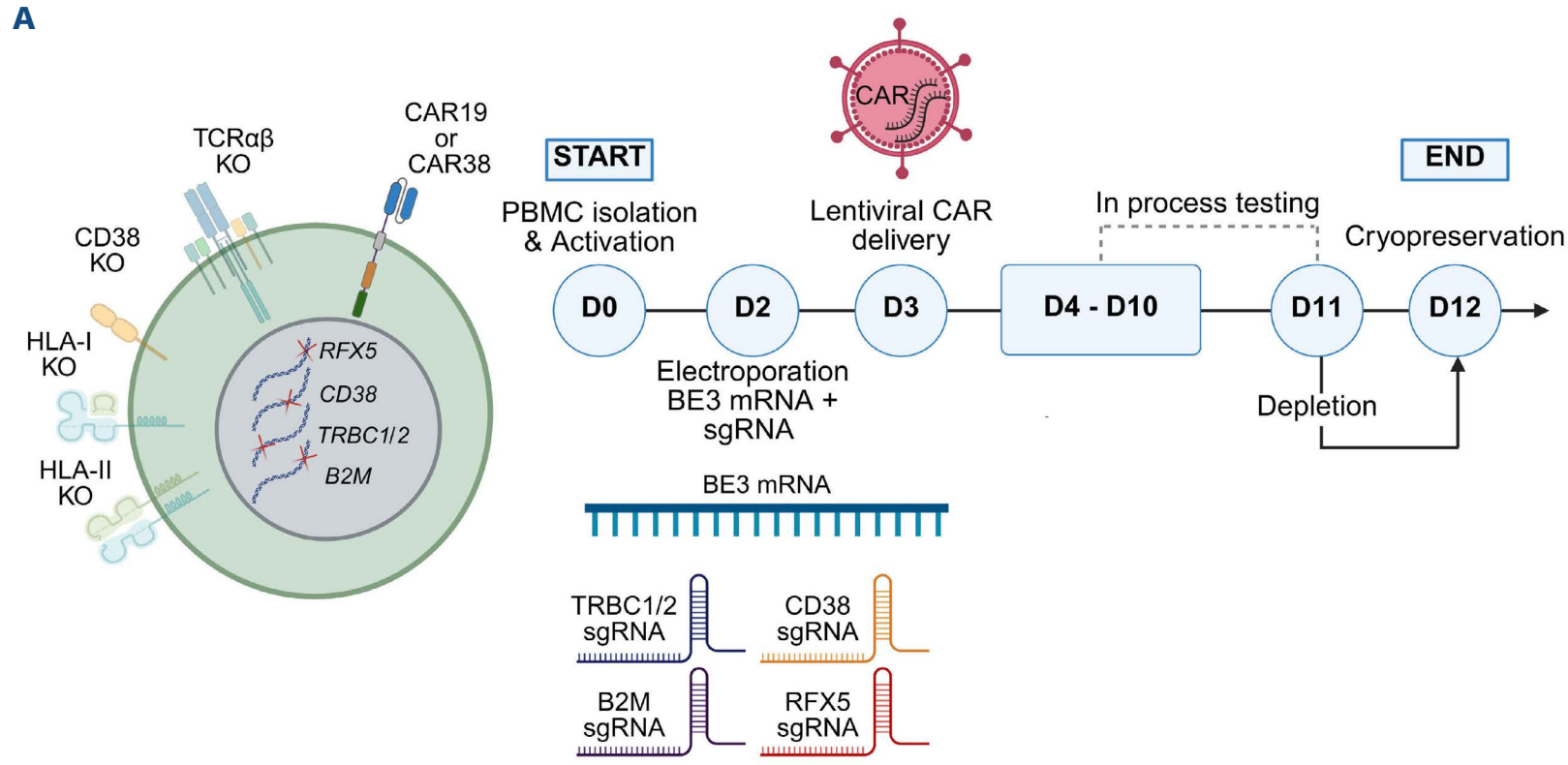
Abstract

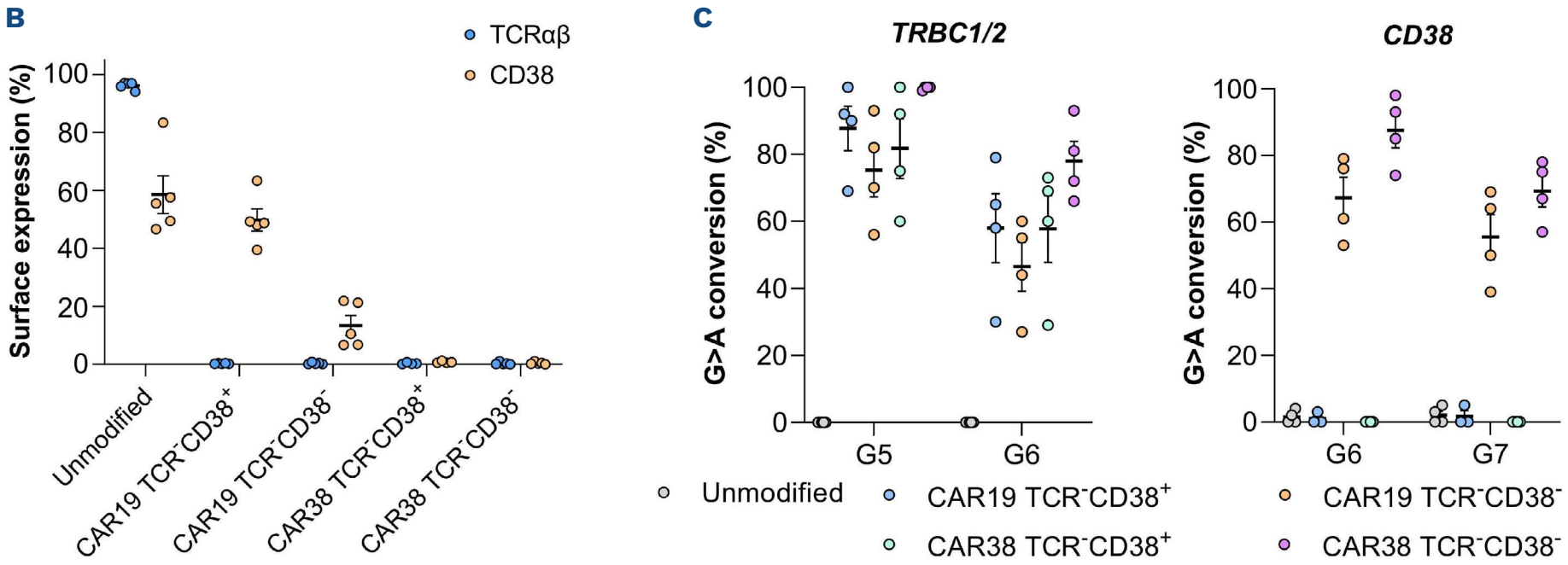
Chimeric antigen receptor (CAR) T-cell therapies are being widely investigated in both autologous and allogeneic settings, with gene editing providing new strategies to address barriers to mismatched cell therapies. Currently 'universal' donor-derived T-cell therapies require intensive lymphodepletion and are still prone to host-mediated rejection. CD38, a transmembrane glycoprotein involved in cell activation and bioenergetics, is a promising immunotherapy target for hematologic 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 sensitized donors and reduced allo-stimulation in mixed lymphocyte cultures, while TCRαβ disruption prevented allo-reactivity. In mixed lymphocyte cultures, CAR38 expression enabled potent 'allo-defense' activity against CD38$^+$ allo-reactive cells. Multiplex base-edited CAR38-T cells exhibited antigen-specific antileukemic activity against human B, T, and myeloid malignancies and inhibited disease progression in humanized murine xenograft models. CAR38-T cells offer a potent 'off-the-shelf' strategy against CD38$^+$ hematologic malignancies and long-lived plasma cells which can be associated with auto-antibody production.

Introduction

Chimeric antigen receptor (CAR) T cells offer new avenues for the treatment of B-cell malignancies including acute lymphoblastic leukemia, with products targeting CD19 or B-cell maturation antigen commercially available.[1,2] Therapeutic applications are also under investigation for autoimmune disorders in which CAR-mediated B-cell elimination has induced remissions of systemic lupus erythematosus 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 allogeneic 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 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-cell acute lymphoblastic leukemia and acute myeloid leukemia, respectively. For all these settings, healthy donor-derived allogeneic CAR (allo-CAR) T cells mediated potent antileukemic effects but relied on intense lymphodepletion with augmented doses of fludarabine and cyclophosphamide as well as alemtuzumab. Here we report that T cells armed with an anti-CD38 CAR, generated using cytidine base editing to first remove CD38 expression,[17] can mediate potent antileukemic 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 the anti-CD38 monoclonal antibodies daratumumab or isatuximab, which have been approved for indications including multiple myeloma,[18] acute lymphoblastic leukemia and acute myeloid leukemia.[19,20] Phase I clinical trials have also reported autologous anti-CD38





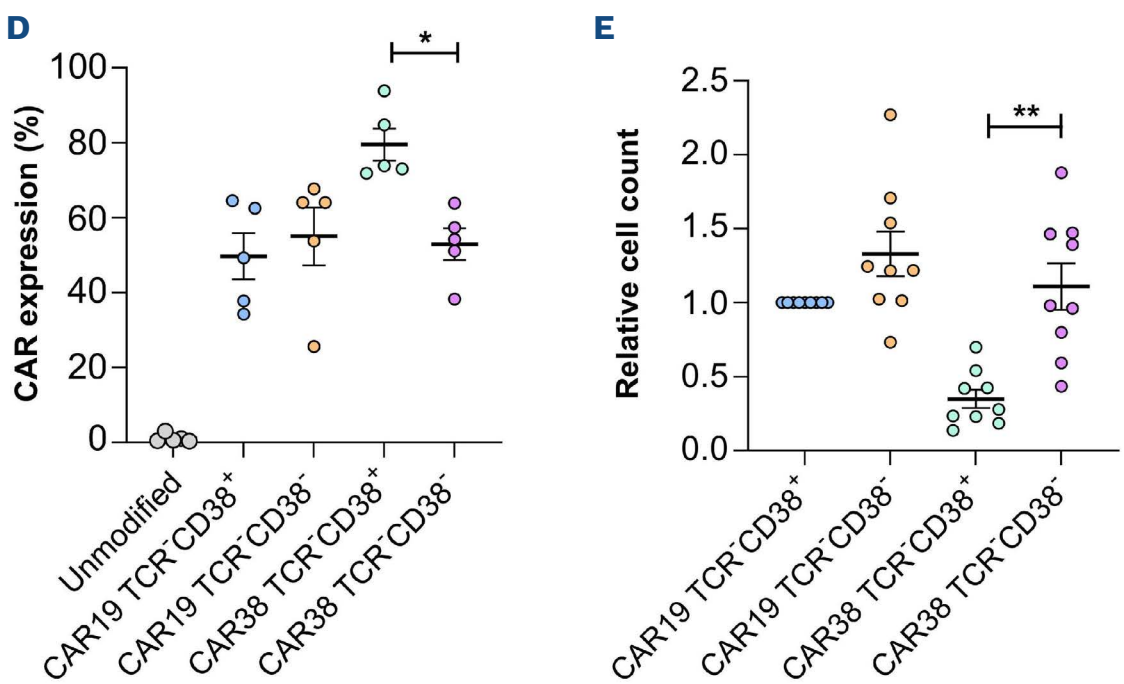


Figure 1. Production of BE-CAR T cells. (A) Timeline of base-edited chimeric antigen receptor (CAR) T-cell manufacture. (B, C) Quantification of TCRαβ and CD38 disruption at the end of manufacture by flow cytometry (N=5) (B) and Sanger sequencing (N=4) (C). (D) CAR-positive cells after magnetic bead-mediated depletion of residual TCRαβ$^+$ cells across multiple donors (N=5). *P≤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≤0.01, paired one-way analysis of variance with a Tukey multiple comparison test. Mean ± standard error of the mean is shown. HLA-II: human leukocyte antigen class II; KO: knockout; HLA-I: human leukocyte antigen class I; CD38: cluster of differentiation 38; TCRαβ: T-cell receptor-αβ; RFX5; regulatory factor X5; TRBC; T-cell receptor beta constant; B2M: beta-2 microglobulin; PBMC: peripheral blood mononuclear cells; D: day; BE3: base editor-3; mRNA: messenger RNA; sgRNA: single-guide RNA.

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 humanized murine models.

Methods

Chimeric antigen receptor T-cell manufacture

Mononuclear cells 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 medium (Miltenyi Biotec) supplemented with 3% heat inactivated human serum (Seralab, Sussex, UK) and 20 ng/mL human recombinant interleukin-2 (Miltenyi Biotec) as described previously.[10] Genome editing used codon-optimized cytidine base editor-3 (BE3) mRNA (TriLink BioTechnologies, San Diego, CA, USA). Delivery and molecular assessments are described in the *Online 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 antihuman antibodies used for immunophenotyping are listed in *Online Supplementary Table S1*.

Chimeric antigen receptor lentiviral vectors

CAR were expressed from third-generation lentiviral vector configurations and transductions performed at a multiplicity of infection of 5.[10,14] Anti-CD38 scFv was derived from daratumumab (heavy-variable-light-variable orientation with a GGGGS3 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 signaling domain (scFv-CD8α-4-1BB-CD3ζ).

Flow-based *in vitro* cytotoxicity assay

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

Mixed lymphocyte cultures

Irradiated BE-CAR T cells (30 Gy) were co-cultured in a 96-well plate (1:1 ratio) with allogeneic mononuclear cells. After 5 days, wells were pulsed with 1 μCi ^{3}H-thymidine (Revvity, Waltham, MA, USA) and incubated for 18-20 hours before transfer of ^{3}H-thymidine-labeled DNA to a Filtermat (Revvity) using a cell harvester (TOMTEC Imaging Systems, Unterschleissheimn, Germany). Meltilex (Revvity) was applied to the Filtermat, and ^{3}H-thymidine incorporation was read using a MicroBeta counter (PerkinElmer, Waltham, MA, 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 antihuman IgG, CD3, and CD19 for 25 minutes before acquisition on a FACSLyric Flow Cytometer (BD).

In vivo chimeric antigen receptor 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, UK). NOD/SCID/γc$^{-/-}$ (NSG) mice (Charles River, The Jackson Laboratory, Bar Harbor, ME, USA), were inoculated by intravenous injection with EGFP$^+$LUC$^+$ Daudi (0.5x10^6), Jurkat (1x10^7), or MOLM14 (1x10^5) cells on day 0. Engraftment was confirmed on day 5 by bioluminescence imaging using an IVIS Lumina III (PerkinElmer, live image version 4.5.18147). On day 6 mice received either 2.5x10^6 unmodified T cells or BE-CAR$^+$ T cells. Tumor inhibition was assessed by serial bioluminescence imaging and bone marrow at necroscopy.

Statistical analysis

Graphs show mean ± standard error of mean. One-way analysis of variance (paired or unpaired) with a Tukey *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 chimeric antigen receptor 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 (*Online Supplementary Figure S1A, B*). Sanger sequencing confirmed appropriate C>T conversions in the anticipated window of deamination with 77% conversions at position C6 (protospacer position six)

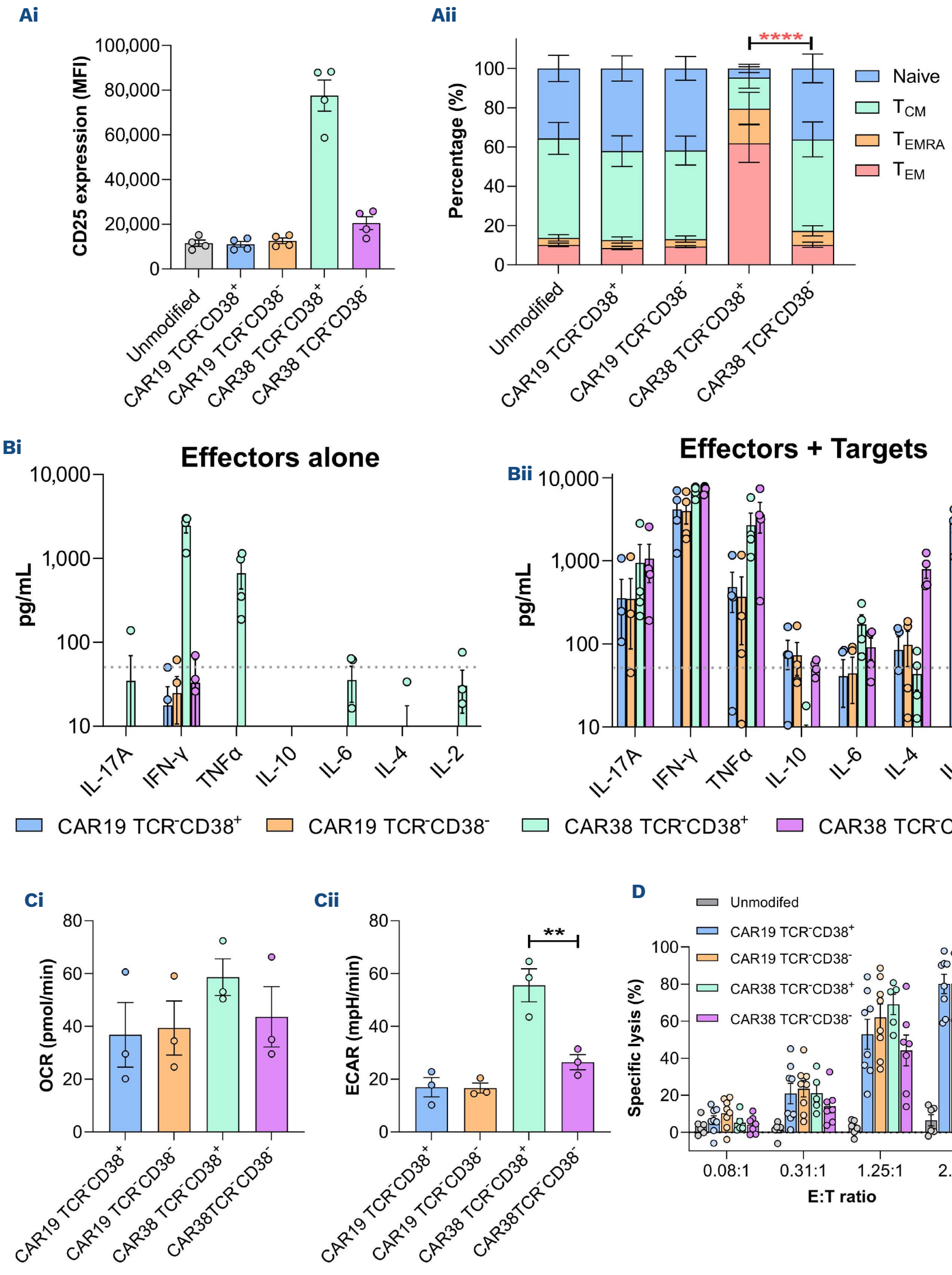


Figure 2. CD38 knockout mitigates against chimeric antigen receptor-mediated activation and fratricidal effects. (A) Immunophenotyping at the end of BE-CAR manufacture (N=4) measuring both (i) activation profile (by CD25 mean fluorescence intensity) and (ii) memory phenotype (by CD45RA and CD62L expression). ****$P \leq 0.0001$ paired one-way analysis of variance (ANOVA) with a Tukey multiple comparison test (analysis between the TEM populations). (B) Cytokine bead array quantifying cytokine

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release (i) in the absence and (ii) in the presence of leukemia target cells (N=3 technical replicates, dotted line indicates the limit of quantification (50 pg/mL). (C) Metabolic activation measured using a Seahorse-XF analyzer (N=3 donors, where each point represents the mean of 4-8 technical replicates, measuring (i) oxygen consumption rate as a surrogate for oxidative phosphorylation and (ii) extracellular acidification rate as a surrogate for glycolysis. $^{**}P \leq 0.01$, paired one-way ANOVA with a Tukey 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 ratios (N=8 donors for all groups except CAR38 TCR$^-$CD38$^+$ which had 5 donors). Mean ± standard error of mean. MFI: mean fluorescence intensity; Naïve: naïve T cells (CD62L$^+$, CD45RA$^+$), T_{CM}: central memory T cells (CD62L$^+$, CD45RA$^-$), T_{EMRA}: effector memory T cells re-expressing CD45RA (CD62L$^-$, CD45RA$^+$); T_{EM}: effector memory T cells (CD62L$^-$, CD45RA$^-$); IL: interleukin; IFN: interferon; TNF: tumor necrosis factor; OCR: oxygen consumption rate; ECAR: extracellular acidification rate; E:T: effector-to-target cell ratio.

and 68% at position C7 (N=3) (*Online Supplementary Figure S1C*). 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 TCRαβ$^-$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 decreased 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 5 bp base-editing window (N=4) (Figure 1C).

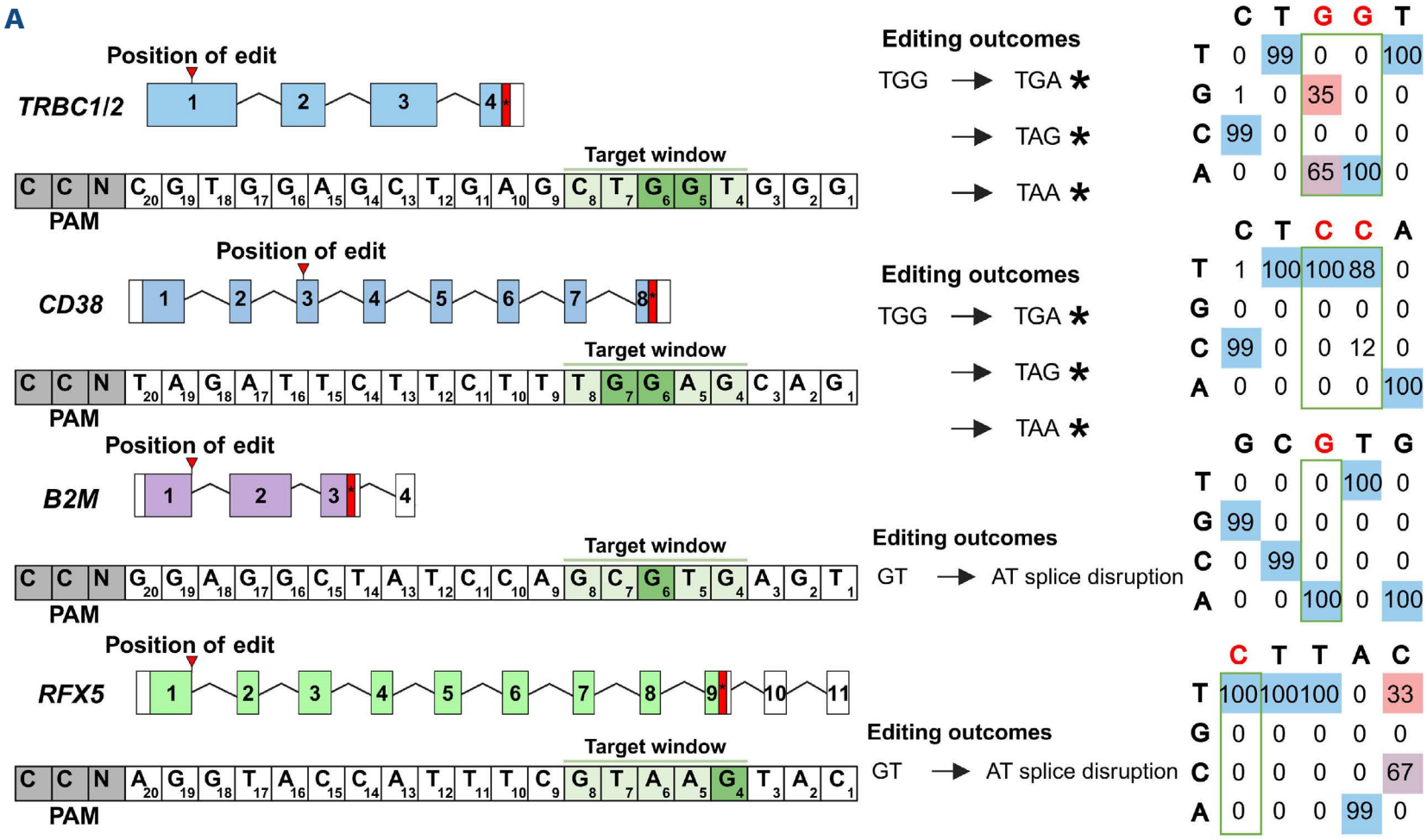
Control BE-CAR19 T cells exhibited similar levels of CAR19 transduction and cell yields irrespective of CD38 base editing (Figure 1D, E) and the phenotype of BE-CAR19 T cells was unaffected by CD38 knockout (Figure 2A, B). In contrast, CAR38 T-cell yields were significantly greater for TCR$^-$CD38$^-$ groups than for 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 2A, B) 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 those of CAR19 controls (N=4) (Figure 2Bii). Favorable metabolic profiles have been reported after CD38 disruption in T cells and natural killer (NK) cells;[27,28] however, immediately after CAR T-cell engineering no significant differences in oxidative phosphorylation (measured by oxygen consumption rate, N=3) (Figure 2Ci) or glycolysis (measured by extra-cellular acidification rate, N=3) (Figure 2Cii) 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 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 graft-*versus*-host disease, and additional editing such as conferring resistance to serotherapy or removal of HLA class I (HLA-I) and II (HLA-II) to tackle host-mediated rejection. The latter was achieved by editing *B2M* for disruption of HLA-I expression, and editing *RFX5*, a major transcriptional factor, for HLA-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-I and HLA-II expressing T cells (Figure 3A). Flow cytometry confirmed knockout and enrichment of highly homogenous TCR$^-$HLAI$^-$ HLAII$^-$ CAR T-cell products (Figure 3Bi, Bii). 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 (*Online Supplementary Figure S2*).

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 sensitization against the complete repertoire of HLA-I and HLA-II molecules expressed by the relevant cell donor (Figure 4A). Both BE-CAR19 and BE-CAR38 T-cell products with intact HLA expression (BE-CAR19$^+$TCR$^-$HLAI$^+$HLAII$^+$ and BE-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 that disruption of both HLA-I and HLA-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 ^{3}H-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 modeled 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



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

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Figure 3. Manufacture of 'universal' chimeric antigen receptor T cells with additional HLA knockouts (TCR$^-$CD38$^-$HLA-I/II$^-$) retain *in vitro* function. (A) Schematic of base-edited loci (*TRBC, CD38, B2M*, and *RFX5*). The red arrow indicates the position of the desired edit; the protospacer sequence is shown below, with numbers indicating protospacer position distal to the protospacer adjacent motif. The 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 analyzed 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 chimeric antigen receptor (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 cell 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 cells (N=3 donors). Each point shows the mean of N=3 technical replicates, with the limit of detection indicated by a dotted line (50 pg/mL). Mean ± standard error of mean. TRBC; T-cell receptor beta constant; PAM: protospacer adjacent motif; CD38: cluster of differentiation 38; B2M: beta-2 microglobulin; RFX5; regulatory factor X5; TCRαβ: T-cell receptor-αβ; KO: knockout; HLA-I: human leukocyte antigen class I; HLA-II: human leukocyte antigen class II; E:T: effector-to-target cell ratio; IL: interleukin; IFN: interferon; TNF: tumor necrosis factor.

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 (*Online Supplementary Figure S3*).

Experiments also investigated possible NK-cell-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 recognized 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-leukemia activity of 'universal' CD38$^-$TCR$^-$ HLA-I/II$^-$ 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 6 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 a 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 bioluminescence imaging, reductions in leukemia 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-acute myeloid leukemia 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 (*Online Supplementary Figure S4A*) or MOLM14 (*Online Supplementary Figure S4B*) cells demonstrated antigen-specific BE-CAR38 T-cell cytotoxicity and cytokine release against CD38$^+$ leukemic lines across effector:target ratios. Responses were comparable to those for BE-CAR7 and BE-CAR33 with no evidence that CD38 editing influenced responses (*Online Supplementary Figure S4A, B*). Comparisons *in vivo* used NSG mice engrafted with either Jurkat or MOLM14 lines and again responses were comparable to those for BE-CAR7 (*Online Supplementary Figure S5A*) or BE-CAR33, respectively (*Online Supplementary Figure S5B*). These findings suggest that BE-CAR38 T cells have potential applicability against a wide variety of CD38$^+$ hematologic malignancies.

Discussion

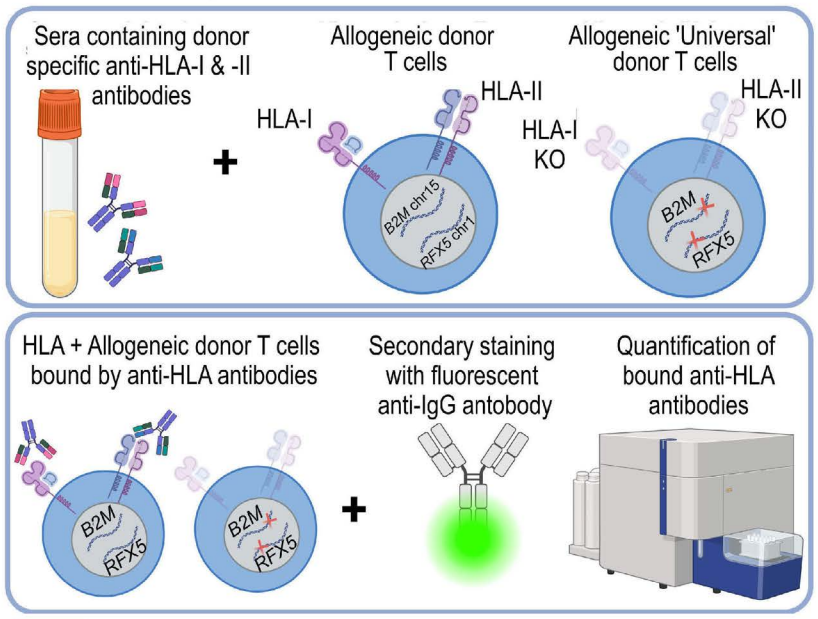
CD38 is a promising candidate for immunotherapy with robust expression in multiple hematologic malignancies[19,20,29,30] but limited expression on healthy tissue beyond the hematopoietic system.[31] Anti-CD38 monoclonal antibodies, exemplified by daratumumab and isatuximab, received Food and Drug Administration approval for treatment of MM due to their efficacy and safety profiles,[32-36] and have also produced encouraging outcomes against acute lymphoblastic leukemia,[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]

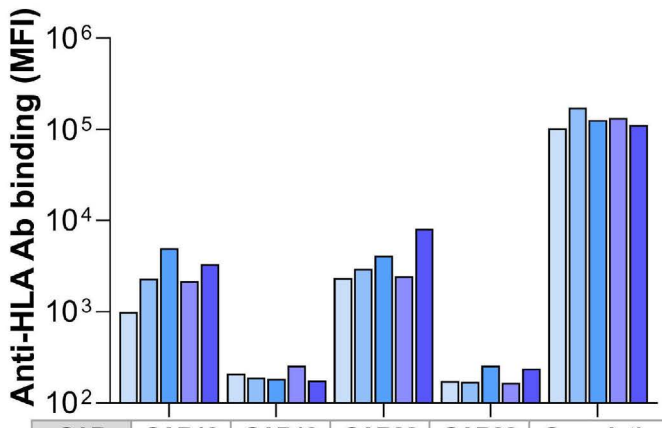
Alternative CAR T-cell-based approaches have also been explored with early clinical trials reporting efficacy and safety data in B-cell acute lymphoblastic leukemia,[21] acute myeloid leukemia,[22] chronic myelogenous leukemia,[24] and multiple myeloma.[23,25] Cytopenia was commonly observed, likely due to CD38 expression across the hematopoietic system, which may require time-limited applications or bridge-to-transplant strategies for donor-derived reconstitution in some settings.

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

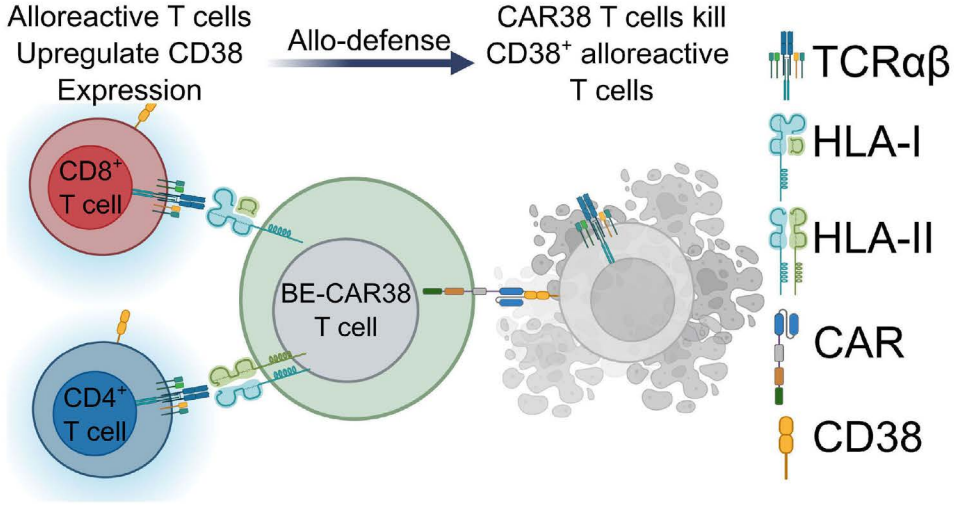
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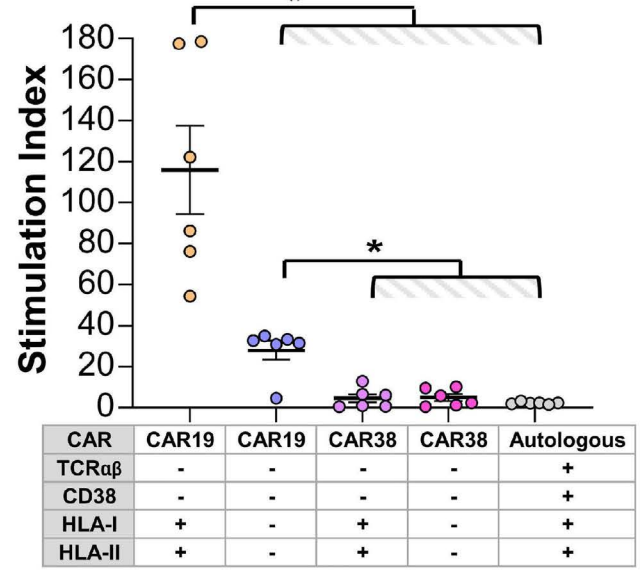
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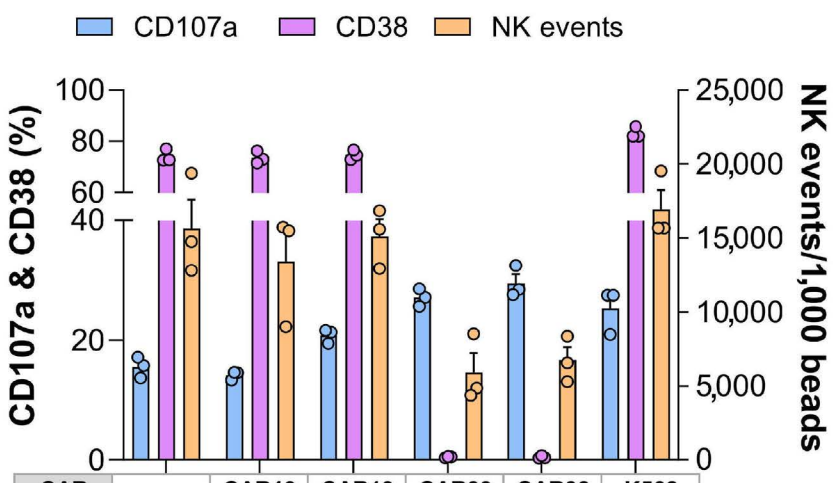
B





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	+	+	-	+	-	-

Figure 4. Base-edited TCR⁻CD38⁻HLA-I/II⁻ chimeric antigen receptor 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 five sera measured by mean fluorescence intensity. (B) Mixed lymphocyte reactions measure thymidine uptake by mismatched allo-mononuclear cells against irradiated (30 Gy) 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 analysis of variance with a Tukey multiple comparison test (*$P \leq 0.05$). (C) Natural killer (NK)-cell degranulation after co-culture with BE-CAR⁺ TCR⁻HLA-I⁻HLA-II⁻ 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⁺ cells. Expression of CD107a (degranulation marker), CD38 (CAR target), and NK events (measured by counting beads) are plotted for each group. The mean ± standard error of mean is shown. CAR: chimeric antigen receptor; HLA: human leukocyte antigen; KO: knockout; Ab: antibody; MFI: mean fluorescence intensity.

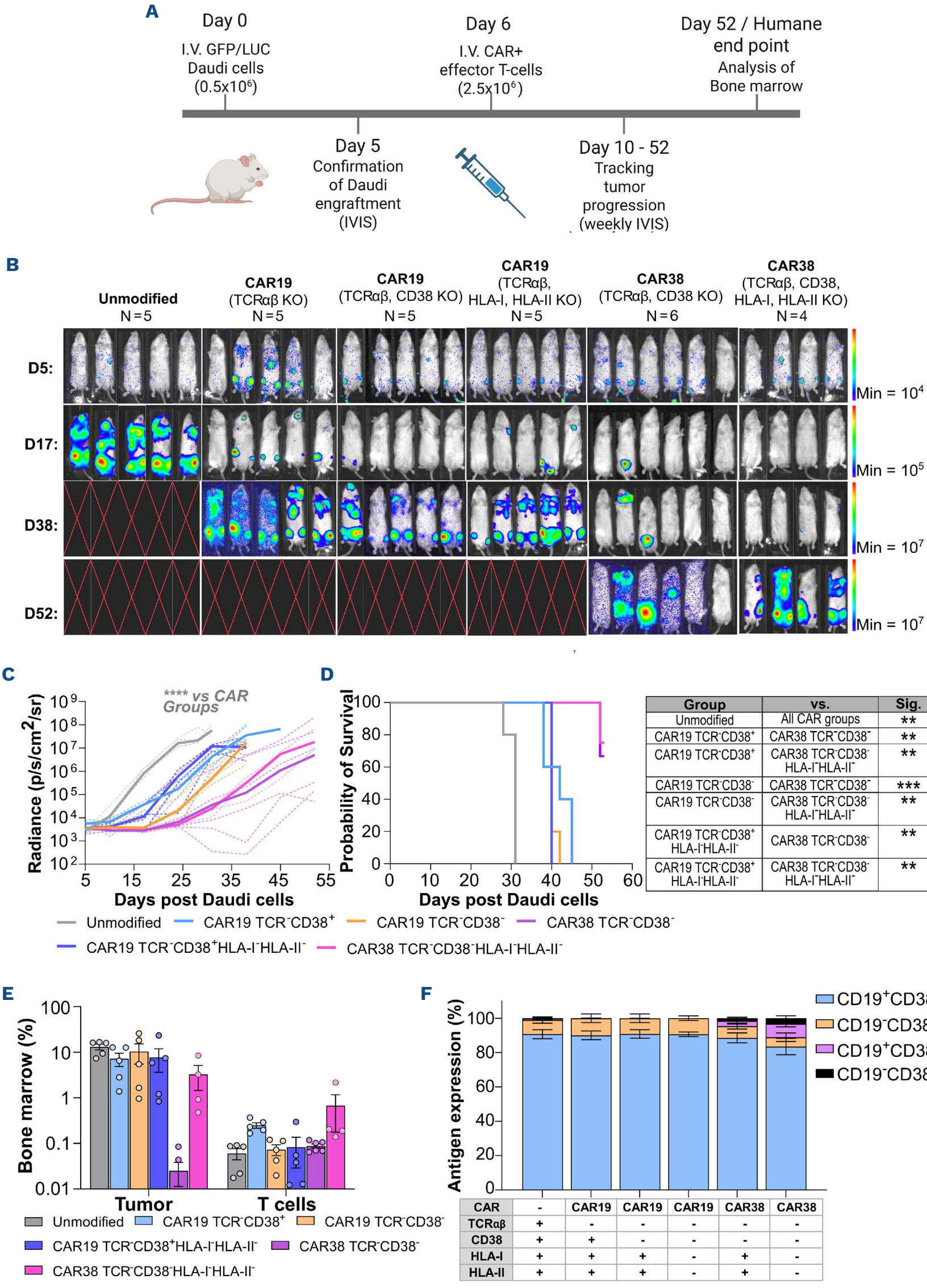


Figure 5. *In vivo* function of TCR⁻CD38⁻HLA-I/II⁻ chimeric antigen receptor T cells after multiplexed base editing. (A) Timeline of an *in vivo* experiment with NSG mice engrafted with Daudi cells expressing green fluorescent protein (GFP) and luciferase. (B)

Continued on following page.

IVIS images confirming tumor engraftment on day 5, prior to chimeric antigen receptor (CAR) T-cell treatment on day 6, and tumor progression over the course of the experiment. Five mice received unmodified, base-edited CAR19 TCR$^-$CD38$^+$, CAR19 TCR$^-$CD38$^-$, and 'universal' CAR19 TCR$^-$HLA-I$^-$II$^-$ T cells. Six mice received CAR38 TCR$^-$CD38$^-$ and four received 'universal' CAR38 TCR$^-$CD38$^-$HLA-I$^-$II$^-$ T cells. (C) Leukemia progression measured by average radiance over the course of the experiment. The 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. $^{**}P \leq 0.01$ and $^{***}P \leq 0.001$. (E) Confirmation of both leukemia 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 ± standard error of mean. I.V.: intravenous; LUC: luciferase; TCRαβ: T-cell receptor-αβ; KO: knockout; CD38: cluster of differentiation 38; HLA-I: human leukocyte antigen class I; HLA-II: human leukocyte antigen class II; D: day.

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 favors 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 a blocking antibody,[44] CRISPR/Cas9 genome editing,[45-47] affinity-optimized scFvs[48] and adaptor-based CAR.[29] We investigated a 'universal' allogeneic 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 graft-*versus*-host disease, 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-I and HLA-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 base 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 in which 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 a 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 both to arm T cells against and to shelter T cells from host immunity. Therapeutic development against a variety of hematologic malignancies and autoimmune conditions using 'universal' 'off-the-shelf' BE-CAR38 T cells is warranted, either alone or in combination with other CD38-edited 'off-the-shelf' CAR T products.

Disclosures

WQ has advised Virocell, Wugen and Galapagos. The other authors have no conflicts of interest to disclose.

Contributions

WQ, RP and CG designed the project. RP, CG, OG, RK, AJ, EC and DK performed experiments and analyzed data. RP and WQ wrote the manuscript. All authors reviewed and approved the manuscript.

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Data-sharing statement

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

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