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CRISPR mediated base conversion allows discriminatory depletion of endogenous T cell receptors for enhanced synthetic immunity.

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Abstract:

Emerging base editing technology exploits CRISPR RNA-guided DNA modification effects for highly specific C>T conversion which has been used to efficiently disrupt gene expression. These tools can enhance synthetic T cell immunity by restricting specificity, addressing HLA barriers and promoting persistence. We report lentiviral delivery of a Hepatitis B virus (HBV) specific recombinant TCR (rTCR) and a linked CRISPR single-guide RNA for simultaneous disruption of endogenous TCRs (eTCR) when combined with transient cytosine deamination. Discriminatory depletion of eTCR and coupled expression of rTCR resulted in enrichment of HBV specific populations from 55% (SEM ± 2.4%) to 95% (SEM ± 0.5%). Intensity of rTCR expression increased 1.8-2.9 fold compared to cells retaining their competing eTCR and increased cytokine production and killing of HBV antigen-expressing hepatoma cells in a 3D microfluidic model was exhibited. Molecular signatures confirmed seamless conversion of C>T (G>A) had created a premature stop codon in TCR beta constant 1/2 loci, with no notable activity at predicted off-target sites. Thus, targeted disruption of eTCR by cytosine deamination and discriminatory enrichment of antigen-specific T cells offers the prospect of enhanced, more specific T cell therapies against HBV associated hepatocellular carcinoma (HCC) as well as other viral and tumour antigens.
Lay Summary:

White blood cells called T cells mediate powerful antiviral effects that can be used to target liver cancers linked to Hepatitis B virus infection. We report new techniques that change the DNA code in T cells and reprogram them to only recognise cells that show a particular Hepatitis-B flag on their surface. Ultimately such approaches could allow banks of healthy donor T cells to be created and used in multiple patients against viruses and certain cancers.
Introduction:

T cells redirected with recombinant T cell receptors (rTCR) are being investigated in early phase human studies \(^1\text{-}^3\). Limitations include unpredictable ‘off-target effects’ due to TCR cross-reactivity, for example cardiac toxicity following therapy with MAGE-A3 rTCR \(^4\text{,}^5\) and concerns that endogenous TCR α and β chains may miss-pair with rTCR chains and give rise to novel dimeric complexes with unpredictable specificities \(^6\text{,}^7\). These limitations have been partially mitigated by predictive modelling of rTCR cross-reactivity and by promoting exclusive rTCR pairing via additional disulfide bonds and other strategies \(^8\text{-}^{11}\). Also of note is the importance of rTCR assembly on the cell surface as a multimeric complex with CD3 chains, as competition from the endogenous TCR (eTCR) for the shared components can limit cell surface expression \(^12\). Competition for such cellular components can be addressed either by overexpression of CD3, disruption of eTCR by RNA interference \(^13\), or nuclease mediated genetic disruption of eTCR chains. Previously, zinc finger nucleases (ZFNs) \(^14\), transcription activator-like effector nucleases (TALENs) \(^15\), and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) have all been used to disrupt one or both TCR α and β chains \(^16\text{-}^{18}\). These genome editing approaches also reduce the likelihood of mispairing, but existing nuclease-based approaches all result in double stranded DNA breaks and may create large insertions/deletions (indels), trigger translocation events, and increase activation of p53 pathways \(^19\text{-}^{23}\). Recently, a report of autologous anti-tumour therapy with T cells edited using Cas9 to disrupt both TCR and PD1 expression noted readily detectable chromosomal translocations in the infused products \(^24\), and similar aberrations were found after TALEN editing of T cells modified to express anti-CD19 chimeric antigen receptors \(^25\).
Here we report the application of emerging cytosine deaminase base editing technology for efficient and seamless base conversion to introduce premature stop codons in homologous regions of T cell receptor beta constant 1 and 2 (TRBC 1/2) chains \(^{26,27}\). BE3 is a CRISPR guided nickase Cas9 (D10A), fused to a rat apolipoprotein B mRNA editing enzyme catalytic polypeptide (rAPOBEC1) deaminase at the N-terminus, which operates within a 4-8bp window distal to the protospacer adjacent motif (PAM) sequence. The inclusion of a C-terminus fusion comprising a uracil-DNA glycosylase inhibitor (UGI) (derived from Bacillus subtilis bacteriophage PBS1) inhibits uracil-DNA glycosylase and blocks uracil excision promoting conversion to thymidine as cells replicate. High levels of C>T conversion and low levels of indels have been reported for this third generation base editor (BE3) \(^{28-30}\). Here we investigate a codon optimized BE3 (coBE3) in the context of engineering T cells against Hepatitis B virus surface antigen, an important target in the treatment of hepatocellular carcinoma (HCC) \(^{31,32}\). HBV viral antigens are processed and presented by major histocompatibility complex (MHC) molecules on the surface of infected cells \(^{33,34}\), and naturally occurring HBV-specific T cells, can engage with peptides presented in the context of HLA, to moderate viral and tumour burdens \(^{35,36}\). Nevertheless, such HBV specific T cell responses can become exhausted during chronic HBV infection \(^{37-39}\) and synthetic HBV-specific T cells can be generated through the expression of rTCRs \(^{40-44}\). The approach has already been tested clinically in HBV associated HCC, \(^{45,46}\) with further studies planned. Lentiviral vector delivery of a rTCR specific for HLA-A2/HBV peptide S183-91, incorporating murine constant regions, and coupled to a CRISPR single guide RNA (sgRNA) targeting TRBC1/2 loci resulted in high levels of targeted cytosine deamination after transient delivery of mRNA encoding coBE3. Thereafter, discriminatory removal of residual eTCR+ cells was achieved by magnetic bead-mediated depletion using the anti-human TCR\(\alpha\beta\) monoclonal
antibody. Consequently rTCR expression was enriched, as the murine constant regions lack the specific epitope recognised by this antibody. Phenotypic and functional assessments, including migration and killing in a 3D microfluidic model verified immunotherapeutic effects following genome editing, and molecular analysis of both DNA and RNA was performed to examine editor effects.

Results:

Base conversion disrupts eTCR expression and allows enrichment of T cells expressing rTCR

A third generation self-inactivating (SIN) lentiviral vector was generated encoding an HLA-A0201 restricted rTCR (S183-91, FLLTRILTI) specific for HBV envelope protein and a linked sgRNA expression cassette targeting TRBC1/2. The latter was embedded within a deleted unique (ΔU3) region of the 3’ long terminal repeat (LTR) under the transcriptional control of an RNA polymerase III human U6 promoter as previously described. This configuration is referred to as terminal-TRBC-S183-91 rTCR (TTRBC-S183-91 rTCR) (Figure 1). Upon electroporation of coBE3 mRNA, the sgRNA mediated highly targeted base conversion of two neighbouring cytosine nucleotides within exon 1 of TRBC1/2 loci. Single or double base conversion produces a premature stop codon within a 4-8bp window distal to the nCas9 (D10A) PAM sequence (Figure 2A). Consequently, disruption of endogenous TCR β chain expression eliminated eTCRαβ assembly, and the inclusion of murine constant regions within the rTCR further addressed any possibility of aberrant cross-pairing between residual recombinant and endogenous chains (Figure 2B). Following the timeline shown in Figure 2C, healthy T cells were readily activated and transduced resulting in 50-60% rTCR expression (Figure 2D and E i). Exposure to coBE3 led to disruption of eTCR expression and
simultaneous emergence of rTCR+ populations, increasing in proportion to approximately 60-65% of the cultures (Figure 2D and 2E i). Furthermore, because eTCR was amenable to detection by anti-TCRαβ monoclonal antibody, magnetic bead-mediated depletion of residual eTCRαβ expressing cells was possible. Notably, rTCR (constructed with murine C domains) was not susceptible to these reagents and thus at the end of production, cells could be enriched for endogenous TCR-/ recombinant TCR+ (eTCR-/rTCR+), resulting in a highly homogenous product (>99% eTCR-/ 95.9% rTCR+) (Figure 2D and Ei). There was also a significant increase in the mean florescence intensity (MFI) of rTCR in eTCR-/rTCR+ cells compared to eTCR+/rTCR±, suggesting enhanced cell surface expression of rTCR in the absence of eTCR, which may otherwise have competed for CD3 chains during assembly (one way ANOVA, p<0.02) (Figure 2E ii).

Hepatitis B antigen specific responses of eTCR-/rTCR+ T cells

Three different in vitro assessments of antigen specific function were undertaken. Firstly, production of cytokines including interferon-γ (IFNγ), tumor necrosis factor-α (TNFα), interleukin-2 (IL-2), and C-C motif chemokine ligand 4 (CCL4) was determined by flow cytometry in T cells responding to HepG2 cells pulsed with the irrelevant control peptide (HBV core C18-27, FLPSDFFPSV) or a gradient of Hepatitis B target surface envelope peptide (S183-91, FLLTRILTI) concentrations. In all three donors tested, cytokine production was higher in eTCR-/rTCR+ T cells in response to target S183-91 peptide (Figure 3A i and ii), with absent response to control C18-27 peptide and no-peptide control (Figure S1). Next, we investigated effector function at different E:T ratios in a previously described XCelligence impedance assay and calculated the relevant normalised cell indices over 72 hours after addition of effector T cells. An increased index indicated HepG2 target cell proliferation,
whereas cell death or apoptosis resulted in a reduced index, signifying higher levels of effector T cell activity (Figure 3B). Control groups included target cells alone (HepG2 alone), and non-transduced effectors (eTCR+/rTCR-), where as expected, there was a progressive increase and plateau in index. In contrast, both effector groups exhibited a transient rise and then decline in index, with more rapid reductions mediated by eTCR-/rTCR+ cells compared to eTCR+/rTCR± T cells at all E:T ratios (Figure 3C i). Overall effector function was calculated by area under the curve as shown in Figure 3C ii, reflecting the increased cytotoxicity by enriched eTCR-/rTCR+ effector cells compared to their unedited, non eTCR depleted counterparts (eTCR+/rTCR±).  

Finally, migration and target cell killing by engineered T cells was determined in a 3D microfluidics device. The system captured migration of effector T cells from a fluidics channel to a collagen gel embedded with target PreS1-GFP-HepG2 cells. Phenotyping of effector T cells confirmed rTCR expression (Figure 4A) and minimal cytokine expression in the absence of stimulation after thawing. Comparable numbers of T cells were observed migrating into the gel between the effector groups (Figure S2) before cytokine expression profiles were compared between cells recovered from inside or outside the gel area (Figure 4B). Both eTCR+/rTCR± and eTCR-/rTCR+ effector groups presented higher levels of IL-2, INFγ, and TNFα expression within the gel. Killing of PreS1-GFP-HepG2 cells by eTCR-/rTCR+ cells was confirmed within 24 hours whereas eTCR+/rTCR± cells at this time point were comparable to control eTCR+/rTCR- indices and the control PreS1-GFP-HepG2 alone groups (Figure 4C). Direct visualisation revealed greater clearance of HepG2 cells after co-culture with eTCR-/rTCR+ T cells (Figure 4D).
The application of novel genome editing tools necessitated further investigation of anticipated and unexpected molecular consequences of T cell engineering. There is an established experience of lentiviral mediated effects, including their propensity to integrate into transcriptionally active genes \(^{57-59}\) and we did not re-examine these aspects. However, base conversion effects of coBE3 were characterised in depth, extending comparisons to the effects of SpCas9 disruption in similar experiments disrupting eTCR expression in T cells engineered to express a CAR against CD19. Both modalities had mediated high levels of TCRαβ disruption (coBE3: 40.4% ± SEM 5.4%, SpCas9: 52.7% ± SEM 6%) (n=4, Figure S3) but as anticipated we found reduced indel frequencies following electroporation of coBE3 (11.9% ± SEM 1.8%) compared to SpCas9 delivery (48.7% ± SEM 6%).

In the context of rTCR delivery, direct sequencing of TRBC 1/2 in TCRαβ depleted eTCR-/rTCR+ T cells was undertaken and analysed using EditR, with cytosines at positions 5 and 6 distal to the PAM of particular interest (Figure 5A i and ii). High levels of C>T conversion (G>A sense strand) were captured at these positions (37.3 ± SEM 3.9% and 24.3 ± SEM 2.2% at C5 and C6 respectively), with little activity at other nearby C residues (5 ± SEM 1.2% C1, 2.3 ± SEM 1% C2, and 4.3 ± SEM 1.8% C3). NGS revealed similar levels of C>T conversion at both positions C5 (40 ± SEM 2.9%) and C6 (32.3 ± SEM 3%) (Figure S4). Although mostly seamless, a minority of reads exhibited small (<10bp, 8.4 ± SEM 1.4%) or large (10-100bp, 8.2 ± SEM 0.7%) indels signatures (Figure 5A iii) as others have noted previously \(^{28-30}\).

\textit{In silico} analysis of sgRNA binding and possible off-target activity was undertaken using Benchling and presented no exonic off-targets with <3 mismatches. Six genomic loci with the highest scores for off-target activity, all of which contained cytosine bases within the BE3 editing window were interrogated directly by NGS in three different donors (Figure 5B). We found very low levels (<1%) of conversion activity at these sites, and only one intronic
site exhibited C>T changes higher than in its respective non-edited control sample. Recent reports in cell lines have also suggested that promiscuous rAPOBEC1 RNA deamination (including by BE3) can arise following plasmid mediated expression of base editors \(^{60-63}\). In the T cell context, and with coBE3 transiently expressed by mRNA electroporation we investigated if regions directing antigen receptor specificity might be affected. Analysis of RNA from T cells exposed to coBE3 focussed on high throughput interrogation of TCR hypervariable regions (TCR\(\alpha\) and TCR\(\beta\) CDR3 regions). Analysis of samples collected at serial time points, from 1-8 days post BE3 mRNA delivery, found no obvious evidence of aberrant deamination compared to controls (99-100\% cysteines unmodified) and intact sequence integrity of HBs183-91 rTCR was verified (Figure 5C). In addition, transcriptomic analysis on these samples detected anticipated effects of T cell activation and transduction over time (Figure S5). Thus the first principal component (PC1), accounted for 76\% of variance when comparing day 5 and day 12. As the second principal component (PC2) accounted for only 13\% of variance, no major transcriptional changes between edited and non-edited cells were noted. *In silico* analysis had identified a further 24 unique sites of possible off-target BE activity in exonic regions. However, these were all found to have low transcriptional activity (averaged < 100 reads) in both edited and non-edited T cells and therefore unlikely to be of importance.

Thus, while on-target deamination and creation of TCR-stop codons was highly efficient, there was no notable activity at sites of potential interest at either the DNA or RNA level for coBE3.

**Discussion:**
T cell immunotherapy against conventional tumour-associated targets such as NY-ESO-1 are being widely investigated, and recent reports indicate autologous T cells with additional CRISPR/Cas9 modifications designed to improve persistence and efficacy can be safely infused. Emerging base editor technologies offer the prospect of highly specific C>T (G>A) base conversion that can be harnessed to create seamless premature stop codons or modify splice sites to disrupt gene expression for advanced T cell engineering.

We previously reported the first therapeutic use of autologous T cells modified to express HBsAg specific T cell receptors in a subject with chemoresistant, extrahepatic, metastatic disease. In that case, HBV antigens were detectable in HCC metastases but not in donor-derived liver (following cadaveric liver transplantation) thereby reducing the risk of T cell-mediated hepatitis. Gene-modified T cells survived, expanded and mediated a reduction in HBsAg levels and whilst efficacy was not established, there was no significant on- or off-target toxicity. A small number of additional subjects have been treated subsequently, although the approach remains highly patient-tailored and extending to larger numbers of patients is logistically challenging and costly. Similar hurdles are being addressed in the arena of haematological malignancies through the generation of ‘universal’ T cells expressing CARs from non-HLA matched healthy donors. As such, depletion of endogenous TCR and other antigens by genome editing has allowed HLA barriers to be circumvented, and ongoing trials suggest that such universal CAR T cells can expand and persist sufficiently to induce molecular remission. The editing tools applied in clinic have included TALENS and CRISPR/Cas9, and rely on targeted DNA cleavage and repair by non-homologous end joining (NHEJ) which results in the creation of indels leading to gene disruption. Application of CRISPR guided base conversion to create stop codons or alter critical splice site to disrupt
gene expression offers the possibility of seamless gene disruption with greatly reduced likelihood of translocations or toxicity. We report the application of APOBEC deaminase technology for the generation of engineered T cells, which are then rendered devoid of endogenous TCRs and uniformly express rTCR specific for an epitope of HBsAg. The resulting product was homogenous and exhibited enhanced rTCR intensity, greater levels of cytokine production and antigen specific functional integrity in models of HCC elimination. An ability to discriminate and selectively process, and deplete eTCR T cells while rTCR populations are untouched provides critical advantages, especially for strategies when allogeneic donor cells bearing potentially alloreactive eTCRs can be eliminated. Non-human protein sequences within constructs have the potential to be immunogenic, although murine TCR constant regions are considered unlikely determinants in the generation of human anti-mouse antibodies \textsuperscript{65}. Likewise, the BE configurations employ bacterial and rodent derived elements, but expression is transient during \textit{ex vivo} culture and unlikely to be problematic \textit{in vivo}.

The rapid development of tools enabling highly targeted base conversion through deamination effects promises tantalising opportunities, although in depth characterisation of desirable and unwanted effects in subsequent therapeutic applications have to be mapped. Existing CRISPR/Cas base-editors employing rAPOBEC1 (including coBE3) are known to mediate off-target DNA edits and transcriptome-wide RNA deamination in both protein-coding and non-coding regions \textsuperscript{60-62}. While these could be problematic, newer variants with more precise DNA restricted editing are already in development and should continue to evolve as ever more efficient, specific and non-toxic editing tools. Our analysis of possible off-target sgRNA activity in three donors found minimal base conversion effects at predicted DNA sites. Importantly, examination of RNA detected no major differences in
gene expression levels between base edited and non-edited T cells, with only very minor perturbations and C>U conversions of the CDR3 variable regions, no greater than in control cells. Such changes could otherwise redirect the specificity of the introduced TCR, and would risk causing autoimmunity or off-target T cell effects.

**Conclusion:**

Removal of eTCR enhances expression of introduced rTCR, reduces the risk of aberrant cross-pairing, and allows discriminatory enrichment of engineered T cells. The strategy also opens the door to generating ‘universal’ allogeneic T cells from healthy HLA-mismatched donors by reducing the risk of graft versus host disease. In the case of the rTCR specific for HBs183-91, blood from healthy HLA-A201 donors could readily be further edited to disrupt mismatched HLA molecules creating immunologically stealthy cells. Additional multiplexed editing of T cell exhaustion markers may promote enhanced persistence and anti-tumour effects. Ultimately, pre-manufactured banks of eTCR-/rTCR+ T cells specific for groups of dominant HLA/peptide combinations could provide treatment options for large numbers of subjects.

**Materials and Methods:**

**CRISPR guide RNA**

Guide sequences compatible with coBE3 targeting homologous sequences in **TRBC1** and **2** were designed using the CRISPR design tool, Benchling (https://benchling.com) and provided an on-target editing score for predicted activity at each cytosine around the editing window. **TRBC1/2:** C₃₄C₁₁C₅₇AC₂₁₉ C₂₁₄AGCUCAGCUCCACG (anti-sense, numbers
indicate predicted editing scores for the specific cytosine base). Predicted exonic off-target binding required at least 3 mismatches within the protospacer.

**Lentiviral construct for rTCR and sgRNA delivery**

Lentiviral design for coupled transgene and guide RNA expression has been previously described. Briefly, rTCR HLA-A0201/HBs183-91 was cloned under the control of an internal human phosphoglycerate kinase 1 (hPGK) promoter and a CRISPR guide expression cassette was embedded in the lentiviral 3’ LTR. This comprised a 5’ RNA polymerase III promoter (U6) and a sgRNA specific for TCRB1/2 with a 5’G for improved transcription. Vector stocks were produced in 293T cells by transient transfection with third generation packaging plasmids and concentrated by ultracentrifugation prior to storage at -80°C.

**Primary human lymphocyte culture and modification**

Peripheral blood mononuclear cells (PBMCs) were isolated by ficoll density gradient and subsequently activated with TransAct reagent (130-111-160, Miltenyi Biotec) at 10µl/ml. TexMACS medium (130-097-196, Miltenyi Biotec) with 3% human AB serum (GEM-100-512-HI, Seralabs) and 100U/ml IL-2 was used for all lymphocyte cell culture. Transduction with lentiviral vector was performed 24 hours post activation at a multiplicity of infection (MOI) of 5. Electroporation of coBE3 mRNA was performed at day 4 post activation, after which cells were cultured in a G-Rex®10 (P/N 80040S, Wilsonwolf). Lymphocytes were cultured for 11 days post activation and magnetically depleted using anti-TCR α/β-biotin (130-098-219, Miltenyi Biotec) followed by incubation with anti-biotin microbeads ultrapure (130-105-637, Miltenyi Biotec) and separation through LD columns (130-042-901, Miltenyi Biotec). Cells were rested overnight before flow cytometry based phenotyping and cryopreservation.
Phenotyping Flow cytometry

Flow cytometry was performed on a 4-laser BD LSRII (BD Biosciences), with subsequent analysis executed using FlowJo v10 (TreeStar). Cells were stained according to manufacturer’s instructions with Mouse TCR β constant-APC (Clone H57-597, Biolegend, Cat 109211), Human TCRα/β-PerCP vio 700 (Clone REA652, Miltenyi Biotec, Cat 130-113-540), PD1-PE (Clone PD1.3.1.3, Miltenyi Biotec, Cat 130-117-384), CD4-VioBlue (Clone REA623, Miltenyi Biotec, Cat 130-114-534), and CD45-VioGreen (Clone REA747, Miltenyi Biotec, Cat 130-110-638).

Antigen specific responses

Target (T) HepG2 cells were pulsed with HBV surface envelope peptide S183-91 (FLLTRILTI, JPT Peptide Technologies) and irrelevant control HBV core peptide C18-27 (FLPSDFFPSV, JPT Peptide Technologies) peptide at gradient concentrations for 1h at 37°C. Cryopreserved effector (E) T cells (eTCR+/rTCR-, eTCR+/rTCR±, and eTCR-/rTCR+) were thawed and cultured at E:T ratio of 1:1 and 0.1μg/ml Brefeldin A (Sigma) was added before overnight co-culture. A Fortessa X20 flow cytometer (BD) was used for cell acquisition, with FlowJo v10 (TreeStar) used to analyse phenotype and function of effector T cells groups. Phenotyping included intracellular staining with TNFα FITC (clone MAb11, BD biosciences, Cat 502906), MIP-1b PE (clone D21-1351, BD biosciences, Cat 550078), IL-2 PerCP-eFlour710 (clone MQ1-17H12, eBioscience, Cat 46-7029-42), GranzymeB AF700 (clone GB11, BD biosciences, Cat 560213), IFNg V450 (clone B27, BD biosciences, Cat 560371), and surface staining with CD3 BUV395 (clone UCHT1, BD biosciences, Cat 563546) and mouse TCR β constant-APC (Clone H57-597, Biolegend, Cat 109211).
Electroporation of base editor mRNA

The BE3 amino acid sequence was sourced from previously published work containing a single C terminus nuclear localisation signal. Additionally, the DNA sequence has been codon optimised by ThermoFisher Scientific, GeneArt. coBE3 mRNA was produced by Trilink, and clean-capped (Cap 1), polyadenylated and purified by high performance liquid chromatography (HPLC). Electroporation used a 100µl tip-kit and Neon transfection system (ThermoFisher Scientific). Cells were electroporated at 20x10⁶ cells/ml in buffer T, using protocol 24 (1600V, 10ms, 3pulses) with 50µg/ml coBE3 mRNA. Following electroporation T cells were incubated overnight at 30°C before restoration to 37°C.

Molecular characterisation of on-target DNA editing

Genomic DNA extraction was performed using DNeasy Blood and Tissue Kit (69504, QIAGEN) and PCR sequencing undertaken using primers for TRBC1/2 loci. TRBC forward: 5’ AGGTCGCTGTGTTTGAGC 3’, TRBC reverse: 5’ CTATCCTGGGTCCACTCGTC 3’. Sanger sequencing data (Eurofins Genomics) was analysed using EditR (https://moriaritylab.shinyapps.io/editr_v10/) In addition amplified products were library prepped for next generation sequencing (NGS) using a Nextera XT kit (Illumina, Cambridge, UK). After the library preparation, individually barcoded samples were pooled and ran in a MiSeq using a 500-V2 nano-cartridge. Demultiplexed fastq files were uploaded to Galaxy for trimming and alignment. NHEJ signatures were analysed using Pindel, haplotypes were analysed using Freebayes. Figures were created in R.
**Molecular characterisation of off-target DNA editing**

Online software, Benchling, was used to predict off-targets for the TRBC guide. Libraries were prepared on the top six off-targets using the same methodology as above (NGS for on-target DNA editing) and combinations of target-specific primers (Supplementary Bioinformatics Methods).

**Characterisation and analysis of the transcriptome**

Sequential RNA samples from engineered T cells were prepared for RNA sequencing using the KAPA mRNA Hyper prep kit (Roche) at UCL Genomics. Initial analysis was performed on a customised Galaxy workflow followed by transcriptomics analysis on iDEP 9.1 (Workflow using R packages). Online software tool, CRISPR RGEN ‘Cas-OFFinder’, predicted 1,071 off-target sites for TRBC guide with parameters set for up to 3 mismatches and a 1 nucleotide bulge). A pipeline was developed for further investigation of these sites in RNAseq data (Supplementary Bioinformatics Methods).

**Screening for rTCR RNA editing effects**

Total RNA was extracted using a QIAamp RNA Blood Mini kit (Qiagen, 52304) for TCR library preparation and sequencing as previously described\(^{52,53}\). rTCR RNA was reverse transcribed using a murine TRBC specific primer (5’ TGGACTTCTTTGCCGTTGAC 3’). Following ligation of an oligonucleotide containing the Illumina SP2 primer and unique molecular identifiers, products were amplified using primers specific to the murine constant alpha and beta chains (5’ CGTTGATCTGGCTGTCGAAG 3’ and 5’ TTGACCCACCAAAGACAGCTC 3’, respectively). Finally, libraries were built in two furthers steps of amplification during which the SP1 sequencing primer, indices and Illumina adaptors were added. Part of the primers used in
these were also specific for the constant regions (5’
ACACTTTTCCCTACACGACGCTCTTCCGATCTNNNNNNGCCAATGCACGTTGATCTGGCTGTCGAA
388 G 3’ and 5’
ACACTTTTCCCTACACGACGCTCTTCCGATCTNNNNNNGCCAATCCGTTGACCCACCAAGACAGCT C 3’).
The final purified libraries were verified using Tapestation (Agilent) and Qubit (Thermo
Fisher Scientific), multiplexed and sequenced on a MiSeq system (Illumina) using 500-V2
cartridges (Illumina). Fastq files were demultiplexed using Demultiplexor (https://github.com/innate2adaptive/Decombinator) 54. Using Galaxy tools 49, the
demultiplexed fastq files were trimmed (Trim Galore and Trimmomatic) and aligned
(Bowtie2) to the relative TCR HBV gene map. Aligned files were interrogated for the
frequency of the reference sequence per base around the complementarity-determining
region 3 (CDR3) (100bp total window).

Data availability
All fastq files will be available on NCBI Sequence Read Archive upon publication (BioProject
ID: PRJNA637371).

Xcelligence impedance assay
Target HepG2 cells were seeded (1x10⁵ per well) in the dedicated device (E-Plate VIEW 16,
ACEA Biosciences Inc.) and cultured for 24 hours. Impedance measurement was acquired
with an interval of 15 minutes by an array of electrodes located at the bottom of the plate.
Different T cell preparations and E:T ratios were added in the well after 24 hours, and the
impedance signal was recorded for the subsequent 72 hours. Three different donors were
tested in triplicate conditions.
Briefly, dissociated PreS1-GFP-HepG2 target cells were mixed with collagen type I gel (rat tail, Corning) and injected into the dedicated region of the 3D cell culture chip (DAX-1, AIM Biotech), before gel polymerization, following a previously developed protocol. R10 media with 3µM of DRAQ7 (Biolegend) cell-impermeable nuclear dye was then added to the media channels to hydrate the gel, and chips were incubated at 37°C. T cells were stained with 3µM Cell-Tracker Violet BMQC (Thermo Fisher Scientific) and were injected into one of two media channels flanking the gel region before overnight incubation. 3D confocal images were acquired daily with a high content imaging system (Phenix, Perkin Elmer). T cells from the liquid channel were collected by manual pipetting; after, collagenase solution was injected into the device to retrieve the immune cells migrating in the hydrogel region for flow cytometer analysis on a 4-laser BD LSRII (BD Biosciences).

Statistics

Statistical analysis was performed using GraphPad Prism software, version 8.0.0.

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WQ holds interests unrelated to this project in Autolus Ltd.

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**Figure 1: Terminal-CRISPR lentiviral vector configuration coupling HBV rTCR, and CRISPR TRBC1/2 sgRNA delivery.** Lentiviral plasmid configuration, coupling the expression of a recombinant T cell receptor (rTCR) against the hepatitis B virus (HBV) envelope surface antigen 183-91 (S183-91) and a T cell receptor beta constant (TRBC)-specific single guide RNA (sgRNA). The S183-91 rTCR is placed under the transcriptional control of an internal
human phosphoglycerate kinase 1 (hPGK) promoter, while TRBC1/2 sgRNA is expressed via
a human U6 promoter. The rTCR is expressed as a single transcript with the rTCR α chain
first, followed by the rTCR β chain separated by a porcine teschovirus-1 2A (P2A) self-
cleavage sequence. These recombinant chains are composed of the T cell receptor α
variable 34 (TRAV34), and the T cell receptor β variable 28 (TRBV28) domains, as well as
either murine TRAC (muTRAC), or murine TRBC 1 (muTRBC1). The rTCR chains contained an
additional cysteine-cysteine disulfide bond between murine constant regions. CMV:
Cytomegalovirus, cPPT: central polypurine tract, WPRE: woodchuck post-transcriptional
regulatory element, LTR: long terminal repeat, ΔU3: deleted unique 3’, R: repeat, U5: unique
5’, ψ: Psi, D: diversity region, J: joining region.

Figure 2: Generation of eTCR-/rTCR+ T cells using coupled cytosine deaminase base editing.
A) Schematic representation of base editor 3 (BE3) targeting exon 1 of the TRBC 1/2 loci.
Editing window (blue) of the BE3 ranged from 4-8bp distal to PAM (red) with conversion of
Tryptophan (Trp) codons to create premature stop codons. B) Theoretical TCR chain pairing
when introducing a rTCR with or without knockout of the endogenous TCR β chain.
Incorporation of murine constant regions with an additional disulfide bridge in the
recombinant α and β (rα-rβ) chains reduced the potential for mispairing shown in the
middle panel and disruption of eTCR further reduces likelihood of mispairing. C) Schema of
cell production. Human peripheral blood lymphocytes were isolated and activated with
TransAct™ (anti-CD3/CD28) (day 0) before transduction (day 1) and electroporation with
codon optimised (co) BE3 mRNA (day 4). After overnight hypothermic culture at 30°C, cells
were expanded in G-Rex®10 flasks for seven days. Discriminatory depletion of residual
endogenous TCR (eTCR)-expressing cells was carried out (day 11), before cryopreservation
on day 14. D) Representative flow cytometry phenotyping of unmodified and Terminal-TRBC-S183-91 rTCR (TTRBC-S183-91 rTCR) transduced cells. Delivery of coBE3 mRNA by electroporation caused reduction of eTCR expression (38.1%) and emergence of eTCR-/rTCR+ cells (Red box). Magnetic bead mediated depletion of residual eTCR+ T cells enriched eTCR- populations, resulting in >99% eTCR-/ 95.9% rTCR+ (gated on CD45+). E) Expression of S183-91 rTCR in three healthy donors. i) Histogram of rTCR (183-91) expression exhibiting transduction ranging from 59.8%-63.9% in cells exposed to both vector and BE3, which following TCRαβ bead-mediated depletion resulted in enrichment of genome edited cells, with rTCR levels increased to 93.9%-96.1%. Three colours represent different donors. ii) Levels of cell surface rTCR expression measured by mean fluorescence intensity (MFI) (n=3) showed increased eTCR-/rTCR+ compared to eTCR+/rTCR± cells (gated on CD45+>rTCR+ population). One way ANOVA with Tukey’s multiple comparison test, \( p < 0.02 \), error bars ± 1 standard error of the mean (SEM). nCas9: nickase CRISPR associated protein 9, UGI: uracil DNA glycosylase inhibitor, rAPOBEC1: rat apolipoprotein B mRNA editing enzyme catalytic polypeptide 1.

Figure 3: Anti-HBV responsiveness of eTCR+/rTCR±, compared to base edited eTCR-/rTCR+ effector T cells. A) Cytokine responses of effector T cells to HepG2 cell line pulsed with target HBV surface peptide (S183-91, FLLTRILTI) n=3. i) Histograms of tumour necrosis factor α (TNFα) responses to HepG2 target cells pulsed with 1µM target peptide. Both eTCR+/rTCR± and eTCR-/rTCR+ effector groups are gated on CD45+>CD3+>rTCR+>CD8+, whereas unmodified eTCR+/rTCR- effectors are gated on CD45+>CD3+>rTCR->CD8+. Three different colours represent results from three donors. ii) Cytokine responsiveness at different concentrations of target peptide (S183-91). HepG2 target cells were pulsed with
1µM of control peptide (C18-27) to ensure specificity of response, showing comparable cytokine responsiveness to the no peptide control. Effector groups eTCR+/rTCR± and eTCR+/rTCR+ are gated on CD45+>CD3+>rTCR+>CD8+; whereas unmodified cells are gated on CD45+>CD3+>rTCR->CD8+. Error bars ± 1 SEM. **B)** Schematic depiction of XCelligence impedance assay showing cancer cells (green) seeded in wells with micro electrode array (yellow), in the presence of effector T cells (blue). Where T cells recognise cancer cells, this leads to cell death (brown) and reduced impedance resulting in lower cell index values, and area under the curve (AUC). **C)** XCelligence data across different effector: target (E:T) ratios (1:1, 1:2, and 1:4). (i) Visualisation of normalised cell index (NCI) over time, all donors showed increased NCI with decreased E:T ratio. Both HepG2 alone (red), and eTCR+/rTCR- (orange) show steadily increasing NCI over time. Whereas eTCR+/rTCR± (purple) and eTCR-/rTCR+ (green) groups show an initially increased NCI, followed by a marked decline. Normalised to time point prior to effector T cell addition. (ii) Summary data of AUC. Increased AUC values were observed at the lower E:T ratios, with eTCR-/rTCR+ consistently presenting with the lowest AUC values. Error bars ± 1 SEM. IFNγ: Interferon-γ, IL-2: interleukin-2, CCL4: C-C motif chemokine ligand 4.
and 25th/75th quartiles (dotted black lines). PreS1-GFP-HepG2 alone (orange) and 20% DMSO (red) were used as negative and positive controls respectively. Increased cytotoxicity was observed with eTCR-/rTCR+ (purple) effectors, compared to PreS1-GFP-HepG2 alone ($p < 0.0001$), eTCR+/rTCR- (green, $p < 0.002$), and eTCR+/rTCR± (blue, $p = 0.0001$). Each point represents a section of a 3D microfluidics device from n=3 technical replicates (3 sections analysed per device). One way ANOVA with Tukey’s multiple comparison test.

Visualisation of a region within the collagen gel. Addition of 20% DMSO resulted in cell death (red), while PreS1-GFP-HepG2 target cells alone resulted in high viability (green). Addition of effector T cells (blue), resulted in different degrees of target cell killing between different effector groups (scale bar= 100µm).

**Figure 5: Molecular analysis of on-/off-target DNA editing, and fidelity of CDR3α and β regions within rTCR mRNA transcripts.**

**A)** Sanger sequencing of on-target editing at TRBC 1/2 loci in eTCR-/rTCR+ cells. i) Representative EditR analysis with wild type sequence (top) and four possible bases (side) shown at each position. Target G>A conversions (red box) generate a premature stop codon (Trp>). ii) Summary of EditR data for three donors at cytosine positions 5 and 6 distal to the PAM, presented as C>T changes (black), non C>T changes (grey) and no editing (white). Error bars ± 1 SEM. iii) NGS sequencing analysis of on-target editing of TRBC 1/2 loci, quantification and characterisation of indels after BE3 editing found only low levels of small (<10bp, black) or large indels (10-100bp, grey) with the majority of reads presenting with no indels (white). Error bars ± 1 SEM.

**B)** Box plots showing off-target editing detected by NGS analysis at the top 6 in silico predicted off-target sites for the TRBC 1/2 sgRNA, with comparison of unedited eTCR+/rTCR- and edited eTCR-/rTCR+ groups (n=3). Larger dots represent outliers, in all cases ≤1.3% conversion. Two-tailed
independent t-test between unmodified (eTCR+/rTCR-), and edited (eTCR-/rTCR+) samples shown for donor 1 \( p > 0.5 \), donor 2 \( p = 0.001 \), and donor 3 \( p > 0.1 \). C) Serial examination of RNA from rTCR HBs183-91 for 8 days post coBE3 mRNA delivery (days 5-12 post activation) found no evidence of promiscuous deamination, with fidelity of CDR3\( \alpha \) and CDR3\( \beta \) regions maintained. Amplicon positions are marked above for C residues and schematic highlights hypervariable CDR3\( \alpha \) and CDR3\( \beta \) regions that confer HLA-peptide specificity. CDR3 regions were mapped as a Heatmap in R using the gplots library for C>T conversion rates at the marked sites (TCR Clone software: TCRmodel\(^{66}\)).
The authors deploy cytosine deamination mediated base editing to genetically disrupt endogenous T cell receptors (eTCR), thereby reduce competition with recombinant TCRs (rTCR) and allowing enrichment of engineered T cells for immunotherapy against Hepatitis B driven hepatocellular carcinoma.
