APOBEC3B expression in 293T viral producer cells drives mutations in chimeric antigen receptors and reduces CAR T cell efficacy

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Chimeric antigen receptor (CAR) T cells are a clinically approved therapy for blood cancers. To produce clinical-grade CAR T cells, a retroviral or lentiviral vector is used to deliver the CAR and associated genes to patient T cells. Apolipoprotein B editing enzyme, catalytic polypeptide 3 (APOBEC3) enzymes are known to be upregulated after transfection and retroviral infection and to deaminate cytidine to uracil in nucleic acids, resulting in cytidine-to-thymine mutations in DNA. Here, we hypothesized that APOBEC3 enzymes, induced during the production of CAR T cells, impact the efficacy of the resulting CAR T cells. We demonstrated that APOBEC3 family member APOBEC3B was upregulated at the RNA and protein levels after transfection of HEK293T cells with plasmids to make lentivirus, and that APOBEC3 signature mutations were present in the CAR construct. APOBEC3B overexpression in HEK293T cells led to further mutations in the resulting CAR T cells, and significantly decreased CAR T cell killing. APOBEC3B knockout in HEK293T cells led to reduced mutations in the CAR construct and significantly increased in CAR T cell killing. These results suggest that generation of CAR-expressing viruses from producer cell lines deficient in genome-modifying proteins such as APOBEC3B could enhance the quality of CAR T cell production.

INTRODUCTION

Chimeric antigen receptor (CAR) T cells are a clinically approved therapy for human blood cancers, including leukemias, lymphomas, and multiple myeloma. In clinically approved CAR T cell therapies, autologous T cells are isolated from patient blood and transduced with a retrovirus or lentivirus encoding an antigen binding region, spacer, and transmembrane and T cell signaling domains, then reintroduced into the patient (reviewed by Sterner and Sterner, 2021).[1,2] In the decade since the first CAR T cell clinical trials, long-term complete responses have been achieved in a majority of patients with B cell lymphomas (reviewed by Cappell and Kochenderfer, 2023).[2] Despite the successes of CAR T cells in blood cancers, CAR T cell clinical trials in solid tumors have been less successful due to factors such as the immunosuppressive microenvironment, lack of tumor antigens, lack of T cell persistence, and lack of T cell ability to infiltrate into the tumor (reviewed by Wagner et al., 2020).[3] Therefore, to optimize CAR T cell therapies in both hematological and solid tumors, it is important to improve the efficiency and efficacy of CAR T cell therapies. In this respect, we hypothesized here that the quality of the CAR might be affected by the process in which the CAR is generated *ex vivo* due to the activation of cellular mutator pathways, such as apolipoprotein B editing complex proteins (APOBECs).

APOBECs are a family of cytidine deaminase proteins which drive cytidine to uracil (C-U) mutations, which manifest as cytidine to thymine (C-T) mutations in DNA.[4] The members of the APOBEC family includes AID, APOBEC1, APOBEC2, APOBEC3A-3G (7 family members), and APOBEC4 (reviewed by Salter et al., 2016).[4] A well-described function of APOBEC3 enzymes, including human APOBEC3B[5] and APOBEC3G,[6] is restriction of retroviruses, including HIV.[7,8] APOBEC3A, APOBEC3B, APOBEC3C, APOBEC3D, APOBEC3F, and APOBEC3H drive mutations that typically manifest as a 5′ TC 3′ phenotype, where a thymine (T) base is upstream of the mutated cytidine (C). In contrast, APOBEC3G mutations typically manifest as 5′ CC, where a C is upstream of the mutated C (reviewed by Harris and Dudley, 2015).[8] APOBEC3-mediated mutations have been shown to preferentially occur on the lagging, or minus strand, of DNA, leading to guanine to adenine mutations (G-A) being observed upon sequencing of the forward strand of APOBEC-mutated targets.[9–11] APOBEC3 enzyme transcripts have been shown to be upregulated upon interferon (IFN) stimulation in hepatocytes, dendritic cells, and peripheral blood mononuclear cells[12–14]; upon stimulation with single-stranded CpG DNA, a known TLR9 ligand[15]; upon HIV lentiviral transduction[16]; and upon genotoxic stress.[17] APOBEC3B and APOBEC3 signature mutations have also been shown to be upregulated in human cancers.[18–21]

We have previously shown that expression of human APOBEC3B drives resistance to several tumor immunotherapies. One such
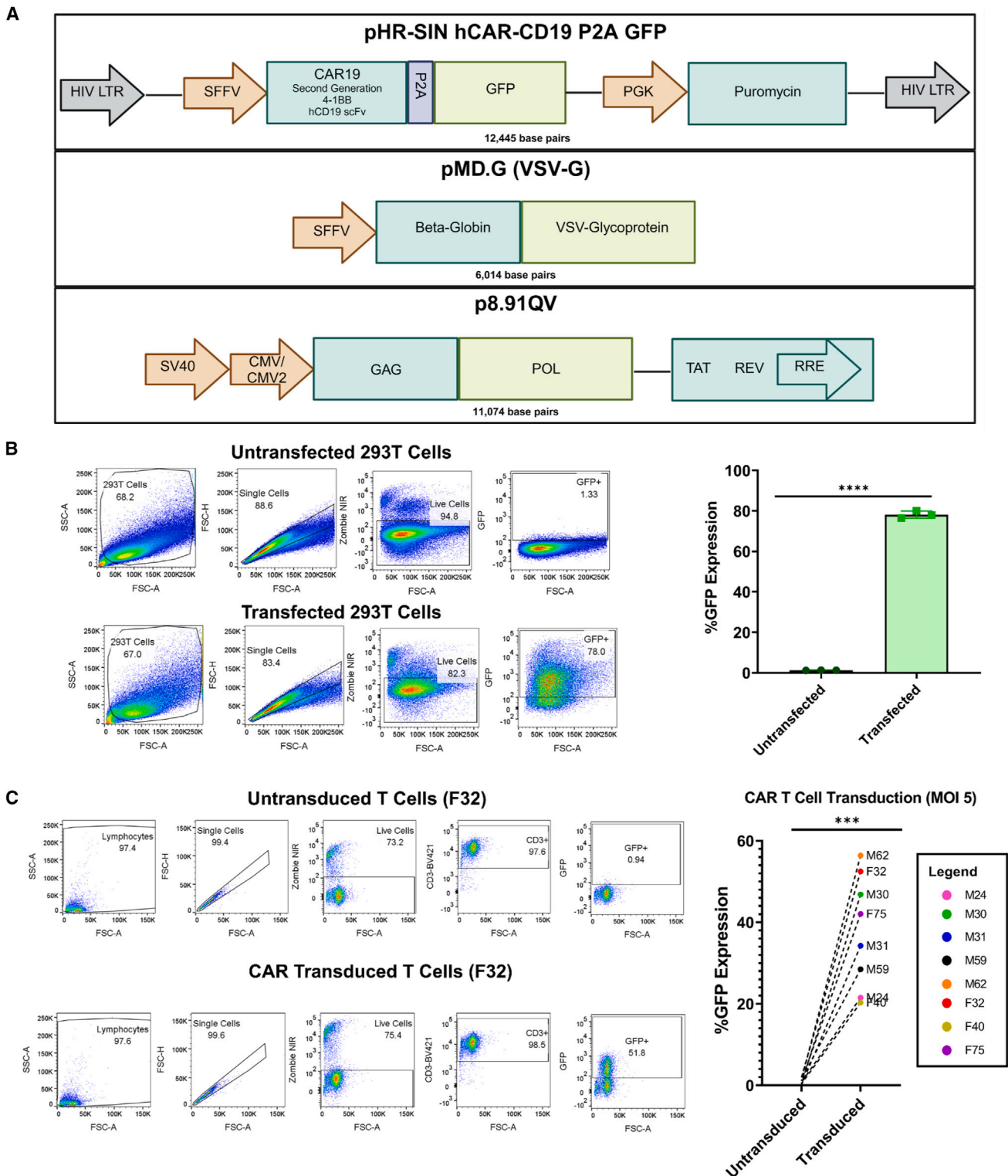
Figure 1. Process for production of virus and transduction of CAR T cells

(A) Diagram of plasmids used to transfect 293T cells to make lentivirus for CAR T cell transduction. (B) Representative flow cytometry gaiting for 293T cells, single cells, live cells (Zombie NIR), and GFP, showing GFP expression in 293T cells at the point of viral harvest (3 days after transfection). A two-tailed unpaired t test ($p < 0.05$) was performed

(legend continued on next page)

therapy that APOBEC3B mediates escape from and resistance to is oncolytic vesicular stomatitis virus (VSV) therapy,[22] which is mediated by a mutation in the RNA-binding cold-shock domain containing E1 protein, a regulator of RNA translation.[23] Furthermore, we have shown that human APOBEC3B expression in melanoma tumors expressing thymidine kinase drives escape from ganciclovir.[24] However, we have also shown that APOBEC3B can drive the generation of heteroclitic neoepitopes for T cell recognition and increased sensitivity to PD1 checkpoint blockade in murine tumors.[25]

Because many clinically approved CAR T cell therapies make use of retroviral and lentiviral vectors that induce anti-viral responses, we hypothesized that the mutagenic activity of APOBEC3 enzymes would reduce the efficiency of CAR T cell production and the overall quality of the CAR T cell product. Our data here suggest that targeting APOBEC3B in producer cells used to generate CAR-encoding viral vectors could prevent mutations in the CAR T cell production process and increase the quality of the final CAR T cell product.

RESULTS

Production of second-generation 4-1BB CAR T cells targeting human CD19

After transfection of 293T producer cells by constructs to produce lentivirus for second generation 4-1BB CAR T cell production (Figure 1A), between 75% and 100% of 293T cells were positive for GFP (Figure 1B) 3 days after transfection. GFP was visibly expressed between 8 and 24 h after 293T transfection by constructs to produce lentivirus for CAR T cells production, and GFP expression increased between 24 and 48 h after transfection, and between 48 and 72 h after transfection (Figure S1). T cells from both male and female donors ranging in age from 24 to 75, transduced by the resulting lentivirus, typically yielded between 25% and 60% transduction efficiency (Figure 1C) as measured by GFP expression (downstream of the single-chain variable fragment and signaling domain).

APOBEC3 signature mutations and APOBEC3B upregulation are present in 293T cells after transfection with plasmids to make lentivirus

To determine whether high-frequency mutations (mutations occurring in >50% of sequenced reads) were present in 293T cells after transfection with plasmids used to generate the CAR lentivirus stock, or in CAR T cells after transduction of CAR lentivirus into T cells from a 32-year-old female donor, PCR was run for a fragment of the GFP gene in the CAR construct. PCR, followed by standard Sanger sequencing, of the GFP gene did not detect any dominant mutations in either the transfected 293T cells or in the human CD19 CAR T cells (Figure S2). To determine if lower frequency mutations

were present in 293T cells after transfection and in CAR T cells from a 32-year-old female donor following transduction, the PCR product of a fragment of the single chain variable fragment of the human CD19 CAR was cloned into a topoisomerase vector. Sanger sequencing indicated progressively increasing accumulation of mutations as CAR T cell production progressed from the parental CAR plasmid, through viral production in 293T cells, to transduction of CAR T cells, including guanine to adenine (G-to-A) and cytosine to thymine (C-to-T) mutations characteristic of an APOBEC3 signature (Table S1).

With further investigation of the low-frequency mutations observed in the CAR T cell production process, differential DNA denaturing (3D)-PCR (Figure 2A) clearly showed that lower frequency mutations were present in the GFP gene after plasmid transfection into 293T cells (Figure 2B). A 1°–2° decrease in the denaturing temperature required for PCR to be successful was observed in the GFP PCR product from the transfected 293T cells 48 and 72 h after transfection, indicating an increase in the proportion of A and T bases (Figure 2B). Sanger sequencing of the 91°C 3D-PCR product from the pHR-SIN hCAR-CD19 P2A GFP plasmid, and the 90°C 3D-PCR product from the 293T cells 72 h post transfection showed exclusively G-to-A mutations in the transfected plasmid, suggestive of APOBEC3 (C→T)-mediated mutations in the DNA lagging strand (Table S2). Nearly all the observed mutations were of the mutational signature preferred by APOBECA, APOBEC3B, APOBEC3C, APOBEC3D, APOBEC3F, and APOBEC3H, in which a G base is mutated to A, with an A base immediately downstream of the mutated base (C to T, with a T base immediately upstream of the mutated base in the leading strand) (Table 1).

Although no differences in APOBEC3 mRNA levels were observed in the 293T cells at 6 and 24 h after transfection, transcript levels of APOBEC3B and APOBEC3F were significantly enhanced 48 and 72 h after transfection (Figures 2C and S3). At 72 h after transfection, transcript levels of APOBEC3C and APOBEC3G were also significantly enhanced (Figure S3). Western blot showed that APOBEC3B, but not APOBEC3A or APOBEC3G, increased 24 h after transfection and further increased in magnitude 48 and 72 h after transfection (Figure 2D). Taken together, these data show APOBEC3 signature mutations were present in the CAR vector after transfection into 293T cells, coupled with an observed upregulation of APOBEC3B.

APOBEC3B proteins are present in CAR lentiviral particles

To investigate whether APOBEC3B was incorporated into lentiviral particles from the producer cells, lentivirus was produced from parental 293T cells or from 293T cells over-expressing APOBEC3B (Figures 3A and 3B). APOBEC3B was detected at low levels in

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to compare untransfected and transfected samples. (C) Representative flow cytometry gaiting for lymphocytes, single cells, live cells, CD3, and GFP, in T cells from a 32-year-old female donor showing GFP expression in human CAR T cells transduced with lentivirus at a volume of 30 μL at the point of CAR T cell harvest (day 10 of CAR T cell preparation) (left), transduction of eight representative donors with a volume of 30 μL at the point of CAR T cell harvest (day 10 of CAR T cell preparation) (right). A two-tailed paired t test ($p < 0.05$) was performed to compare untransduced and transduced samples. ****, $p < 0.0001$; ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$; ns, not significant.
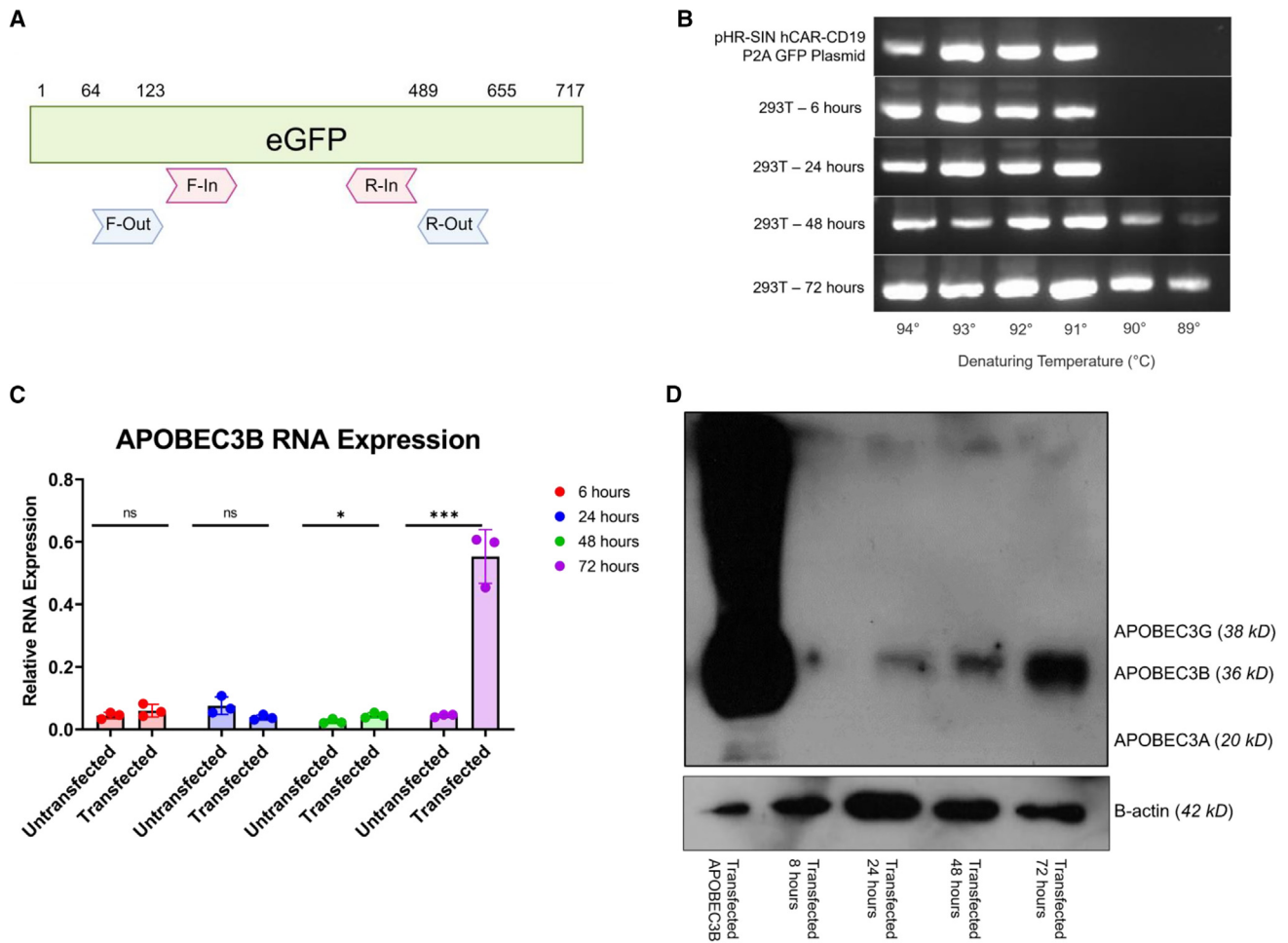
Figure 2. APOBEC3B upregulation in 293T cells after transfection with plasmids to make lentivirus

(A) Schematic of primer binding regions in target DNA. Primers for the first (outside, 5′GTAAACGGCCACAAGTTCAG3′ - forward and 5′CCATGTGATCGCGCTTCT3′ - reverse) PCR are in blue, and primers for the second (inside, 3D primers, 5′GCTGACCCTGAAGTTCATCTG3′ - forward and 5′CACCTTGATGCCGTTCTTCT3′ - reverse) PCR are in red. (B) 3D-PCR for plasmid DNA, and DNA from transfected 293T cells 6, 24, 48, and 72 h after transfection of plasmids to make lentivirus with denaturing temperatures of 94°, 93°, 92°, 91°, 90°, and 89°C. (C) RT-qPCR for APOBEC3B 6, 24, and 48 and 72 h after transfection of pMD.G (VSV-G), p8.91QV (*gag/pol*), and pHR SIN (hCAR-CD19 P2A GFP) plasmids into 293T cells to make lentivirus. A two-tailed unpaired t test ($p < 0.05$) was used for statistical comparison at each time point. (D) Western blot for APOBEC3B overexpressing cells, and APOBEC3B 8, 24, 48, and 72 h after transfection of pMD.G (VSV-G), p8.91QV (*gag/pol*), and pHR SIN (hCAR-CD19 P2A GFP) plasmids into 293T cells to make lentivirus. ****, $p < 0.0001$; ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$; ns, not signficant.

lentiviral particles produced from parental 293T cells, and at high levels in lentiviral particles produced from APOBEC3B-overexpressing 293 T cells (Figure 3C). In this study, lentivirus was purified by filtering virus through a 0.45-μm filter to remove cells, followed by a high-speed centrifugation. To maximize viral purification, virus was filtered through a 0.45-μm filter, and a sucrose gradient was performed.[26] APOBEC3B and VSV-G were present in viral lysates purified with a 0.45-μm filter and a high-speed centrifugation. However, beta-actin, a cytoskeletal component, was also present in viral lysates purified using this method. In viral lysates purified by filtration through a 0.45-μm filter, followed by a sucrose gradient, APOBEC3B and VSV-G, but not beta-actin were detected, confirming that APOBEC3B is present in viral particles (Figure 3D). Consistent

with the carryover of APOBEC3B protein into released lentiviral particles, 3D-PCR of the GFP gene showed that extensive APOBEC3B signature mutations were present in RNA from the lentiviral particles, and that the minimum denaturing temperature required for successful PCR was lower in the lentiviral cDNA than the CAR T cell DNA (Figure 3E). However, 3D-PCR products from lentiviral particles were unable to be Sanger sequenced with confidence due to high background in the sequenced PCR products (Figure S4). Taken together, these data show that APOBEC3B derived from the 293T producer cells was incorporated into CAR lentiviral particles and that extensive mutations were present in the viral particles suggesting that APOBEC, or other mutagenic activities, were operative within the viral particles.

Table 1. Signature of mutations detected in 3D-PCR of GFP in 293T cells after transfection

Downstream base G*X*	Mutations observed
G*G*	1/17
G*A*	16/17
G*T*	0/17
G*C*	0/17

The underlined base represents the base being mutated. The base in *italics* represents the base downstream of the mutated base.

APOBEC3 signature mutations are present in transduced CAR T cells

CAR T cells generated from a 32-year-old female donor contained exclusively G-to-A mutations characteristic of APOBEC3 signatures in the GFP gene of the CAR vector by Sanger sequencing of 3D-PCR products. The majority of the mutations in the CAR vector in the CAR T cell product were the same as those detected in the 293T cells used to generate the CAR vector (Figure 4A; Table 2). In many cases we observed that a dominant mutation in the CAR vector was present at sub-dominant levels in the 293T producer cells (Figure 4B). In addition, a subset of mutations was found to be common across different donors produced from multiple different viral preps (Tables S3 and S4), indicating some common mutational targets exist within the CAR vector during the process of CAR T cell generation.

No differences in APOBEC3 transcript levels, including APOBEC3B, were observed 8, 24, or 48 h after lentiviral transduction in CAR T cells from a 24-year-old male donor (Figure S5). An increase in APOBEC3B transcripts 24 h after transduction in CAR T cells from a 40-year-old female was observed, but no other increases in APOBEC3 levels were present at any other time point (Figure S6). Western blot showed detectable levels of an APOBEC3 species, likely APOBEC3G, at all timepoints in T cells, but no observable differences in the APOBEC3 species in the CAR T cells of the 24-year-old male or the 40-year-old female donors were evident via Western blot (Figures 4C and 4D). A lower molecular weight APOBEC3 species, likely APOBEC3A, was also present at high levels in the M24 donor, but not the M40 donor (Figures 4C and 4D). Analysis of the purified CD3 T cell cultures by flow cytometry indicated that up to 10% of cells may have been CD3-negative depending on the donor, so there is a possibility that some of the APOBEC3A detected in these experiments was not necessarily T cell associated. Therefore, these data suggest that human T cells often express relatively high levels of APOBEC enzymes, that transduction with CAR lentiviral vectors does not greatly impact these levels, and that the mutagenic consequences on the incoming CAR vector while appreciable are not highly significant. Taken together, these data show that the majority of the G-to-A mutations in the CAR vector which are observed in the final CAR T cell product are seeded by mutagenic activity of APOBEC3B and possibly other mutagens in the producer 293T cells, that the majority of those mutations are reproducibly carried into the CAR T cells

via the lentivirus vector, and that some become increasingly dominant in the CAR T cells. However, a large majority of mutations induced within the virus itself seem to be incompatible with infection/transduction and are not represented in the final CAR vector.

APOBEC3B overexpression in 293T cells correlates with reduced CAR T cell function

Lentivirus produced from APOBEC3B-overexpressing 293T cells had a significantly lower titer than virus produced from parental 293T cells (Figures S7A and S7B). Because it was possible that mutations introduced into the GFP gene in the CAR would artificially decrease the apparent titer of virus produced from APOBEC3B overexpressing 293 T cells, human T cells were transduced with an equivalent volume of virus, rather than an equivalent multiplicity of infection (MOI) of virus. GFP expression levels were consistently lower in the CAR T cells transduced with virus produced from the APOBEC3B overexpressing 293T cells compared with those transduced with virus produced from the parental 293T cells (Figure 5A).

DNA harvested from human CAR T cells transduced with virus from APOBEC3B-overexpressing 293T cells contained additional mutations in the GFP gene compared with CAR T cells generated by transduction with virus produced from parental 293T cells (Figure 5B) (Table S5). Consistent with this, IFN-γ levels were significantly lower 24 h after CAR T cell challenge of human melanoma cells overexpressing CD19 (MEL888-CD19) when the CAR T cells were prepared with virus produced from APOBEC3B-overexpressed 293T cells. (Figure 5C). In addition, significantly more tumor cells survived 5 days after CAR T cell challenge when the CAR T cells were produced from APOBEC3B-overexpressing 293T producer cells. (Figure 5D). To confirm the effects were not solely due to the reduced transduction efficiency described in Figure 5A, human T cells were transduced with an equivalent MOI of virus produced from either parental 293T cells or 293T cells overexpressing APOBEC3B (Figure 5E). Once again, significantly more tumor cells survived 5 days after CAR T cell challenge in the group challenged with CAR T cells produced from lentivirus from APOBEC3B overexpressing 293T cells (Figure 5F). Taken together, these data show that APOBEC3B expression in the lentiviral producer cells is associated with both an increased level of mutation within the CAR vector, and a corresponding reduced functional activity of the CAR T cells.

APOBEC3B knockout in 293T cells correlates with improved CAR T cell function

APOBEC3B knockout in 293T cells using CRISPR-Cas9 was validated following stimulation with either the APOBEC3B agonist phorbol-myristic acid (PMA)[27] or lentiviral transduction (Figure 6A). Lentivirus produced from either parental 293T cells or APOBEC3B knockout 293T cells had similar titers (Figure S8). When human T cells were transduced with an equivalent volume of lentivirus from either the parental 293T cells or the APOBEC3B knockout 293T cells, no significant difference in GFP expression was observed (Figure 6B, left). From all of the donors transduced, CAR T cells
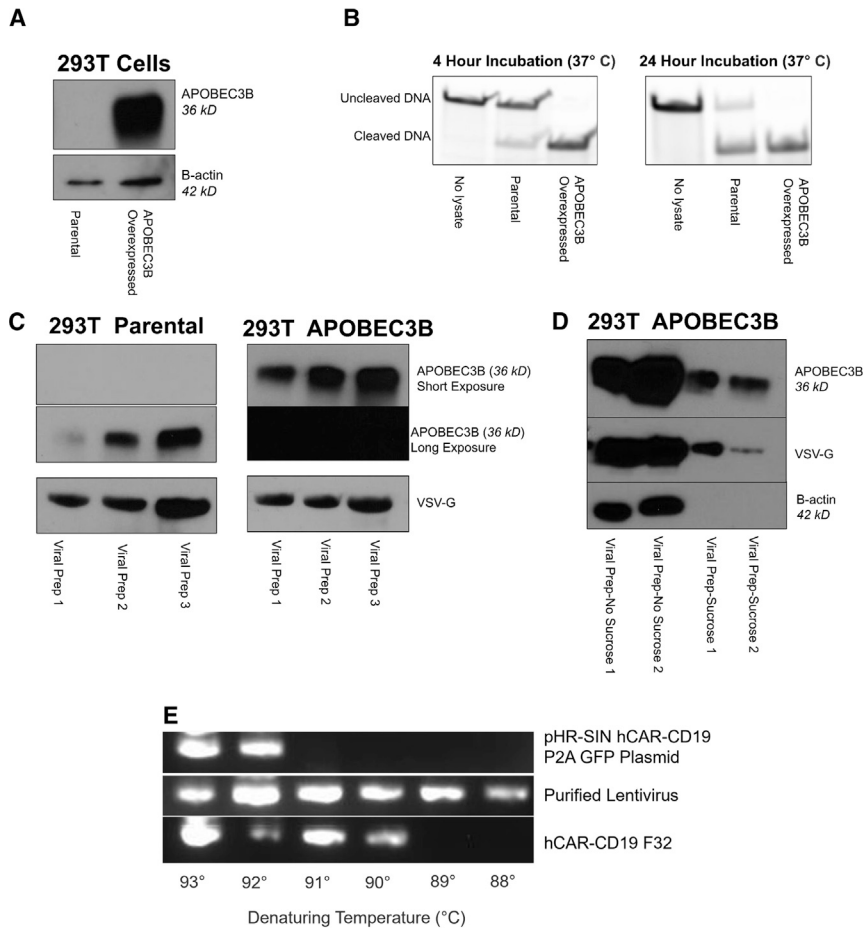
Figure 3. APOBEC3B proteins are present in CAR lentiviral particles

(A) Western blot showing APOBEC3B expression in parental 293T cells cultured in DMEM + 10% FBS and in 293T cells stably overexpressing APOBEC3B via lentiviral vector, cultured in DMEM + 10% FBS + 1.25 mg/mL puromycin. Beta-actin is used as a loading control. (B) APOBEC3 activity assay using lysate from parental 293T cells cultured in DMEM + 10% FBS and 293T cells stably overexpressing APOBEC3B via lentiviral vector, cultured in DMEM + 10% FBS + 1.25 mg/mL puromycin. Samples were incubated at 37°C for either 4 h or 24 h. (C) Western blot showing APOBEC3B expression in purified lentiviral particles harvested 3 days after transfection of pMD.G (VSV-G), p8.91QV (*gag/pol*), and pHR-SIN (hCAR-CD19 P2A GFP) plasmids. VSV-G is used as a loading control. (D) Western blot showing APOBEC3B, VSV-G, and beta-actin expression in purified lentiviral particles, purified using centrifugation (lanes 1 and 2), and centrifugation plus sucrose gradient (lanes 3 and 4) harvested 3 days after transfection of pMD.G (VSV-G), p8.91QV (*gag/pol*), and pHR-SIN (hCAR-CD19 P2A GFP) plasmids. (E) 3D-PCR in the GFP gene for plasmid DNA, cDNA from purified lentivirus harvested 3 days after transfection, and DNA isolated from human CD19 CAR T cells harvested 10 days after transduction of lentivirus, with denaturing temperatures of lentivirus with denaturing temperatures of 93°C, 92°C, 91°C, 90°C, 89°C, and 88°C.

produced with virus from APOBEC3B knockout 293T cells had reduced mutations compared with the CAR T cells produced with virus from parental 293T cells using either the equivalent MOI or the equivalent volume of virus (Figure 6C; Table S6). IFN-γ levels were significantly higher 24 h after CAR T cell challenge from CAR T cells produced with lentivirus from APOBEC3B knockout 293T producer cells (Figure 6D). Significantly fewer tumor cells survived 5 days after CAR T cell challenge (Figure 6E), from CAR T cells generated from APOBEC3B knockout 293T producer cells compared with CAR T cells generated from parental 293T cells.

Finally, human T cells were transduced with an equivalent MOI of lentivirus produced in either the parental 293T cells or the APOBEC3B knockout 293T cells. A slight, but significant, difference in GFP expression was observed between the CAR T cells transduced with virus from parental 293T cells and the CAR T cells transduced with virus from APOBEC3B knockout 293T cells (Figure 6B, right). IFN-γ levels were again significantly higher 24 h after CAR T cell challenge in the group challenged with CAR T cells produced with lentivirus from APOBEC3B knockout 293T cells. (Figure 6F). Significantly fewer tumor cells survived 5 days after CAR T cell challenge in the group challenged with CAR T cells from APOBEC3B knockout

293T cells (Figure 6G). Taken together, these data show that APOBEC3B knockout in the lentiviral producer cells was associated with both a decreased mutational load, and increased functional activity of the resultant CAR T cell product.

DISCUSSION

Here, we demonstrate that APOBEC3B drives mutations during CAR T cell production and impacts the overall quality of the resulting CAR T cells. This work has the potential to impact and improve already approved CAR T cell therapies for blood cancers, CAR T cells for solid tumors, and CAR T cell combination therapies. We show that the levels of APOBEC3B were elevated upon transfection of 293T cells with the plasmids used to make self-inactivating CAR-encoding lentivirus. Induction of APOBEC3B was associated with mutations in the CAR vector. Most of the same mutations, as well as a few additional mutations, were also present in the CAR T cells after transduction with lentivirus, although viral transduction of the T cells was not associated with significant changes in levels of activation of APOBEC3B or any human APOBEC3 protein. Decreased killing and IFN-γ production upon recognition of target cells were observed with CAR T cells produced from APOBEC3B-overexpressing 293T cells compared with CAR T cells produced from parental 293T cells. In contrast, increased killing and IFN-γ production were observed with CAR T cells produced from APOBEC3B knockout 293T cells.
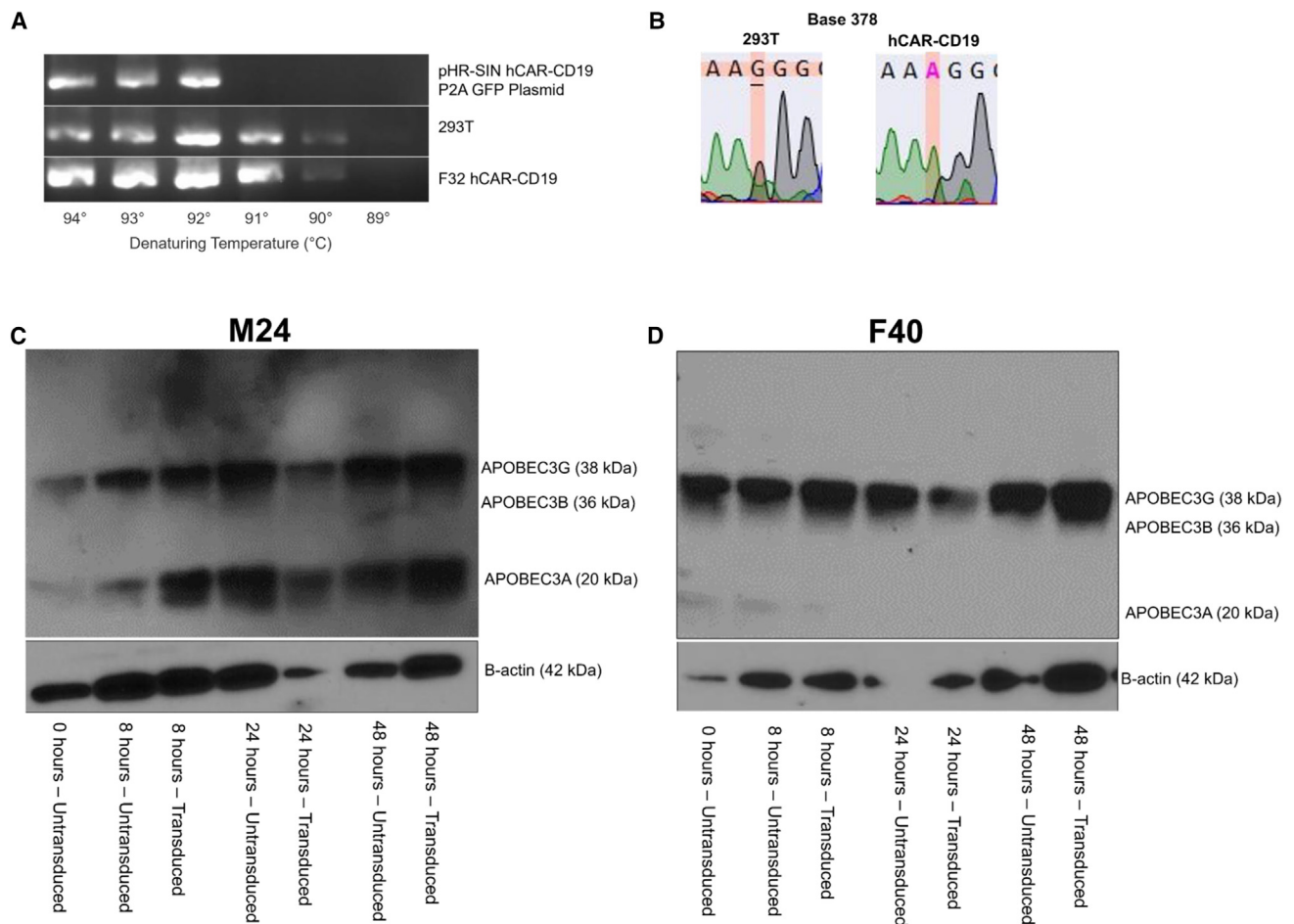
Figure 4. APOBEC3 signature mutations with little change in APOBEC3B levels are present in transduced CAR T cells

(A) 3D-PCR in the GFP gene for plasmid DNA. DNA was isolated from 293T cells harvested 3 days after transfection of pMD.G (VSV-G), p8.91QV (*gag/pol*), and pHR-SIN (hCAR-CD19 P2A GFP) plasmids and from F32 CD19 CAR T cells harvested 10 days after transduction of lentivirus. (B) Sample Sanger sequencing, showing an example of a non-dominant adenine peak (G dominant, A non-dominant) at base 378 in the GFP sequence in DNA isolated from 293T cells 3 days after transfection with pMD.G (VSV-G), p8.91QV (*gag/pol*), and pHR-SIN (hCAR-CD19 P2A GFP) plasmids, and a dominant adenine peak (G non-dominant, A dominant) in F32 CD19 CAR T cells harvested 10 days after transduction of lentivirus into 32-year-old female donor T cells. (C) Western blot showing APOBEC3A, APOBEC3B, and APOBEC3G expression in T cells from a 24-year-old male donor harvested at the time of transduction (untransduced), 8 h after transduction (transduced and untransduced), 24 h after transduction (transduced and untransduced), and 48 h after transduction (transduced and untransduced). (D) Western blot showing APOBEC3A, APOBEC3B, and APOBEC3G expression in T cells from a 40-year-old female donor harvested at the time of transduction (untransduced), 8 h after transduction (transduced and untransduced), 24 h after transduction (transduced and untransduced), and 48 h after transduction (transduced and untransduced).

To our knowledge, this study is one of the first to show the role of transfection-induced APOBEC3 on the quality of a CAR T cell product. Several recent studies highlight a role for APOBEC3B and other APOBEC3 enzymes in clearing foreign DNA upon transfection into cells. APOBEC3A has been shown to deaminate foreign DNA and reduce the levels of foreign DNA plasmid levels.[15] Overexpressed APOBEC3A and APOBEC3B in 293T cells correlated with decreased expression of a transfected plasmid in 293T cells.[28] APOBEC3G has been shown to clear CRISPR plasmid DNA upon transfection into keratinocytes.[29] Our data, therefore, expand on previous studies by showing that APOBEC3B induction upon transfection of foreign DNA mutates lentiviral vectors, which can poten-

tially lead to a significant inhibition of the quality of the resultant CAR cell product.

We used a three-plasmid system encoding the pVSV-G glycoprotein envelope, *gag* and *pol*, and the CAR, also encoding GFP, to produce CAR T cells (Figure 1).[30] GFP, downstream of the single chain variable fragment region, was highly expressed at the time of harvest of virus for concentration in the 293T cells (80% transfection in 293T cells), but was expressed at different levels in the transduced CAR T cells depending on the donor (between 20% and 60%), possibly due to variable levels of expression of the low-density lipoprotein receptor for VSV-G on different populations of T cells.[31] We show that

Table 2. Comparison of mutations detected in 3D-PCR of GFP in 293T cells and human CAR T cells

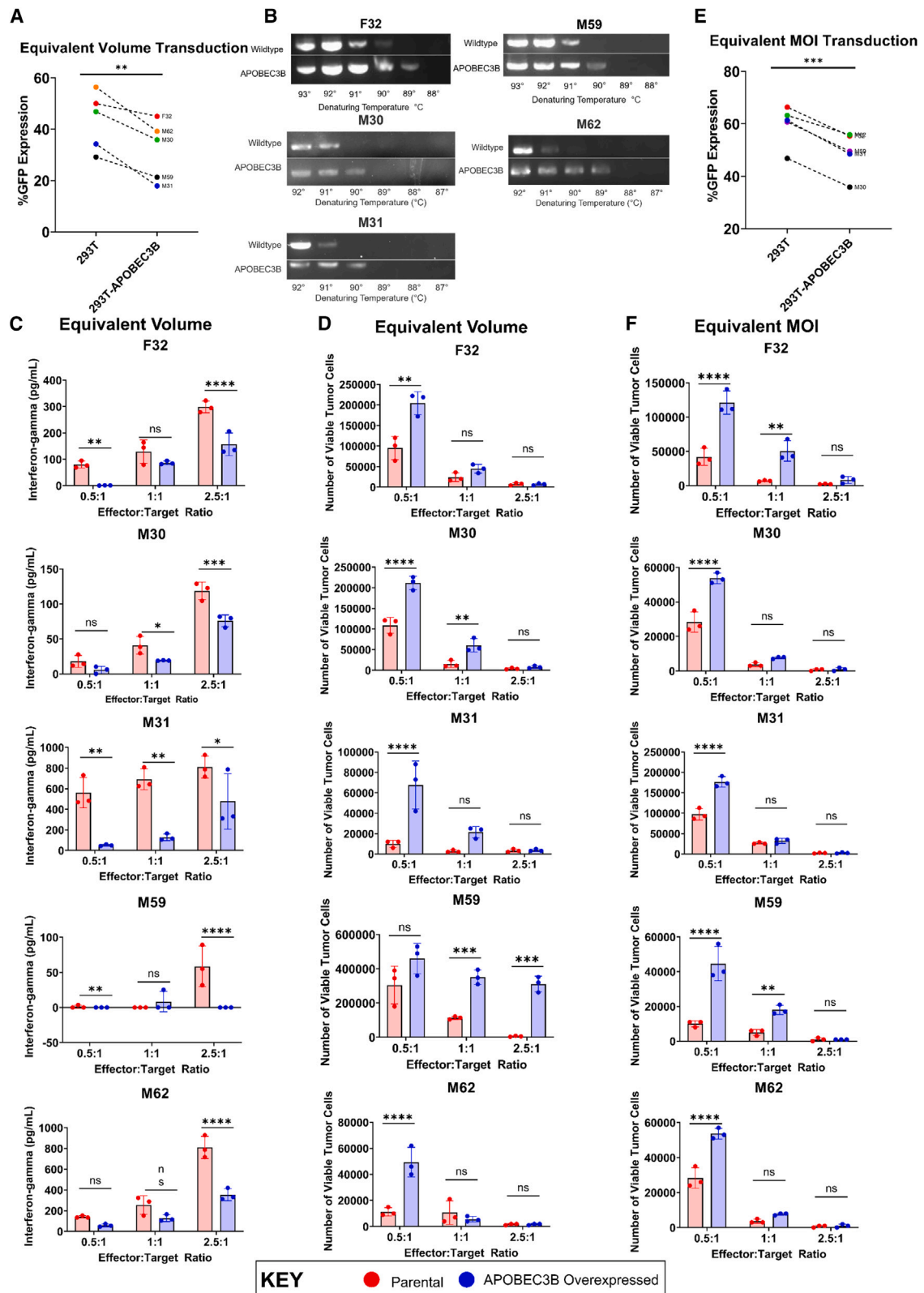| 293T cells			F32 hCAR-CD19 cells		
Nucleotide position	Parental base	Mutated base	Nucleotide position	Parental base	Mutated base
192	G	A	192	G	A
226	G	A	226	G	A
234	G	A	234	G	A
244 ■	G	A	268	G	A
268	G	A	282	G	A
282	G	A	304*	G	A
336	G	A	307*	G	A
343	G	A	321*	G	A
360	G	A	336	G	A
370	G	A	343	G	A
375	G	A	349	G	A
385	G	A	360	G	A
424	G	A	370	G	A
463 ■	G	A	375	G	A
			378*	G	A
			385	G	A
			423	G	A
			424	G	A

Squares (■) represent mutations that are dominant in the 293T sequencing, but not the CAR T cell sequencing. Asterisks (*) represent mutations that are dominant in the CAR T cell sequencing, but not the 293T sequencing.

mutations were induced in the CAR vector within 48 h of transfection of the plasmids into 293T cells (Figure 2; Table S2). Deaminations by APOBEC3 enzymes, including APOBEC3B, have previously been shown to be favored on the lagging strand during DNA replication in both tumor and yeast models. Therefore, the G-to-A mutations that we observed are consistent with a model in which APOBEC3B is active after producer cell transfection.[9,32] Furthermore, APOBEC3 mutations, for all APOBEC3 enzymes except for APOBEC3G, preferentially have a thymine base (T) upstream of the mutated cytidine base (C), which is being deaminated to uracil (Reviewed by Salter et al., 2016).[4] In the lagging strand, this means that an adenine base (A) downstream of the mutated guanine base (G), would be the preferential mutational signature. In 16 of the 17 mutations observed via 3D-PCR in the 293T cells, this APOBEC3 signature was present (Table 1). Consistent with the hypothesis that transfection of producer cells induces mutator pathways of which APOBEC3B is an important player, APOBEC3B transcripts were highly upregulated in the 293T cells after transfection with the plasmids to make lentivirus, indicating that APOBEC3 enzyme activation was triggered in the time frame in which the mutations were observed (Figure S3).

GFP, an indicator of plasmid protein translation after transfection, was detected between 8 and 24 h after transfection, and increased in intensity between 24 and 72 h after transfection (Figure S1). The induction of APOBEC3B mirrored this increase between 8 and 72 h, suggesting that APOBEC3B expression was causally associated with proteins expressed after plasmid transfection (Figure 2), which include VSV glycoprotein and lentiviral GAG and POL (Figure 1A), which may be inducing APOBECB expression as a result of engagement with Toll-like receptors or other pattern recognition receptors on the transfected cells.[33,34]

It is unclear whether APOBEC3B alone is responsible for the generation of the mutations that we describe here in the 293T cells. All of the mutations observed via Sanger sequencing of the 3D-PCR products were G-to-A mutations (Table S2), although mutations observed in TOPO cloning sequencing were not all of the APOBEC phenotype (Table S1). Our RT-qPCR data (Figures 2C and S3) show that the majority of the human APOBEC3 enzymes were transcriptionally upregulated upon transfection of 293T cells with plasmids to make lentivirus. These results suggest that other APOBEC3 enzymes may play at least some role in driving the mutations that we describe here. In this respect, although increased levels of APOBECA or APOBEC3G were not detected by Western blotting using a monoclonal antibody that detects APOBEC3A, APOBEC3B, and APOBEC3G after transfection, we have confirmed induction of APOBECC3F (but not APOBEC3C) (Figure 2D). RNA sequencing and protein expression studies are currently underway to determine the full range of mutator pathways, in addition to the APOBECs, which are activated at each step of the virus production and infection protocol, such as ADAR, an adenosine deaminase

enzyme,[35] which has been previously shown to be present in HIV particles.[36]

Endogenous APOBEC3B (from both parental 293T and APOBEC3B-overexpressing 293T cells) was present in lentivirus particles purified both by passage through a 0.45-μm filter (which does not exclude added cellular debris) as well as in sucrose gradient purified virus produced from 293T cells overexpressing APOBEC3B, consistent with previous reports[37] (Figure 3). We observed multiple mutations in the CAR vector within the lentiviral particles, of which only a small subset were retained in the CAR within transduced CAR T cells. One possible explanation is that the hypermutated lentiviral particles render some lentivirus clones unable to infect human T cells due to associated mutations elsewhere in the CAR vector (such as in the long terminal repeats needed for integration). These results are significant in that they highlight a potentially significant source of loss of CAR integrity during the process of making clinically used CAR T cells. Therefore, investigating ways to reduce this step of CAR vector mutation represents an important avenue for increasing quality control.

After transduction of T cells from human donors with CAR-encoding lentivirus, a similar set of mutations was detected in the 293T cells and the CAR T cells, with a few additional mutations present in the CAR T cells (Figure 4; Table 2). In some cases, sub-dominant sequencing signals were observed as mutations present in the 293T cells, which then became dominant following transduction of the lentivirus into the human T cells, perhaps due to increased transduction efficiency of CAR T cells with those mutation-containing vectors. It is also possible that additional, unique mutations to the CAR vector developed in the CAR T cells upon viral transduction, consistent with the high expression of APOBECs (such as APOBEC3G) observed in both untransduced and transduced T cells (Figures 4C and 4D), and in previous reports.[13]

APOBEC3B overexpression in the producer 293T cells led to multiple additional G-to-A mutations in the CAR T cells and significantly reduced levels of both CAR T cell killing and IFN-γ production (Figure 5; Table S5). APOBEC3B knockout in the 293T cells using a CRISPR-Cas9 system led to reduced G-to-A mutations in the CAR T cells, significantly increased CAR T cell killing, and significantly increased IFN-γ production (Figure 6; Table S6). There was no significant change in IFN-γ production in APOBEC3B knockout produced CAR T cells in two of the donors transduced with an equivalent MOI of virus (M30, M59), despite a significant decrease in surviving tumor cells after challenge with APOBEC3B knockout produced CAR T cells from all five studied donors. This may reflect the fact that effector mechanisms other than IFN-γ, such as tumor necrosis factor-α or granzyme, may be the critical effector mechanism of CAR T cell killing. Alternatively, this disconnect may be explained by the difference in timing between the measurement of IFN-γ and cell killing. Thus, whereas IFN-γ levels were measured early (24 h after CAR T cell challenge), cytotoxicity was measured late (5 days after CAR T cell challenge), by which time cytokine levels may no longer correlate with CAR T cell activity (Figures 6F and 6G). While APOBEC3 enzymes have long been known to show a role in viral restriction,[5–8] we show here that APOBEC3B mutationally inhibits lentiviral production in viral producer cells, with significant implications for the quality of the final CAR T cell product.

Our findings here are significant because they show that the quality of a CAR T cell product can be significantly reduced by cellular mutator pathways, which lead to mutation of the CAR vector at several stages of the production process. This opens the door to strategies to improve the quality of clinically used CAR T cell products by targeting these mutator pathways. In this respect, direct inhibition of APOBEC3 enzymes in viral producer cells might be possible through by delivery of cytidine nucleoside analogues.[38,39] In addition, specific knockdown of individual APOBEC enzymes in clinical grade viral producer cells could alleviate the mutational load induced in the resulting CAR T cells. Therefore, we would argue that testing producer cell lines that are routinely used for both clinical, and pre-clinical, CAR T cell vector production for APOBEC3B, other APOBECs, and other potential anti-viral mutator pathways would represent an important step in increasing the efficiency of CAR T cell therapies.[40]

In summary, we show here that, in the model 293T producer cell system, multiple mutations are imprinted into the CAR vector upon initial transfection of viral production plasmids into the producer cell line. Many of these mutations are also present in the final CAR

Figure 5. APOBEC3B overexpression in 293T cells correlates with reduced CAR T cell function
(A) GFP expression in F32, M30, M31, M59, and M62 CD19 CAR T cells transduced with an equivalent volume of lentivirus (30 μL) produced from parental 293T cells or 293T cells stably overexpressing APOBEC3B via lentiviral vector. A paired t test ($p < 0.05$) was used for statistical comparison. (B) The 3D-PCR in the GFP gene for DNA isolated from F32, M30, M31, M59, and M62 CD19 CAR T cells 10 days after transduction produced with either lentivirus produced from parental 293T cells or 293T cells overexpressing APOBEC3B via lentiviral vector. (C) IFN-γ ELISA 24 h after challenge of MEL888-CD19 cells with CD19 CAR T cells at effector:target ratios of 0.5:1, 1:1, and 2.5:1 produced with equivalent volume of lentivirus produced from parental 293T cells or 293T cells overexpressing APOBEC3B via lentiviral vector. A two-way ANOVA ($p < 0.05$) and Sidak's multiple comparison test were used for statistical comparison. (D) Count of surviving tumor cells 5 days after challenge of MEL888-CD19 cells with F32, M30, M31, M59, and M62 CD19 CAR T cells at effector:target ratios of 0.5:1, 1:1, 2.5:1 produced with equivalent volume (30 μL) of lentivirus produced from parental 293T cells or 293T cells overexpressing APOBEC3B via lentiviral vector. A two-way ANOVA ($p < 0.05$) and Sidak's multiple comparison test were used for statistical comparison. (E) GFP expression in F32, M30, M31, M59, and M62 CD19 CAR T cells transduced with an equivalent MOI (5) of lentivirus produced from parental 293T cells or 293T cells overexpressing APOBEC3B via lentiviral vector. A paired t test ($p < 0.05$) was used for statistical comparison. (F) Count of surviving tumor cells 5 days after challenge of MEL888-CD19 cells with F32, M30, M31, M59, and M62 CD19 CAR T cells at effector:target ratios of 0.5:1, 1:1, and 2.5:1 produced with equivalent MOI (5) of lentivirus produced from parental 293T cells or 293T cells overexpressing APOBEC3B via lentiviral vector. A two-way ANOVA ($p < 0.05$) and Sidak's multiple comparison test were used for statistical comparison. ****, $p < 0.0001$; ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$; ns, not significant.
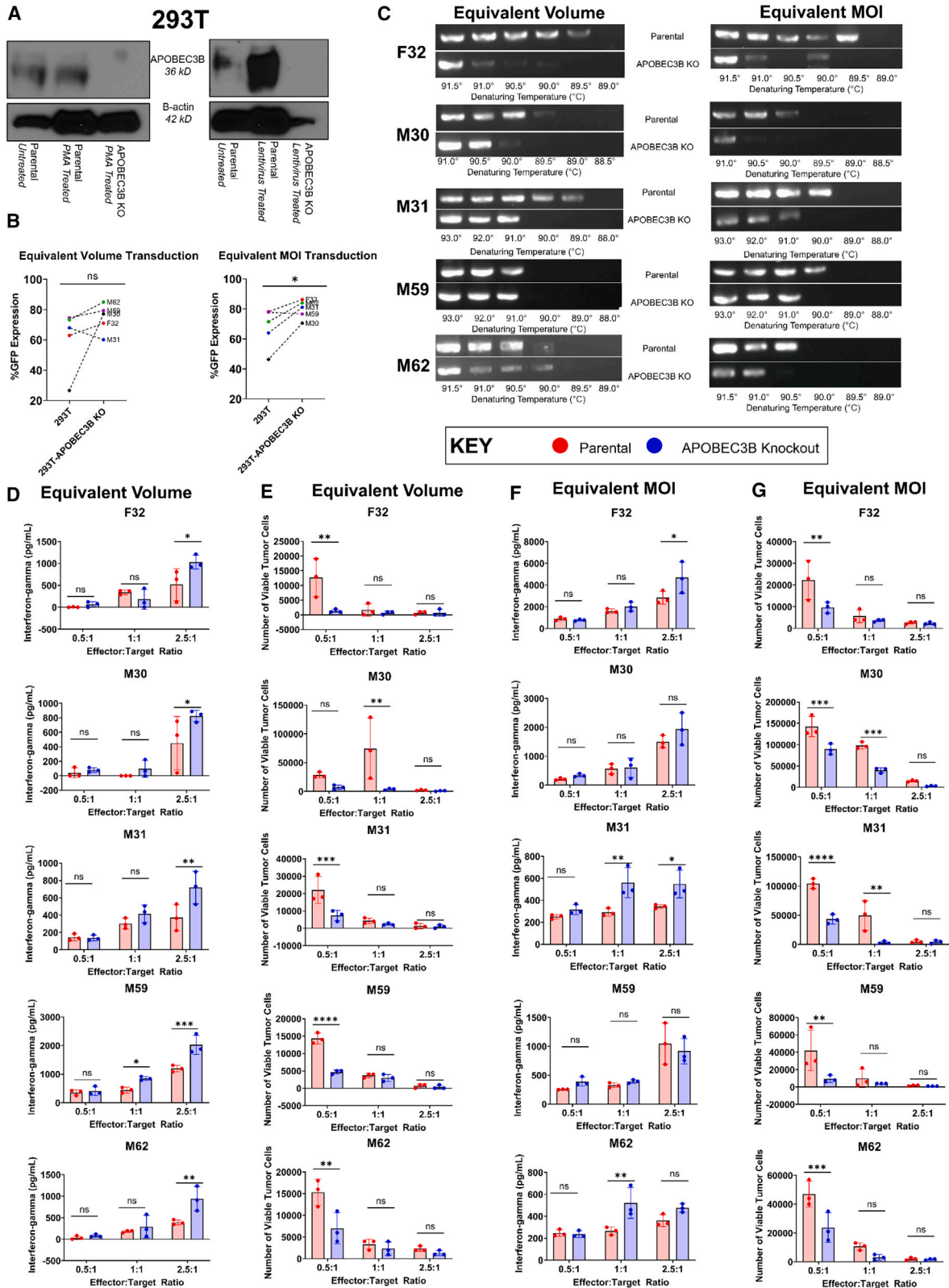
T cell product. Many of these mutations bear the signature of APOBEC3B and other APOBEC enzymes, consistent with our demonstration of the induction of APOBEC3B principally at the plasmid transfection stage of the process. After overexpression of APOBEC3B in the producer cells, CAR T cells were significantly less functional in killing target cells; similarly, following knockout of APOBEC3B in the producer cells, CAR T cells were significantly more functional in killing target cells, indicating that APOBEC3B is likely an important factor in regulating the quality of CAR T cells. Therefore, we suggest that further screening of CAR T cell production protocols for the induction of mutator pathways could be an important approach by which the quality of both pre-clinical and clinical CAR T cell production could be further improved. Identification and subsequent manipulation, or knock out, of these mutator proteins and pathways, such as APOBEC3B, could then be undertaken to generate better quality of CAR T cell products.

MATERIALS AND METHODS

Cell culture

Human 293T cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) (Gibco Thermo Fisher Scientific, Waltham, MA, Cat. A525670). The 293T-APOBEC3B cell line was derived from 293T cells, stably transduced with a lentivirus encoding APOBEC3B, and cultured in DMEM supplemented with 10% FBS and 1.25 mg/mL puromycin. The 293T-APOBEC3B KO cell line was generated by CRISPR knockout of APOBEC3B in single cell clones, and cultured in DMEM supplemented with 10% FBS. The MEL888-CD19 cell line was derived from MEL888 parental cells and was stably transfected to express human CD19 (previously described by Evgin et al., 2022[41]) and cultured in DMEM supplemented with 10% FBS and 1.25 mg/mL puromycin. Cell lines were maintained at 37°C at 5% CO_2 and were regularly shown to be free of infection by *Mycoplasma*.

Virus generation and titer

Lentiviral vectors were generated using the following plasmids: pHR-SIN (containing the vector and the transgene to be overexpressed), p8.91QV (containing *gag* and *pol*), and pMD-G (containing pVSV-G). The pHR-SIN plasmid contained either a second-generation CAR T cell construct (described in section on the generation of human CAR T cells), or human APOBEC3B. To make lentivirus, 293T cells were seeded at approximately 5e6 cells per 10 cm plate in DMEM supplemented with 10% FBS, allowed to settle, and transfected with the plasmids described using lipofectamine as a transfection reagent. For certain CAR T cell preps, 293T cells overexpressing APOBEC3B or with APOBEC3B knocked out were used for the transfection step and compared with CAR T cell preps with normal 293T cells. The day after transfection, the media was removed and was replaced with fresh media. Three days after transfection, media from the 293T cell cultures was harvested, filtered through a 0.45-μm filter, centrifuged at 25,000 RPM for 2 h to concentrate the vector, and frozen at −80°C until the date of use. The titer of the lentivirus with the CAR T cell construct was determined by transducing 0.1 μL, 1 μL, and 10 μL of virus onto 100,000 293T cells in a 24-well plate, harvesting the 293T cells for flow cytometry for Zombie NIR (Biolegend, San Diego, CA, Cat. 423105) and GFP 72 h after transduction, and determining titer using cells with approximately 20%–30% GFP expression.

For the viral preparations in which a sucrose gradient was used, media from the 293T cell cultures was harvested and filtered through a 0.45-μm filter. A sucrose gradient with 70% sucrose in PBS, 60% sucrose in DMEM, 30% sucrose in DMEM, and 20% sucrose in PBS was prepared, and the viral supernatant was overlaid on the sucrose gradient according to Brown et al.[26] The sucrose gradient and viral supernatant was centrifuged at $70,000 \times g$ for 2 h with minimal acceleration and deceleration to concentrate the vector, and the 30% and 60% sucrose layers containing the lentivirus were extracted after centrifugation. The lentivirus was centrifuged at $70,000 \times g$ for 2 h with minimal acceleration and deceleration with a 20% sucrose cushion in PBS, and frozen at −80°C until the date of use.

Generation of human CAR T cells

Cones of peripheral blood mononuclear cells (PBMCs) were obtained from the Mayo Clinic Blood Donor Center. Blood was drained from the cones, diluted with PBS (Corning, Corning, NY, Cat. 21-031-CV),

Figure 6. APOBEC3B knockout in 293T cells improved with reduced CAR T cell function
(A) Western blot showing APOBEC3B expression in parental 293T and 293T cells with APOBEC3B knocked out via CRISPR-Cas9 after treatment with PMA (harvested 8 h after challenge) or a lentivirus (harvested 24 h after challenge, MOI of approximately 10). (B) GFP expression in F32, M30, M31, M59, and M62 CD19CAR T cells transduced with an equivalent volume (30 μL) or an equivalent MOI (5) of lentivirus produced from parental 293T cells or 293T cells with APOBEC3B knocked out via CRISPR-Cas9. A paired t test ($p < 0.05$) was used for statistical comparison. (C) 3D-PCR in the GFP gene for DNA isolated from F32, M30, M31, M59, and M62 CD19 CAR T cells produced with either lentivirus produced from parental 293T cells or 293T cells with APOBEC3B knocked out via CRISPR-Cas9. (D) IFN-γ ELISA 24 h after challenge of MEL888-CD19 cells with F32, M30, M31, M59, and M62 CD19 CAR T cells at effector:target ratios of 0.5:1, 1:1, and 2.5:1 produced with equivalent volume of lentivirus produced from parental 293T cells or 293T cells with APOBEC3B knocked out. A two-way ANOVA ($p < 0.05$) and Sidak's multiple comparison test were used for statistical comparison. (E) Count of surviving tumor cells 5 days after challenge of MEL888-CD19 cells with F32, M30, M31, M59, and M62 CD19 CAR T cells at effector:target ratios of 0.5:1, 1:1, and 2.5:1 produced with equivalent volume of lentivirus produced from parental 293T cells or 293T cells with APOBEC3B knocked out. A two-way ANOVA ($p < 0.05$) and Sidak's multiple comparison test were used for statistical comparison. (F) IFN-γ ELISA 24 h after challenge of MEL888-CD19 cells with CAR T cells at effector:target ratios of 0.5:1, 1:1, and 2.5:1 produced with equivalent MOI of lentivirus produced from parental 293T cells or 293T cells with APOBEC3B knocked out. A two-way ANOVA ($p < 0.05$) and Sidak's multiple comparison test were used for statistical comparison. (G) Count of surviving tumor cells 5 days after challenge of MEL888-CD19 cells with F32, M30, M31, M59, and M62 CD19 CAR T cells at effector:target ratios of 0.5:1, 1:1, and 2.5:1 produced with equivalent MOI of lentivirus produced from parental 293T cells or 293T cells with APOBEC3B knocked out. A two-way ANOVA ($p < 0.05$) and Sidak's multiple comparison test were used for statistical comparison. ****, $p < 0.0001$; ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$; ns, not signficant.

and Lympholyte-H (Cedarlane Labs, Burlington, Ontario, Canada, Cat. CL5010) was underlaid. The blood, PBS and Lympholyte-H mixture was centrifuged at 800×*g* for 20 min, and PBMC cells from the PBS/Lympholyte-H interface were collected and counted. The isolated PBMC cells were frozen in liquid nitrogen in 90% FBS/10% DMSO until the date of CAR T cell preparation. On day 0 of CAR T cell preparation, PBMCs were thawed from liquid nitrogen and T cells were isolated using and according to protocol of a Human Pan T cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, North Rhine-Westphalia, Germany, Cat. 130-096-535). T cells were resuspended in T cell media, which consisted of RPMI 1640 1× with L-Glutamine (Corning, Cat.10-040-CV) supplemented with 10% FBS, 50 μM 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, Cat. M3148), 1% PenStrep (Corning, Cat. 30-002-Cl), 1% non-essential amino acids (Gibco Thermo Scientific, Waltham, MA, Cat. 11140-050), and 1% sodium pyruvate (Sigma Aldrich, Cat. S8636). T cells were stimulated with Human T Activator CD3/CD28 Dynabeads (Thermo Fisher Scientific, Cat. 11131D) with 1e6 cells per well in a 24-well plate. On day 1 of CAR T cell preparation, isolated T cells were transduced with lentivirus encoding a single chain variable fragment specific to human CD19, a CD8-derived hinge region, a CD8 transmembrane domain, a 4-1BB costimulatory domain, and a CD3 zeta domain, at an MOI of 5 (or, in the experiments in Figures 5 and 6, an equivalent volume of virus in each group of CAR T cells roughly equivalent to an MOI of 5 in CAR T cells transduced with parental-produced virus). eGFP was separated from the CD3z domain by a P2A site. On days 2, 3, and 4 of CAR T cell preparation, 1 mL of T cell media was added to each well. On day 5, the CD3/CD28 Dynabeads were removed and cells were counted and normalized to 1e6 cells/mL. On days 6, 7, 8, and 9, CAR T cells were counted and normalized to 8e5 cells/mL. On day 10, CAR T cells were counted and analyzed for transduction via flow cytometry for GFP. Remaining CAR T cells were frozen in liquid nitrogen in 90% FBS/10% DMSO until the time of use.

Antibodies

To detect APOBEC3A, APOBEC3B, and APOBEC3G, a monoclonal antibody described by Brown et al.[42] was obtained from the National Institutes of Health (NIH) HIV Reagent Program, Division of AIDS, NIAID, NIH. The antibody was Anti-Human APOBEC3B Monoclonal (5210-87-13), ARP-12397, contributed by Dr. Reuben Harris. Anti-human APOBEC3B monoclonal antibody was used at approximately 1:100 for western blot. The secondary antibody used for the anti-human APOBEC3B antibody was ECL anti-rabbit IgG HA peroxidase linked whole antibody. Secondary antibody was used at approximately 1:2,000 for western blot. As a control for western blot, anti beta-actin peroxidase antibody (Sigma Aldrich, Cat. A3854) was used at 1:2-000–1:10,000. To detect CD3 in flow cytometry experiments, a BV421 anti-human CD3 mouse IgG-kappa (Biolegend, Clone UCHT1, Cat. 300434) was used at 1:200.

CRISPR-Cas9 knockout of APOBEC3B

We transfected 100,000 293T cells with 0.1 μg of APOBEC3B CRISPR-Cas9 KO plasmid (h) (Santa Cruz Biotechnology, Santa Cruz, CA, sc-401700) in a 24-well plate. At 2 days after transfection, cells were replated on a 10-cm dish. Single-cell clones were identified via the presence of GFP, and single-cell clones were isolated by picking colonies of sufficient size, and growing the colonies individually. To validate APOBEC3B knockout, a western blot was performed according to the protein expression analysis section. Clones were challenged with either 500 ng/mL PMA or a high titer (MOI 10) lentivirus. PMA-treated clones were harvested 8 h after challenge and lentivirus challenged clones were harvested 24 h after challenge. APOBEC3B-expressing 293T cells were also treated with PMA and lentivirus as a control.

Flow cytometric analysis

All flow cytometry experiments were run on a Benton Dickinson FACSCantoX (Franklin Lakes, NJ) machine at the Mayo Clinic Flow Cytometry Core. All analysis was completed in FlowJo, version 10.8.1.

3D-PCR analysis

All PCR reactions were performed using ExTaq (Mg^{2+} Free Buffer) (Takara, Kusatsu, Shiga, Japan, Cat:RR01AM). For 3D-PCR, an initial round of PCR for GFP was run on an AnalytikJena (Jena, Germany) T Professional Trio Thermocycler, using *5′ GTAAAC GGCCACA AGTTCAG 3′* (forward) and *5′ CCATGTGATCGCGCTTCT 3′* (reverse) primers from Integrated DNA Technologies (Coralville, IA). After the initial round of PCR, the PCR products were diluted 1:4. A second round of PCR was run using 1 μL of the initial PCR product as the template DNA on an Applied Biosciences Thermo Fisher Scientific Veriti 96 well thermocycler, using *5′GCTG ACCCTGAAGTTCATCTG 3′* (forward) and *5′ CACCTTGATGCC GTTCTTCT 3′* (reverse) primers. To confirm PCR reactions were successful, reactions were run on a 2% agarose gel, prepared with 50× Tris/acetic acid/EDTA, diluted to 1× (BioRad, Hercules, CA, Cat. 1610743) and 2% SeaKem GTG Agarose (Lonza, Basel, Switzerland, Cat. 50074).

Sequencing of PCR products

DNA was harvested from cell pellets and isolated using a DNeasy Blood & Tissue Kit from Qiagen (Hilden, Germany) (Cat. 69504). A normalized concentration of DNA was used for PCR. PCR reaction products were run on 2% agarose gels, and the PCR product was extracted from the gel. Gel purification was performed using a QIAquick Gel Extraction Kit (Qiagen, Cat. 28704). Sequencing was performed by mixing 5 μL of DNA and 10 μL of either the forward or reverse PCR primer diluted 1/20, with the primers described in the 3D-PCR analysis section and sending the samples to Genewiz/Azenta for Sanger sequencing. Sequence alignments of the.ab1 files was performed using DNA Dynamo.

RT-qPCR

RNA was harvested from cell pellets and isolated using a RNeasy Plus MiniKit (Qiagen, Cat. 74134). A normalized concentration of RNA was converted to cDNA using a Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland, Cat. 04379012201). RT-qPCR

mixes were prepared using a LightCycler 480 SYBR Green I Master Kit (Roche, Cat. 0407516601). RT-qPCR for APOBEC3 transcripts was performed using primers described by Refsland et al.[13] with GAPDH as the housekeeping gene, using a Roche (LightCycler 480 II).

Protein expression analysis

Protein was harvested from cell pellets by sonicating the cells in RIPA buffer and centrifuging the sonicated protein at $13,000 \times g$ for 10 min. Remaining protein concentrations were normalized using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Cat. 23225 and 23227). For western blots using the anti-human monoclonal APOBEC3B antibody (5210-87-13), samples were denatured using 2-mercaptoethanol (BioRad, Cat. 161–0710) and $4\times$ Laemmli Sample Buffer (BioRad, Cat. 1610747), with 0.8 M urea (Sigma, Cat. U5378). Samples were run on Mini-PROTEAN TGX gels (BioRad, Cat. 4561043) and transferred via semi-dry transfer for 30 min, 84 mA per gel, with five sheets of Whatman 3mm CHR chromatography paper (Cytiva, Marlborough, MA, Cat. 3030-866) paper soaked in TGM buffer onto a Immobilon Transfer Membrane (Millipore, Burlington, MA, Cat. IPVH30F40). TGM buffer consisted of 100 mL $10\times$ Tris/Glycine (BioRad, Cat. 1610732), 200 mL methanol, and 700 mL water. Membranes were blocked for at least 1 h, then incubated with primary antibody overnight at $4°C$. The following day, membranes were washed in TBS-T. TBS-T consisted of 900 mL water, 100 mL $10\times$ TBS (BioRad, Cat. 1706435), and 1 mL Tween 20 (Sigma, Cat. 9005-64-5) ($1\times$ for 2 min, $3\times$ for 15 min), incubated with secondary antibody for 2 h at room temperature, then washed with TBS-T ($1\times$ for 2 min, $3\times$ for 15 min). The membrane was developed using Super Signal West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific, Cat. 34580) on HyBlot CL Autoradiography Film (LabForce Powered by Thomas Scientific, Chadds Ford Township, PA, Cat.1141351).

ELISA

MEL888-CD19 tumor cells were plated at 20,000–25,000 cells/well in 24-well plates. Then, 8–16 h later, human CAR T cells were thawed from liquid nitrogen, added to 10 mL of T cell media, and centrifuged at 1,500 rpm for 5 min, and plated with the MEL888-CD19 tumor cells at 0.5:1, 1:1, and 2.5:1 effector:target ratios ($3\times$ wells/condition). We harvested 100 μL of media for ELISA from each well 24 h after challenge with CAR T cells and frozen at $-20°C$, until the date of the ELISA. For IFN-γ ELISAs, a DuoSet Human IFN-Gamma kit (R&D Systems, Minneapolis, MN DY285B-05) was used according to the protocols from the manufacturer.

Cell viability assay

MEL888-CD19 tumor cells were plated at 20,000–25,000 cells/well in 24-well plates. At 8–16 h later, human CAR T cells were thawed from liquid nitrogen, added to 10 mL of T cell media, and centrifuged at 1,500 rpm for 5 min, and plated with the MEL888-CD19 tumor cells at 0.5:1, 1:1, and 2.5:1 effector:target ratios ($3\times$ wells/condition). At 5 days after challenge with CAR T cells, the media in the wells was aspirated off, and each well was washed $2\times$ with PBS to remove residual CAR T cells and dead cells. Surviving cells were dissociated using

trypsin/EDTA (Corning, Cat. 25–053). Cells were resuspended in 200 μL of CAR T cell media and counted using TrypanBlue dye (Gibco Thermo Fisher Scientific) (either a 1:1 or a 1:10 ratio of cells/Trypan blue).

APOBEC3 activity assay

The protocol for the activity assay was adopted from previously described protocols.[43–45] Cell extracts and deamination reactions were prepared as described by Akre et al.[45] Deamination reactions were incubated for 1 h, 4 h, and overnight at $37°C$. We added 1 M NaOH after the reaction concluded and incubated at $95°C$ for 10 min in a thermocycler. We added $2\times$ RNA loading dye to each reaction, the reactions were loaded in to 15% TBE urea gels (BioRad, Cat. 4566055) and imaged.

Statistical analysis

All statistical analysis was completed in GraphPad Prism. The p value used for all statistical tests was $p = 0.05$. Student t tests, one-way ANOVA, two-way ANOVA, and Sidak's multiple comparison tests were used to analyze the results of experiments as appropriate. The p value used for all statistical tests was $p = 0.05$. For all statistical tests, the following notation was used to visual p values: ****, $p < 0.0001$; ***, $p < 0.001$; **, $p < 0.01$; and *, $p < 0.05$.

DATA AND CODE AVAILABILITY

Data supporting the conclusions are available within the contents of the article and the supplemental information.

AUTHOR CONTRIBUTIONS

Conceptualization and design: J.S., J.Tonne, and R.V.; Development of methodology: J.S., J.Tonne, and R.V.; Acquisition of data: J.S., J.Tonne, T.S., M.M., and R.V.; Analysis and interpretation of data: J.S., J.Tonne, J. Thompson, T.S., B.K., O.L., M.M., and R.V.; Writing – review & editing: J.S., J.Tonne, J. Thompson, T.S., B.K., O.L., M.M., and R.V.; Study supervision: R.V.

DECLARATION OF INTERESTS

The authors declare no conflicts of interest.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omton.2024. 200873.

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