Sub-optimal T Cell Therapy Drives a Tumor Cell Mutator Phenotype That Promotes Escape from Frontline Treatment

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Running title

T cell activation of APOBEC3 promotes therapeutic escape

Keywords

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Abstract

Anti-tumor T cell responses raised by frontline therapies such as chemotherapy, radiation, tumor cell vaccines, and viroimmunotherapy, are generally likely to be weak both quantitatively (low frequency) and qualitatively (low affinity). We show here that T cells which recognize tumor-associated antigens (TAAs) can directly kill tumor cells if used at high effector to target ratios. However, when these tumor reactive T cells were present at suboptimal ratios, direct T cell mediated tumor cell killing was reduced, and surprisingly, the ability of tumor cells to evolve away from a co-applied therapy (oncolytic or suicide gene therapy) was promoted. This T cell mediated increase in therapeutic resistance was associated with C to T transition mutations that are characteristic of APOBEC3 cytosine deaminase activity, and was induced through a TNFα and PKC dependent pathway. Short hairpin RNA inhibition of endogenous APOBEC3 returned the level of tumor escape from oncolytic virus or suicide gene therapy to that seen in the absence of anti-tumor T cell co-culture. Conversely, overexpression of human APOBEC3B in tumor cells led to significantly enhanced escape from suicide gene therapy and oncolytic virus therapy both *in vitro* and *in vivo*. Our data raise the possibility that weak affinity/low frequency T cell responses against tumor antigens may actively contribute to the ability of tumor cells to evolve away from frontline therapies. Therefore, immunotherapies need to be optimized as early as possible so that, if they do not kill the tumor completely, they do not promote treatment resistance.

Introduction

Tumor cells readily escape from a variety of frontline therapies through multiple genetic and epigenetic mechanisms that are derived from the genetic instability inherent to cancer. This genetic instability confers phenotypic plasticity and allows for the selection and expansion of therapeutically resistant subclones. Dysregulation of the APOBEC3 family of cytosine deaminase enzymes represents an endogenous source of DNA damage that has recently been implicated as an important mediator of the evolution of cancer[1, 2]. Originally identified as innate antiviral restriction factors[3], the APOBEC3 enzymes catalyze cytosine to uracil deamination of ssDNA to generate C to T transitions, and to a lesser frequency C to G transversion mutations. Evidence of the APOBEC3A and B mutation motifs exists in approximately half of all human cancers and correlates with high levels of APOBEC3A and B expression [1, 4, 5]. The presence of the APOBEC3B mutation signature has been associated with poor prognosis, and there is some evidence that it may be associated with therapeutic resistance in multiple cancer types[6-8]. In the context of estrogen receptor positive breast cancer, APOBEC3B expression levels inversely correlated with tamoxifen benefit both pre-clinically and clinically[7]. APOBEC3B overexpression has been attributed to protein kinase C (PKC) mediated activation of the NFκB pathway[9, 10], however, it remains unclear which cell types or soluble mediators in the tumor microenvironment may play an important role in modulating its expression in tumor cells.

Although the human genome encodes seven distinct APOBEC3 enzymes (APOBEC3A though H), the mouse genome encodes a single gene[11, 12]. It is not immediately clear which of the human APOBEC3 activities or specificities that mouse APOBEC3 (mAPOBEC3) mimics with respect to inducing cancer promoting mutations. In the current study, we have investigated whether mAPOBEC3 may have similar

activities to those of human APOBEC3B (hAPOBEC3B) as an inducer of tumor cell heterogeneity which contributes to cancer evolution.

Adoptive T cell therapies, immune checkpoint inhibition, viroimmunotherapies, and cancer vaccines, seek to raise adaptive anti-tumor immune responses. There is also evidence that tumor reactive T cells can be primed and expanded in patients in response to the induction of immunogenic cell death by standard frontline therapies such as radiation and chemotherapy[13]. Although radiation and chemotherapy lead to direct killing of tumor cells, their capacity to induce DNA damage can introduce mutations that may also promote resistance. Several evolutionary mechanisms to evade adoptive T cell therapy, oncolytic viroimmunotherapy, and suicide gene therapy have been described in murine models[14-16].

The overarching view is that the direct, or indirect, generation of a tumor- specific adaptive immune response of any magnitude is beneficial to the patient, even if the tumor reactive T cells are at low frequency or bear low affinity T cell receptors for their cognate tumor antigens. Herein however, we show that the effector cytokine TNFα produced by tumor reactive T cells acts on target tumor cells by upregulating the mAPOBEC3 enzyme which can lead to an increased resistance to therapy. When tumor reactive T cells were co-cultured with tumor cells at low effector to target ratios in the presence of additional selective pressures such as an oncolytic virus or suicide gene therapy, mAPOBEC3 upregulation increased the rate of treatment-resistant clonal outgrowth. In an immune competent mouse model of HSV TK driven suicide gene therapy, APOBEC3B overexpression promoted the accumulation of silencing mutations in the TK gene, and significantly reduced median survival. Therefore, suboptimal immunotherapies may indirectly act as tumor cell mutators, in the same way as other mutagenic therapies such as radiation and chemotherapy, and should be optimized to prevent a direct enhancement of tumor cell escape.

Materials and Methods

Cell lines. B16 murine melanoma cells were obtained from the ATCC prior to being modified with the relevant transgenes. Cell lines were authenticated by morphology, growth characteristics, PCR for melanoma specific gene expression (gp100, TYRP-1 and TYRP-2) and biologic behavior, tested mycoplasma-free and frozen. Cells were cultured less than 3 months after thawing. The B16OVA cell line was derived from a B16.F1 clone transfected with a pcDNA3.1ova plasmid obtained from Dr. Esteban Celis in 2000 [17, 18]. B16OVA cells were grown in DMEM (HyClone, Logan, UT, USA) + 10% FBS (Life Technologies) + 5 mg/mL G418 (Mediatech, Manassas, VA, USA) until challenge. GL261 cells were obtained from Dr. Aaron Johnson (Mayo Clinic) in 2014. GL261OVA was obtained by transfection of parental GL261 cells with pcDNA3.1 OVA in 2015. LLC cells were obtained from Professor Ian Hart (ICRF, London) in 1998. LLCOVA was obtained by transfection of parental LLC cells with pcDNA3.1 OVA in 2015. B16TK cells were derived from a B16.F1 clone transfected with a plasmid expressing the Herpes Simplex Virus thymidine kinase (HSV-1 TK) gene in 1997/1998[19]. Following stable selection in 1.25 µg/mL puromycin, these cells were shown to be sensitive to Ganciclovir (Cymevene) at 5µg/ml[20-22]. Cells were tested for mycoplasma using the MycoAlert Mycoplasma Detection Kit (Lonza Rockland, Inc. ME, USA).

Mice. 6-8 week old female C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, Maine). The OT-I mouse strain is on a C57Bl/6 background (H-2K^b) and expresses a transgenic T cell receptor Vα2/Vβ5 specific for the SIINFEKL peptide of ovalbumin in the context of MHC class I, H-2K^b as previously described[23] and were bred at Mayo Clinic.

Viruses. Wild-type Reovirus type 3 (Dearing strain) was obtained from Oncolytics Biotech (Calgary, AB, Canada) and stock titers were measured by plaque assay on L929

cells.

Viability assays. B16TK cells were seeded in 96 well plates in triplicate and treated with reovirus (MOI 0.1) or with GCV (Cymevene) at 5μg/ml. Cell titer blue (Promega, Madison, WI) was added to wells at 10% v/v and fluorescence was measured after approximately 4 hours incubation ($560_{Ex}/590_{EM}$). Relative viability of experimental conditions was normalized to untreated cells.

CD8 T cell preparation. Spleens were immediately excised from euthanized C57Bl/6, OT-I mice and dissociated *in vitro* to achieve single-cell suspensions. Red blood cells were lysed with ACK lysis buffer. CD8 T cells were prepared using the CD8α T Cell Isolation kit (Miltenyi, Auburn, CA) and co-cultured with target tumor cells at various effector to target ratios as described in the text. Supernatants were assayed for IFNγ by ELISA as directed in the manufacturer's instructions (Mouse IFN-γ ELISA Kit, OptEIA, BD Biosciences, San Diego, CA).

***In vitro* T cell activation.** OT-I T cell were activated in IMDM (Gibco, Grand Island, NY, USA) + 5% FBS + 1% Pen/Strep + 40 μM 2-ME. Media was supplemented with the SIINFEKL peptide at 1 μg/mL and human IL2 at 50 U/mL. Cells were used for *in vitro* assays following 4 days of activation.

Generation of tumor experienced B16TK (T.E.) CD8 T cells. CD8 T cells were prepared as described above from C57BL/6 mice that had been cured of subcutaneous B16TK tumors following three weekly courses of GCV (50 mg/kg on days 5-9, 12-16, and 19-23). Cells were harvested between 60 and 80 days post tumor implantation.

***In vitro* selection of therapy resistant populations.** B16TK or B16OVA cells were plated in triplicate wells in the presence of GCV (Cymevene) at 5μg/ml, reovirus (MOI 0.1) or 4-day *in vitro* activated OT-I CD8T cells or T.E. CD8 T cells (E:T ratio of 5:1) for 7 days in Iscove's Modified Dulbecco's Medium (IMDM; Gibco, Grand Island, NY) + 5% FBS + 1% Pen-Strep + 40 μM β-mercaptoethanol. Wells were washed 3 times with PBS

and cultured in normal medium for a further 7 days. Surviving cells were then cultured again in the presence of PBS, GCV, reovirus (MOI 0.1) or 4-day *in vitro* activated OT-I CD8 T cells or T.E. CD8 T cells (various effector to target ratios) for 7 days.

These co-culture systems were also performed with the anti-H-2K^b antibody (AF6-88.5; 0.5 µg/mL) (Biolegend, San Diego, CA), the inhibitor of PKC signaling (AEB071; 10µM) (MedChemExpress, Monmouth Junction, NJ) or the anti-TNFα antibody (AF-410-NA; 0.5µg/ml) (R&D Systems; Minneapolis, MN) or the anti-IFNγ antibody (MAB485; 0.5µg/ml) (R&D Systems; Minneapolis, MN).

Quantitative RT-PCR and sequencing. RNA was prepared with the QIAGEN-RNeasy-MiniKit (Qiagen, Valencia, CA). 1µg total RNA was reverse-transcribed in a 20µl volume using oligo-(dT) primers using the First Strand cDNA Synthesis Kit (Roche, Indianapolis, IN). A cDNA equivalent of 1ng RNA was amplified by PCR with gene-specific primers using GAPDH as loading control (mgapdh sense: TCATGACCACAGTCCATGCC; mgapdh antisense: TCAGCTCTGGGATGACCTTG; APOBEC3 sense: ATGGGACCATTCTGTCTGGGA; APOBEC3 antisense: TCAAGACACGGGGGTCCAAG). qRT-PCR was carried out using a LightCycler480 SYBRGreenI Master kit and a LightCycler480 instrument (Roche) according to the manufacturer's instructions. The $\Delta\Delta C_T$ method was used to calculate the fold change in expression level of APOBEC3 and GAPDH as an endogenous control for all treated samples relative to an untreated calibrator sample.

The OVA transgene was sequenced using the following primers:

Sense:ATGGGCTCCATCGGCGCAGCand antisense: CCGTCTACACAAAGGGGAATT and aligned to the reference sequence CAA23682.1. The HSV TK transgene was sequenced using the following primers: CACGCAGATGCAGTCGGGGCGGCG (Downstream of the EcoR1 site in the 5'UTR), CTGGTGGCCCTGGGTTCGCGCGA,

GCGTTCGTGGCCCTCATCCC, GCCTGGGCCTTGGACGTCTTGG, and AGGGCGCAACGCCGTACGTCG and aligned to the reference sequence AB009254.2.

APOBEC3 and HSV TK protein quantification. Murine APOBEC3 was measured by western blotting with a rabbit polyclonal (PA511430, Thermo Fisher) or a rabbit monoclonal anti-human APOBEC3B (184990, Abcam, San Fransisco, CA) which react with both human APOBEC3B and murine APOBEC3 (Thermo Fisher) or by ELISA according to the manufacturer's instructions (Antibody Research Corporation, St Charles, MO). B16TK cells were treated with recombinant murine TNFα (R&D Systems, Minneapolis, MN). HSV TK protein was detected by western blotting tumor cell lysates with a goat polyclonal antibody (28038; Santa Cruz, Dallas, TX). β-actin was detected using an HRP conjugated mouse monoclonal antibody (clone AC-15; Sigma, St. Louis, MO).

APOBEC3 knockdown and overexpression. Four separate mouse unique 29mer shRNA retroviral constructs (Origene Technologies, Rockville, MD) were tested individually, or as a combination, for their ability to reduce expression of murine APOBEC3 in B16 cells compared to a single scrambled shRNA encoding retroviral construct. Optimal knockdown for periods of more than two weeks in culture was achieved using all four constructs pre-packaged as retroviral particles in the GP+E86 ecotropic packaging cell line and used to infect B16 cells at an MOI of ~10 per retroviral construct. In addition, a single scrambled negative control non-effective shRNA cassette was similarly packaged and used to infect B16TK cells to generate B16TK (scrambled shRNA) cells.

B16TK cells were infected with a retroviral vector encoding either full length functional APOBEC3B or a mutated, non-functional form of APOBEC3B as a negative control obtained from Reuben Harris (University of Minnesota, MN). Infected populations were

selected for 7 days in hygromycin to generate B16TK (APOBEC3B) or B16TK (APOBEC3B MUT) cell lines and used for experiments as described. In populations of B16TK (APOBEC3B) cells selected for more than 7-10 days in hygromycin expression of APOBEC3B returned to basal levels associated with the toxicity of prolonged APOBEC3B expression. Murine APOBEC3 (Accession: BC003314) was expressed from the pCMV-SPORT6 plasmid obtained from Dharmacon, Lafayette, CO)

***In vivo* experiments.** All *in vivo* studies were approved by the Institutional Animal Care and Use Committee at Mayo Clinic. Mice were challenged subcutaneously with $2x10^5$ B16TK melanoma cells, in 100 µL PBS (HyClone, Logan, UT, USA) or with $1x10^4$ cells in 2 uL intracranially into the frontal lobe as previously reported[24]. Subcutaneous tumors were treated with a two or three-week course of GCV (50 mg/kg) administered IP daily. Tumors were measured 3 times per week, and mice were euthanized when tumors reached 1.0 cm in diameter. Intracranial tumors were treated with a three- week courses of GCV (50 mg/kg) administered IP daily. Mice were sacrificed upon emergence of neurological symptoms or weight loss.

Statistics. Survival curves were analyzed by the Log-Rank test. Student's T tests, one-way ANOVA and two-way ANOVA were applied for *in vitro* assays as appropriate. Statistical significance was set at $p<0.05$ for all experiments.

Results

Tumor cell escape from therapy is enhanced by the presence of tumor reactive CD8 T cells.

B16 cells expressing the HSV-1 thymidine kinase (B16TK) were sensitive to treatment with ganciclovir (GCV) at 5 µg/mL (**Fig 1A**) or reovirus at an MOI of 0.1 (**Fig 1B**). However, despite the induction of significant cell death, a small proportion of cells survived as escape variants following two consecutive weekly cycles of treatment with

GCV or reovirus (**Fig 1C, D**). We have previously shown that clearance of B16TK tumors by GCV in immune competent mice is dependent upon CD8 T cells, and that tumor-cured mice have significant CD8 T cell responses against parental B16 cells[20-22]. Purified CD8 T cells from mice which had rejected B16TK tumors following GCV therapy (tumor experienced CD8; T.E. CD8) killed target B16TK cells and produced IFNγ at low levels *in vitro* when cultured at an effector to target (E:T) ratio of 10:1. (**Fig 1E**). We therefore reasoned that the combination of GCV, or reovirus, with T.E. CD8 T cells would lead to enhanced cumulative cell killing. However, to our surprise, when purified T.E. CD8 T cells were co-cultured with B16TK cells at an E:T ratio of 10:1 at the time of treatment with GCV or infection with reovirus, we observed a significant increase in the number of B16TK cells which survived compared to those treated in the absence of T cells. In contrast, we did not observe this enhanced outgrowth when B16TK cells were treated with GCV or reovirus in the presence of CD8 T cells purified from naïve C57/BL6 mice (**Fig 1F**). We confirmed that the functionality of the T.E. CD8 T cells was not compromised by GCV. The cytotoxicity of the T.E. CD8 T cells was unaffected, and the secretion of IFNγ was only modestly reduced in the presence of GCV when they were co-cultured with parental B16 cells, as opposed to B16TK cells (**Supplemental Fig 1A**). In addition, the activation of OT-I CD8 T cells was increased in the presence of reovirus (**Supplemental Fig 1B**) showing that the virus does not diminish CD8 T cell function and cannot explain the increased survival of the target cells.

A CD8 T cell mutator phenotype is associated with C-T mutation of target antigen

To investigate this phenomenon in a model with a defined antigenic target, we evaluated the potential of B16OVA cells to escape therapy when co-cultured with *in vitro* activated CD8 OT-I T cells. At high E:T ratios (50:1 and 10:1), no discrete surviving escape colonies of B16OVA were observed (**Supplemental Table 1**). At lower ratios

(5:1 and 1:1) individual colonies of B16OVA cells could be isolated as escape variants, and were subsequently resistant to further OT-I killing, even at high E:T ratios (**Supplemental Fig 2**). B16TK cells do not express the *ova* gene and were therefore not targeted by OT-I T cells. We then introduced either naïve or T.E. CD8 T cells into the OT-I CD8 T cell- B16OVA co-culture system at an T.E. effector to OT-I effector to target ratio of 10:10:1, and observed escape variant B16OVA clones emerge (**Table 1**, timeline outlined in **Fig. 2A**). In contrast, when B16OVA cells were co-cultured with activated CD8 OT-I T cells, or activated CD8 OT-I T cells in combination with naïve CD8 T cells at an E:E:T ratio of 10:10:1, complete target cell killing was observed. We isolated and expanded 15 clones from the T.E. CD8 and OT-I co-culture condition. Ten of these clones showed complete loss of the *ova* gene, consistent with our previous findings[15, 17]. However, 5 of 15 escape B16OVA clones retained the *ova* gene. Sequencing revealed that 4 of 5 clones contained a TC to TT conversion in two locations (positions 406 and 457), both of which generated a premature STOP codon upstream of the immunodominant MHC class I binding SIINFEKL epitope (**Fig 2B**). The B16OVA cells used in the experiment of **Fig 2** and **Table 1** were originally derived from a single cell clone of B16OVA selected for high level recognition by OT-I T cells and with a fully sequenced *ova* gene. In addition, none of the mutant OVA-containing clones emerged from co-culture with OT-I alone or OT-I with naïve CD8 T cells (**Table 1**). Taken together, these data suggest that these OVA mutant-containing B16OVA cells were selected for by a gain of mutation induced in the OT-I/T.E. CD8 T co-cultures.

Weak T cell responses induce APOBEC3 expression

The first hotspot C to T transition mutation in the *ova* gene was consistent with the previously reported murine APOBEC3 motif TXC, and both hotspots were consistent with that of the APOBEC3B cytosine deaminase with an A in the +1 position (TCA) [4, 12, 25-27]. We therefore hypothesized that T cell interaction may induce an equivalent

murine APOBEC3B-like activity in tumor cells, which plays a role in generating cellular mutations that allow for escape from therapy. We therefore evaluated the expression of mAPOBEC3 by qRT-PCR in tumor cells following co-culture with tumor reactive T cells at effector to target ratios at which escape variants were observed.

mAPOBEC3 mRNA expression rose sharply after 12 hours of co-culture with OT-I or T.E. CD8 T cells, as well as following treatment with the PKC activator PMA[9] (**Fig 3A**). Similarly, mAPOBEC3 protein was induced in B16OVA cells at suboptimal E:T ratios with OT-I CD8 T cells (5:1 and 1:1), but not at a high E:T ratio (10:1) (**Fig 3B**), consistent with the outgrowth of escape variants (**Table 1**). This same effect was observed in B16TK cells co-cultured with T.E. CD8 T cells at low E:T ratios, with maximal upregulation of mAPOBEC3 at a ratio of 10:1 (**Fig 3C**). The different T.E. CD8 and OT-I E:T ratios required for maximal mAPOBEC3 induction likely reflects the lower frequency of antigen-specific T cells in the T.E. CD8 population. We also confirmed the upregulation of mAPOBEC3 by western blot at the respective suboptimal T cell effector to target ratios (**Fig 3D**).

Upregulation of APOBEC3 in tumor cells at suboptimal effector to target ratios inversely correlated with the secretion of the effector cytokine IFNγ (**Fig 3E**). Co-culture of CD8 OT-I cells with B16OVA cells at an E:T ratio of 10:1 induced robust IFN-γ production, but little mAPOBEC3 expression, and as the ratio was reduced, APOBEC3 expression rose. Naïve OT-I cells that had not previously been activated *in vitro,* but which express a transgenic TCR specific for the SIINFEKL epitope of OVA, produced low levels of IFNγ when co-cultured with B16OVA cells (which present SIINFEKL at low levels to the OT-I T cells), and stimulated high levels of mAPOBEC3 expression from the target cells. Conversely, naïve OT-I cells co-cultured with B16OVA cells in the presence of exogenous SIINFEKL peptide produced high IFNγ, and low levels of mAPOBEC3

induction. High E:T ratios were also associated with high levels of TNFα secretion from OT-I T cells (**Fig 3F**), which decreased as the number of activated T cells was reduced.

To show the relationship between the E:T ratio of T cell killing, TNF-α levels, and mAPOBEC3 induction, we used a transwell co-culture system in which B16OVA target tumor cells were co-cultured with effector OT-I T cells in the upper chambers, and (bystander) B16OVA cells were plated in the lower chambers (**Fig 3G**). In the upper chamber, at the high E:T ratio of 10:1 OT-I: tumor cells, most of the target B16OVA tumor cells were killed (**Fig 3H**), with correspondingly high levels of T cell activation-associated TNFα in the cultures (**Fig 3I**), such that levels of mAPOBEC3 could not be measured because of a lack of surviving cells (**Fig 3J**). At the lower E:T ratios of 5:1 and 1:1 direct T cell-mediated tumor cell killing was greatly reduced (**Fig 3H**). However, in these surviving tumor cells, levels of APOBEC3 were significantly elevated (**Fig 3J**), and were associated with high levels of TNFα induced by the T cell co-cultures (even though these E:T ratios were not able directly to kill B16OVA at high levels) (**Fig 3I**). At all E:T ratios of T cell co-cultures in the upper chambers, the bystander B16OVA tumor cells not directly exposed to T cells in the lower chambers survived at high levels (**Fig 3H**). However, as a result of the T cell activity in the upper chambers, TNFα was still detected at significant levels above background in the lower chambers (**Fig 3I**), and this was associated with induction of mAPOBEC3 in the bystander B16OVA cells (**Fig 3J**).

Those bystander B16OVA cells which survived in the lower chambers following exposure to TNFα, and in which mAPOBEC3 had been induced (**Figs 3H-J**), were significantly more resistant to killing by OT-I T cells when re-plated in fresh co-cultures than were parental B16OVA cells (**Fig 3K**). The bystander B16OVA cells recovered from the lower chambers of the E:T 10:1 co-cultures were the most resistant to subsequent OT-I cell killing (**Fig 3K**). These bystander B16OVA cells had been exposed to the

highest levels of TNFα as a result of T cell activation and killing in the upper chambers (**Fig 3I**, lower chambers) and had the highest levels of mAPOBEC3 induction (**Fig 3J**, lower chambers). The levels of resistance to OT-I T cell killing in these bystander B16OVA cells were equivalent to that induced by *de-novo* over-expression of mAPOBEC3 in B16OVA cells (**Fig 3K&L**), suggesting that mAPOBEC3 is a major mediator of the T cell induced, bystander tumor cell escape from therapy. Taken together, these data show that when ineffective T cell killing clears only a proportion of tumor cells, bystander cells, either directly in contact with T cell activity, or which are physically separated from the T cells themselves yet are exposed to T cell- derived factors such as TNFα, can upregulate mAPOBEC3 expression and acquire mutations which may provide them with a selective advantage.

We also confirmed that a low E:T ratio of tumor antigen specific T cells to tumor cells induced mAPOBEC3 not only in the B16 cell line but also in both the GL261 glioma line (**Supplemental Fig 3A**) and the lung carcinoma LLC line (**Supplemental Fig 3B**). Taken together, these data show that there is a threshold where suboptimal T cell activation and limited effector function induces APOBEC3 upregulation in tumor cells.

mAPOBEC3 induction and tumor cell outgrowth from GCV and T cell therapy was dependent on MHC class I recognition of tumor cells, TNFα secretion, and activation of PKC signaling[9], as antibody blockade of H-2K^b, TNFα, or pharmacologic inhibition of PKC by AEB071 ablated the effect (**Figs 4A, B**). In contrast, an IFNγ blocking antibody had no significant effect on tumor cell mAPOBEC3 expression following CD8 T cell co-culture (**Fig 4C**). mAPOBEC3 induction in B16TK cells was not affected by the DMSO solvent used to dilute the AEB071 drug or by the IgG control antibody (**Supplemental Fig 4A**). None of the treatments in **Fig.4A** significantly inhibited the growth of B16TK cells alone (**Supplemental Fig 4B**). Consistent with a role for

TNFα in T cell mediated mAPOBEC3 induction in tumor cells, exogenous TNFα was sufficient to induce mAPOBEC3 (**Fig 4D and E**), an effect which was almost completely inhibited by AEB071 action upon the tumor cells themselves (**Fig 4E**). Although the cytotoxicity of the T.E. CD8 T cells was not significantly altered in the presence of AEB071 compared to PBS, the levels of IFNγ were significantly decreased in the presence of AEB071 (**Supplemental Fig 4C**). Therefore, the attenuated induction of mAPOBEC3 seen in the presence of T.E. CD8 T cells and AEB071 in **Fig 4A** may indeed be the result of reduced induction of APOBEC3 in the tumor cells through inhibition of PKC signaling (**Fig 4E**), and/or the result of partial inhibition of CD8 T cell function by AEB071.

The CD8 T cell induced mutator activity is dependent on APOBEC3

To confirm that mAPOBEC3 was required for the outgrowth of escape variants in this model, we generated a stable B16TK cell line expressing 4 unique 29mer shRNA constructs targeting mAPOBEC3 as well as a stable B16TK cell line with a single scrambled shRNA construct. mAPOBEC3 expression was significantly reduced in B16TK shRNA mAPOBEC3 cells, both at basal levels, and upon induction with PMA (**Fig 5A**). In cultures treated with no CD8 T cells, or with naïve CD8 T cells, fewer B16TK sh mAPOBEC3 cells survived either GCV or reovirus treatments compared to parental B16TK or B16TK (scrambled shRNA) cells (Fig.5B & C). These data are consistent with a role for mAPOBEC3 expression in mediating mutagenesis which facilitates escape from either of these therapies. We did not observe a statistically significant increase in the number of surviving B16TK sh mAPOBEC3 cells treated with GCV or reovirus when cells were co-cultured with T.E. CD8 T cells according the schedule in **Fig 2A (Fig 5B & C)**. B16TK parental or B16TK scrambled shRNA cells treated with GCV and co-cultured with T.E. CD8 exhibited the pattern of enhanced escape, in contrast to co-culture with

naïve CD8 T cells. These results were recapitulated with reovirus infection, where more treatment resistant clones arose from parental B16TK and B16TK cells with scrambled shRNA than from B16TK sh mAPOBEC3 cells co-cultured with T.E. CD8 T cells (**Fig 5C**). The three B16TK, B16TK sh mAPOBEC3 and B16TK scrambled shRNA cell lines grew at similar rates *in vitro* (**Supplemental Fig 5A**), therefore confirming that these results were not attributable to different rates of cell growth. In addition, more treatment-resistant clones could be isolated from parental B16OVA and from B16OVA cells with scrambled shRNA, than from B16OVA sh mAPOBEC3 cells co-cultured with OT-I CD8 T cells (**Supplemental Fig 5B**).

These results were validated *in vivo* where B16TK cells transduced with the scrambled shRNA or the mAPOBEC3- targeting shRNA were implanted subcutaneously and treated with a suboptimal course of GCV. While B16TK (scrambled shRNA) tumors all eventually escaped therapy (0/7 long term survivors) (**Fig 5D**), recurrence was delayed, or did not occur, in B16TK tumors transduced with mAPOBEC3 shRNA (4/7 long term survivors) (**Fig 5E**). These results could not be attributed to different growth rates of the tumors, as untreated tumors which expressed either scrambled shRNA or sh mAPOBEC3 grew at equivalent rates (**Supplemental Fig 5C**). This confirms that mAPOBEC3 expression after sub-optimal therapies can contribute to the generation of treatment- resistant clones *in vivo*.

As the knockdown of mAPOBEC3 significantly reduced the ability of B16TK cells to escape GCV treatment (**Fig 5B-E**), we evaluated how the overexpression of mAPOBEC3 could promote therapeutic escape in the previously described 21-day treatment cycle (**Fig 5F**). The T cell mediated induction of a TCA-TTA mutation in the *Ova* gene which allowed escape from OT-I T cell therapy (**Fig 2**) suggested that mAPOBEC3 may have overlapping functionality and specificity with its human APOBEC3B counterpart, and therefore we additionally overexpressed hAPOBEC3B in

B16TK cells. Consistent with this hypothesis, we observed that overexpression of either mAPOBEC3 or hAPOBEC3B both promoted the outgrowth of treatment resistant clones compared to unmodified B16TK cells. Moreover, overexpressed mAPOBEC3 or hAPOBEC3B was able to rescue the phenotype induced by shRNA knockdown of APOBEC3 in B16TK cells treated with GCV. In contrast, overexpression of a catalytically inactive form of the protein, hAPOBEC3B MUT, did not promote resistance. Taken together, these data confirm that mouse APOBEC3 activity, as shown to be induced through T cell derived TNFα (**Fig 3**), can explain the induction of resistance to GCV killing that we observed in **Figs 1-4**.

We further investigated the mechanism of GCV escape by sequencing the bulk populations from each condition in **Fig 5F**. B16TK parental cells that escaped GCV treatment *in vitro* retained the wild type *HSV-TK* sequence, suggesting that failure to eradicate these cells was not due to mutation of the therapeutic gene (**Fig 5G**). In contrast, B16TK cells engineered to overexpress either hAPOBEC3B, or mAPOBEC3, both selected populations of cells in which the *HSV-TK* gene contained an ATCA-ATTA mutation (**Fig 5G**). This mutation introduced a STOP codon in the first 8 amino acids of the protein, thereby preventing expression of functional HSV-TK protein to maintain susceptibility to GCV therapy. These data provide strong evidence that (1) mAPOBEC3 has overlapping mutational specificity with hAPOBEC3B and (2) provides a direct mechanism by which the acquired resistance to GCV therapy that we observe upon low E:T ratio T cell activity can be attributed to mAPOBEC3 activity.

hAPOBEC3B overexpression drives tumor escape

We further evaluated the role of APOBEC3B in the acquisition of therapeutic resistance using the retroviral overexpression system of human APOBEC3B used in Fig 5F. B16 tumor cell lines were engineered to overexpress hAPOBEC3B, or a catalytically

inactive form of the protein hAPOBEC3B MUT. 48 hours post transduction, bulk populations of cells were selected in hygromycin for 2 weeks and used for experiments. We have observed that over-expression of hAPOBEC3B is toxic in that elevated levels of hAPOBEC3B are seen within 72 hours post transfection/infection and then return to similar levels to that seen in parental unmodified cells. We believe that this is because mutagenesis by hAPOBEC3B is tolerable to the cell up to a certain threshold, and then in cells where critical mutations are induced, this can be lethal. In other cells, overexpression of hAPOBEC3B may not reach the threshold, or mutations may not be induced in critical genes, allowing those cells to survive carrying the hAPOBEC3B induced mutations. The increased frequency of GCV resistant tumor cell outgrowth that had previously been seen when B16TK cells were co-cultured with T.E. CD8 T cells was recapitulated by the overexpression of hAPOBEC3B in B16TK cells (**Fig 6A**). In contrast, we did not observe an increase in the number of treatment-resistant cells derived from B16TK cells expressing a catalytically inactive mutant hAPOBEC3B. In parallel, hAPOBEC3B overexpression in B16TK cells increased the rate of outgrowth following reovirus infection (**Fig 6B**). Together, these experiments demonstrate that hAPOBEC3B function is sufficient to support therapeutic escape.

Finally, when implanted intracranially in a model of metastatic melanoma, B16TK-hAPOBEC3B tumors grew significantly more quickly following GCV treatment compared to B16TK-hAPOBEC3B MUT tumors (**Fig 6C**). These results could not be attributed to different growth rates of the tumors, as untreated tumors which overexpressed either hAPOBEC3B or mutated APOBEC3B grew at equivalent rates (**Supplemental Fig 5D**). Western blot analysis of intracranial tumors recovered from mice showed that 4/4 B16TK-hAPOBEC3B MUT tumors continued to express the HSV TK protein, despite eventually failing therapy, whereas 0/4 B16TK hAPOBEC3B tumors continued to express HSV TK protein (**Fig 6D**). Sequencing of the HSV TK gene

identified a C to T mutation in the coding region at position 22 within the characteristic hAPOBEC3B TCA motif in all four B16TK hAPOBEC3B tumors that was not observed in the parental B16TK cell line (**Fig 6E**). This mutation converted a (CAA) glutamine to a (TAA) stop codon within the first 10 amino acids of the protein and was the same mutation observed *in vitro* (**Fig 5F**). None of the B16TK-APOBEC3B MUT tumors contained this mutation, or any detectable characteristic APOBEC3 C to T mutation within the TCW motif in the HSV TK gene. Finally, B16TK-hAPOBEC3B subcutaneous tumors also grew significantly more quickly following GCV treatment compared to parental B16TK or B16TK-hAPOBEC3B MUT tumors, even though untreated tumors grew at equivalent rates (**Fig 6F**). These data strongly support the hypothesis that hAPOBEC3B expression drives a mutator phenotype in tumor cells which allows for selection of a tumor cell clone that can resist treatment and emerge *in vivo* despite powerful treatment pressure.

Discussion

We began our study with the hypothesis that sub-optimal therapies would be improved by combination with T cell therapies in which tumor reactive T cells were used as an adjunct to the frontline treatment. However, we report here a novel mechanism by which CD8 T cells undergoing low E:T ratio interactions with tumor cells can promote an increase in the mutational burden of the tumor cells and drive escape from a co-applied therapy via the induction of mAPOBEC3. We observed that suboptimal effector to target ratios induced mAPOBEC3 expression in tumor cells via a mechanism that was dependent upon interaction with MHC class I, TNFα secretion, and PKC signaling. Blocking any of these molecules, or knocking down endogenous mAPOBEC3 expression, negated the T cell induced mutator phenotype *in vitro* and *in vivo*. Finally,

consistent with the hypothesis that the single murine APOBEC3 protein may, amongst other properties, mimic the cancer-driving activity of the human APOBEC3B protein, over-expressing hAPOBEC3B in tumor cells enhanced escape from either suicide gene therapy or oncolytic virotherapy *in vitro* and *in vivo.*

It is known that threshold levels of T cell interaction with target cells are required for cell killing to occur[28]. Thus, successful T cell killing of target cells results from a combination of sufficient affinity of the TCR for the antigen-MHC complex, as well as the number of engagements made. However, due to the nature of self, or near-self, of tumor associated antigens, most therapies which induce anti-tumor T cell responses either directly (vaccines, oncolytic viro-immunotherapy) or indirectly (chemotherapy, radiation) generate T cells with a low affinity TCR and/or low frequency T cell responses. Despite the fact that the tumor experienced CD8 T cells had a relatively low recognition of target B16TK cells, we still predicted that therapy with GCV, or reovirus virotherapy, would lead to *enhanced* cumulative cell killing in combination with these tumor reactive CD8 T cells. However, to our surprise, we observed the opposite effect at low effector to target ratios. Weakly reactive CD8 T cells promoted tumor cell acquisition of a mutator phenotype that enhanced their ability to acquire resistance to a frontline treatment of chemotherapy or virotherapy (**Fig 1E**).

Using a co-culture system of B16OVA and OT-I T cells, we traced the molecular events occurring in the *Ova* gene that drove tumor cell escape. At low E:T ratios (5:1 and 1:1) B16OVA cells escaped from OT-I T cell therapy *in vitro*, consistent with our previous results in this system[15] (**Supplemental Table 1, Table 1, and Fig 2**). However, at a higher E:T ratio of OT-I: B16OVA (10:1) at which no resistance was seen, the addition of weakly tumor reactive T cells actually promoted tumor cell escape from OT-I therapy. Although most of the resistant clones studied had lost the *ova* gene and accounted for their escape from OT-I killing (**Fig 2**), 5 resistant clones retained the *Ova*

transgene, and of those, 4 had mutations which prevented the translation of the OT-I-recognized SIINFEKL epitope. The two recurrent mutations that were observed in the *Ova* coding sequence (C to T transitions) were consistent with the enzymatic signature of the APOBEC3 family of DNA deaminase enzymes, and suggested that it would be a likely candidate effector mechanism. The mutations observed in the ovalbumin gene highlight the likelihood that APOBEC3 family members may play an important role in tumor immunoediting. We hypothesize that evidence of APOBEC3 enzymatic activity in tumor cells co-cultured with the polyclonal tumor experienced T cell repertoire may be observed more broadly in the tumor genome, including genes involved in antigen processing and presentation. Indeed, the presence of APOBEC3 signature mutations have been associated with the loss of heterozygosity of human leukocyte antigen (HLA) and thus has been proposed as a mechanism of immune-evasion[29]. The expression of a variety of APOBEC3s across multiple cancer types has been associated with lymphocyte infiltration, mutational burden, and PDL-1 and PDL-2 expression, in TCGA datasets [19, 30, 31]. This type of *post-hoc* TCGA analysis cannot distinguish between T cells which were present and responding to neoantigens generated by prior APOBEC3 activity, or the presence of tumor reactive T cells which produce TNFα and in turn promote APOBEC3B activity. Nonetheless, the clinical picture is consistent with a correlation between the presence of T cell activity and APOBEC3 family members playing an important role in increasing the mutagenic fuel that may promote immunoediting.

We have demonstrated that B16 cells can escape from a low MOI reovirus infection over a 21 day culture period *in vitro* (**Figs 1D&F**). This escape is almost certainly mediated by an anti-viral response, which itself may involve APOBEC3 activity against the virus in the B16 tumor cells. We also show that the presence of tumor specific T cells, but not naïve T cells, mediated both increased mAPOBEC3 expression

in the tumor cells (**Fig 3**), and increased resistance to reovirus oncolysis, over and above that induced within the B16 cells themselves (**Fig 1F**). In the case of the OVA or HSV-TK models, a very defined selective pressure enabled us to define a mechanism driven by APOBEC3 whereby the integrity of the transgene is compromised. However, the mechanism by which APOBEC3 impedes oncolytic viral replication, spread, or oncolysis, may include the mutation of any number of cellular innate anti-viral genes, or indeed, the mutation of the virus genome. In order to distinguish between these two non-mutually exclusive possibilities, we are currently sequencing the genomes of the cells and viruses which have escaped treatment as a result of APOBEC3 induction.

The data of **Figure 3** show that, where it is possible to achieve high ratios of effector T cell:target tumor cells, target tumor cells are efficiently killed. However, where these E:T ratios fall below the threshold at which high levels of tumor cell killing are possible, byproducts of T cell activation, such as TNFα, are associated with the induction of APOBEC3 in the surviving, bystander tumor cells. This induction of APOBEC3 may, therefore, serve as a major mediator of the ability of those bystander tumor cells to evolve away from further waves of T cell-, or other types of therapy-mediated-, tumor cell killing through the induction of genomic mutations of the type we observed in **Figures 1&2**. Maximal APOBEC3 expression was induced at lower E:T ratios of OT-I CD8 T cells (1:1) than CD8 T cells from T.E. mice (10:1). This difference is likely due to the much lower frequency of antigen-specific T cells in the T.E. CD8 population and/or because of the difference in affinities of the TCR for their cognate antigens. In this respect, OT-I T cells express a high affinity TCR which recognizes the $OVA_{257–264}$ SIINFEKL epitope presented in the context of H2-K^b. In contrast, the CD8 T cells recovered from tumor experienced C57Bl/6 mice which had rejected B16TK tumors are likely to have only low affinity TCR for B16-expressed self, or near self, tumor

antigens. Further study is warranted to fully characterize how the affinity and avidity of the T cell receptor engagement influence the induction of a mutator phenotype.

T cell interaction induced APOBEC3 expression through a mechanism dependent upon MHC class I, TNFα, and PKC (**Fig 4**), consistent with previous reports of APOBEC3 induction through the PKC pathway [9, 32]. Interestingly, although in our system APOBEC3 expression in tumor cells was not dependent upon IFNγ, other studies have shown that IFNγ produced by tumor reactive T cells can drive tumor heterogeneity through genome editing mechanisms associated with DNA damage response pathways[33]. Therefore, it is clear that the tumor reactive T cells can shape the tumor response to therapy in multiple ways. We hypothesize that APOBEC3 inhibition may in fact be an important adjuvant to all direct and indirect immunotherapeutic platforms to reduce the risk of treatment failure and prevent recurrence. This may be particularly relevant to oncolytic viral therapies, as well the treatment of virally induced cancers associated with HPV[34] or EBV[35] infection, to which APOBEC3 induction is a natural response to restrict viral infection. Although no direct APOBEC3B inhibitors have been identified, indirect inhibition of PKC with AEB071[9], or B-Myb with EGFR inhibitors[36] represent feasible clinical approaches.

Similarly, shRNA-mediated knockdown of APOBEC3 expression in B16TK cells significantly reduced the ability of these cells to develop resistance to either GCV or virotherapy *in vitro* and to suicide gene therapy *in vivo* (**Fig 5**). Our results here show that a burst of *de novo* expression of mouse APOBEC3 (through T cell derived TNFα or through overexpression) increases the resistance of B16TK cells to frontline therapies, such as GCV killing or reovirus oncolysis. However, the results of **Figs 5B&C** show that there were fewer surviving B16TK cells in which mAPOBEC3 was knocked down by shRNA than in parental B16 cells, even with no CD8 T cells or with naïve T cells

present. These data suggest that reducing the steady state levels of endogenous mAPOBEC3 expression still prevents the acquisition of resistance to selection by GCV. The mechanism of this resistance was not through mutation of the *HSV-TK* transgene, since parental B16TK cells, expressing steady state mAPOBEC3, maintained a wild type functional *HSV-TK* sequence through the GCV selection process both *in vitro* (**Fig 5G**) and *in vivo* (**Fig 6E**). Therefore, it may be that endogenous, steady state expression of mAPOBEC3, in addition to induced mAPOBEC3, still contributes to the emergence of resistance, perhaps through the mutation of genes associated with other aspects of GCV transport and/or metabolism by the cell.

hAPOBEC3B overexpression promoted an aggressive tumor phenotype that rapidly acquired resistance to GCV therapy through a mutation that introduced a premature STOP codon in the TK gene (**Fig 6**). Indeed, hAPOBEC3B is a potent source of mutagenesis, and its overexpression could both functionally recapitulate and accelerate the generation and selection of clonal variants subjected to therapeutic pressure that had previously been seen with the tumor experienced CD8 T cells. These data demonstrate that APOBEC3 family expression in B16TK tumor cells contributes significantly to intrinsic mechanisms that promote tumor cell escape from therapy.

Additional cell types in the tumor microenvironment that produce TNFα, such as NK cells or macrophages, could also upregulate APOBEC3. Indeed, the central role of TNFα in APOBEC3 regulation is consistent with our finding that TNFα derived from CD11b cells promoted tumor recurrence[37].

In summary, our data here suggest that the generation of weak affinity and/or low frequency, sub-optimal T cell responses against TAAs may actively drive a mutator phenotype in tumors through APOBEC3 activity and promote the emergence of treatment resistant tumor populations. Hence, whenever potentially immunogenic

frontline therapies are being administered to patients, it will be important to try to optimize the generation of potent CD8 T cell responses, with high frequencies of high affinity circulating anti-tumor T cells in order to reduce any T cell mediated induction of tumor escape and recurrence.

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Disclosure of Potential Conflicts of Interest

Matt Coffey is an employee of Oncolytics Biotech Inc. No potential conflicts of interest were disclosed by the other authors.

References

1. Swanton, C., et al., *APOBEC Enzymes: Mutagenic Fuel for Cancer Evolution and Heterogeneity.* Cancer Discov, 2015. **5**(7): p. 704-12.
2. Mertz, T.M., V. Harcy, and S.A. Roberts, *Risks at the DNA Replication Fork: Effects upon Carcinogenesis and Tumor Heterogeneity.* Genes (Basel), 2017. **8**(1).
3. Harris, R.S. and M.T. Liddament, *Retroviral restriction by APOBEC proteins.* Nat Rev Immunol, 2004. **4**(11): p. 868-77.
4. Roberts, S.A., et al., *An APOBEC cytidine deaminase mutagenesis pattern is widespread in human cancers.* Nat Genet, 2013. **45**(9): p. 970-6.
5. Burns, M.B., et al., *APOBEC3B is an enzymatic source of mutation in breast cancer.* Nature, 2013. **494**(7437): p. 366-70.
6. Walker, B.A., et al., *APOBEC family mutational signatures are associated with poor prognosis translocations in multiple myeloma.* Nat Commun, 2015. **6**: p. 6997.
7. Law, E.K., et al., *The DNA cytosine deaminase APOBEC3B promotes tamoxifen resistance in ER-positive breast cancer.* Sci Adv, 2016. **2**(10): p. e1601737.
8. Cescon, D.W., B. Haibe-Kains, and T.W. Mak, *APOBEC3B expression in breast cancer reflects cellular proliferation, while a deletion polymorphism is associated with immune activation.* Proc Natl Acad Sci U S A, 2015. **112**(9): p. 2841-6.
9. Leonard, B., et al., *The PKC/NF-kappaB signaling pathway induces APOBEC3B expression in multiple human cancers.* Cancer Res, 2015. **75**(21): p. 4538-47.
10. Maruyama, W., et al., *Classical NF-kappaB pathway is responsible for APOBEC3B expression in cancer cells.* Biochem Biophys Res Commun, 2016. **478**(3): p. 1466-71.

11. Conticello, S.G., et al., *Evolution of the AID/APOBEC family of polynucleotide (deoxy)cytidine deaminases.* Mol Biol Evol, 2005. **22**(2): p. 367-77.
12. MacMillan, A.L., R.M. Kohli, and S.R. Ross, *APOBEC3 inhibition of mouse mammary tumor virus infection: the role of cytidine deamination versus inhibition of reverse transcription.* J Virol, 2013. **87**(9): p. 4808-17.
13. Bezu, L., et al., *Combinatorial strategies for the induction of immunogenic cell death.* Front Immunol, 2015. **6**: p. 187.
14. Boisgerault, N., et al., *Functional cloning of recurrence-specific antigens identifies molecular targets to treat tumor relapse.* Mol Ther, 2013. **21**(8): p. 1507-16.
15. Kaluza, K.M., et al., *Adoptive T cell therapy promotes the emergence of genomically altered tumor escape variants.* Int J Cancer, 2012. **131**(4): p. 844-54.
16. Kottke, T., et al., *Detecting and targeting tumor relapse by its resistance to innate effectors at early recurrence.* Nat Med, 2013. **19**(12): p. 1625-31.
17. Kaluza, K., et al., *Adoptive transfer of cytotoxic T lymphocytes targeting two different antigens limits antigen loss and tumor escape.* Hum Gene Ther, 2012. **131**: p. 844-854.
18. Kaluza, K.M., et al., *Adoptive T cell therapy promotes the emergence of genomically altered tumor escape variants.* Int J Cancer, 2012. **Sep 20**: p. [Epub ahead of time].
19. Boichard, A., I.F. Tsigelny, and R. Kurzrock, *High expression of PD-1 ligands is associated with kataegis mutational signature and APOBEC3 alterations.* Oncoimmunology, 2017. **6**(3): p. e1284719.
20. Sanchez-Perez, L., et al., *Synergy of adoptive T-cell therapy with intratumoral suicide gene therapy is mediated by host NK cells.* Gene Therapy, 2007. **14**: p. 998-1009.

21. Vile, R.G., et al., *Generation of an anti-tumour immune response in a non-immunogenic tumour: HSVtk-killing in vivo stimulates a mononuclear cell infiltrate and a Th1-like profile of intratumoural cytokine expression.* Int J Cancer, 1997. **71**: p. 267-274.

22. Vile, R.G., et al., *Systemic gene therapy of murine melanoma using tissue specific expression of the HSVtk gene involves an immune component.* Cancer Research, 1994. **54**: p. 6228-6234.

23. Hogquist, K.A., et al., *T cell receptor antagonistic peptides induce positive selection.* Cell, 1994. **76**: p. 17.

24. Carlson, B.L., et al., *Establishment, maintenance and in vitro and in vivo applications of primary human glioblastoma multiforme (GBM) xenograft models for translational biology studies and drug discovery.* Curr Protoc Pharmacol, 2011. **Chapter 14**: p. Unit 14 16.

25. Shi, K., et al., *Structural basis for targeted DNA cytosine deamination and mutagenesis by APOBEC3A and APOBEC3B.* Nat Struct Mol Biol, 2017. **24**(2): p. 131-139.

26. Nair, S., et al., *Biochemical and biological studies of mouse APOBEC3.* J Virol, 2014. **88**(7): p. 3850-60.

27. Chen, J. and T. MacCarthy, *The preferred nucleotide contexts of the AID/APOBEC cytidine deaminases have differential effects when mutating retrotransposon and virus sequences compared to host genes.* PLoS Comput Biol, 2017. **13**(3): p. e1005471.

28. Schmid, D.A., et al., *Evidence for a TCR affinity threshold delimiting maximal CD8 T cell function.* J Immunol, 2010. **184**(9): p. 4936-46.

29. McGranahan, N., et al., *Allele-Specific HLA Loss and Immune Escape in Lung Cancer Evolution.* Cell, 2017. **171**(6): p. 1259-1271 e11.

30. Leonard, B., et al., *APOBEC3G Expression Correlates with T-Cell Infiltration and Improved Clinical Outcomes in High-grade Serous Ovarian Carcinoma.* Clin Cancer Res, 2016. **22**(18): p. 4746-55.

31. Smid, M., et al., *Breast cancer genome and transcriptome integration implicates specific mutational signatures with immune cell infiltration.* Nat Commun, 2016. **7**: p. 12910.

32. Mehta, H.V., et al., *IFN-alpha and lipopolysaccharide upregulate APOBEC3 mRNA through different signaling pathways.* J Immunol, 2012. **189**(8): p. 4088-103.

33. Takeda, K., et al., *IFN-gamma is required for cytotoxic T cell-dependent cancer genome immunoediting.* Nat Commun, 2017. **8**: p. 14607.

34. Henderson, S., et al., *APOBEC-mediated cytosine deamination links PIK3CA helical domain mutations to human papillomavirus-driven tumor development.* Cell Rep, 2014. **7**(6): p. 1833-41.

35. Hu, H., et al., *Epstein-Barr Virus Infection of Mammary Epithelial Cells Promotes Malignant Transformation.* EBioMedicine, 2016. **9**: p. 148-60.

36. Chou, W.C., et al., *B-Myb Induces APOBEC3B Expression Leading to Somatic Mutation in Multiple Cancers.* Sci Rep, 2017. **7**: p. 44089.

37. Timothy Kottke, L.E., Kevin G. Shim, Diana Rommelfanger, Nicolas Boisgerault, Shane Zaidi, Rosa Maria Diaz, Jill Thompson, Elizabeth Ilett, Matt Coffey, Peter Selby, Hardev Pandha, Kevin Harrington, Alan Melcher and Richard Vile, *Subversion of NK Cell and TNF-alpha Immune Surveillance Drives Tumor Recurrence.* Cancer Immunology Research, 2017.

Tables

Table 1. Generation of B16OVA escape variants in a co-culture system with OT-I T cells and T.E. CD8 T cells

Number of colonies		
OT-I only (E:T is 10:1)	OT-I + Naïve CD8 T cells (E:E:T is 10:10:1)	OT-I + T.E. CD8 T cells (E:E:T is 10:10:1)
0; 0; 0	0; 0; 0	3; 15; 1

Figures

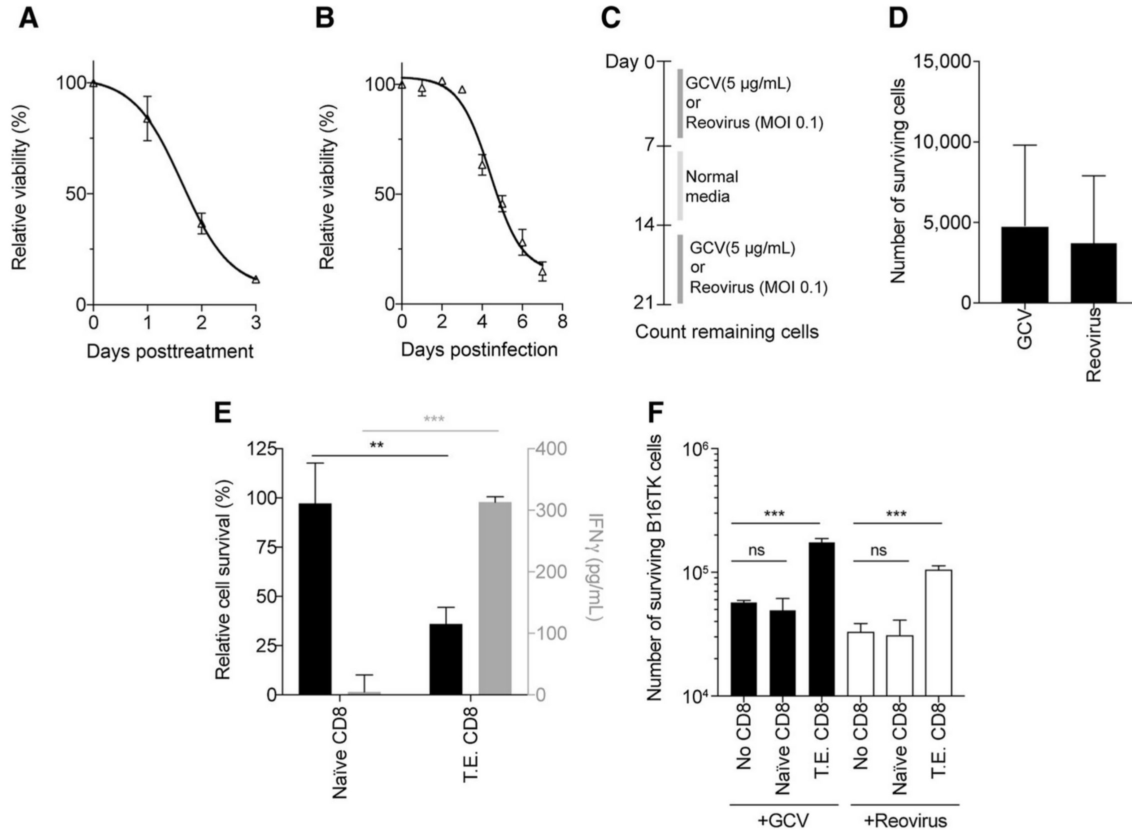

Figure 1. Tumor experienced CD8 T cells enhance escape from therapy.

B16TK cells were treated with 5 µg/mL of ganciclovir (GCV) (**A**) or reovirus at an MOI of 0.1. (**B**) Cell viability was measured using Cell titer blue and normalized to untreated cells. (**C**) Schematic timeline for the generation of escape variants. 10^4 B16TK cells were plated in the presence of GCV (5µg/ml) or reovirus (MOI 0.1) for 7 days. Wells were washed with PBS, cultured in medium for 7 days, then treated with GCV or reovirus, respectively, for a further 7 days. (**D**) B16TK cells treated according to **Fig 1C** were counted on day 21. (**E**) B16TK cells were co-cultured for 72 hours with purified CD8 T cells from untreated C57BL/6 mice (Naïve) or from mice that had previously rejected B16TK tumors following treatment with GCV (Tumor Experienced; T.E.) at an E:T ratio of 10:1. Surviving tumor cells counted (left y-axis). IFNγ in the supernatant was measured by ELISA (right y-axis). (**F**) B16TK cells were cultured according to **Fig 1C**

either with no added T cells, or with purified naïve or T.E. CD8 T cells at an E:T ratio of 10:1. Surviving tumor cells were counted at the end of the 21 day culture period. Mean ± SD of triplicate wells per treatment is shown for all panels. ns $P > 0.05$; *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$.

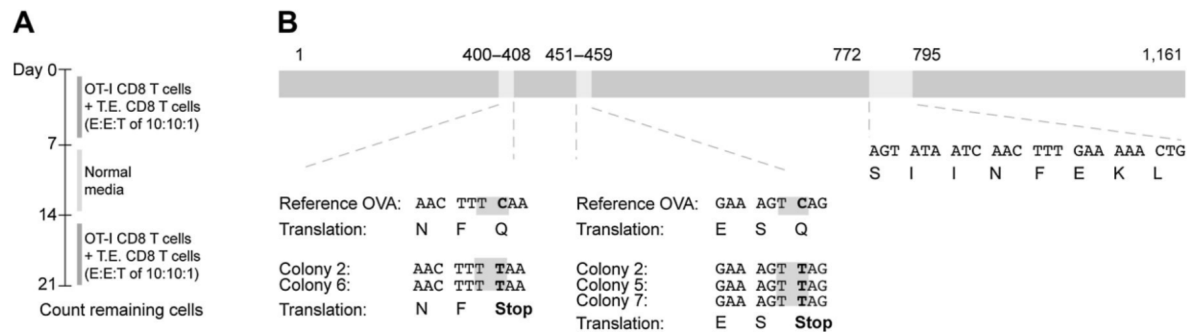

Figure 2. The T cell mutator phenotype is associated with C to T mutation

(**A**) Timeline for the generation of B16OVA escape variants. B16OVA cells were plated in the presence of *in vitro* activated OT-I CD8 T cells and purified T.E. CD8 T cells at an effector to effector to target (E:E:T) ratio of 10:10:1 for 7 days. Wells were washed 3 times with PBS and cultured in normal medium for a further 5 days. Surviving cells were then cultured again in the presence of 4-day *in vitro* activated OT-I CD8 T cells and T.E. CD8 T cells (E:E:T ratio 10:10:1) for 7 days. (**B**) The ovalbumin gene was sequenced from discrete colonies of surviving cells on day 21 following treatment from **Fig 2A**.

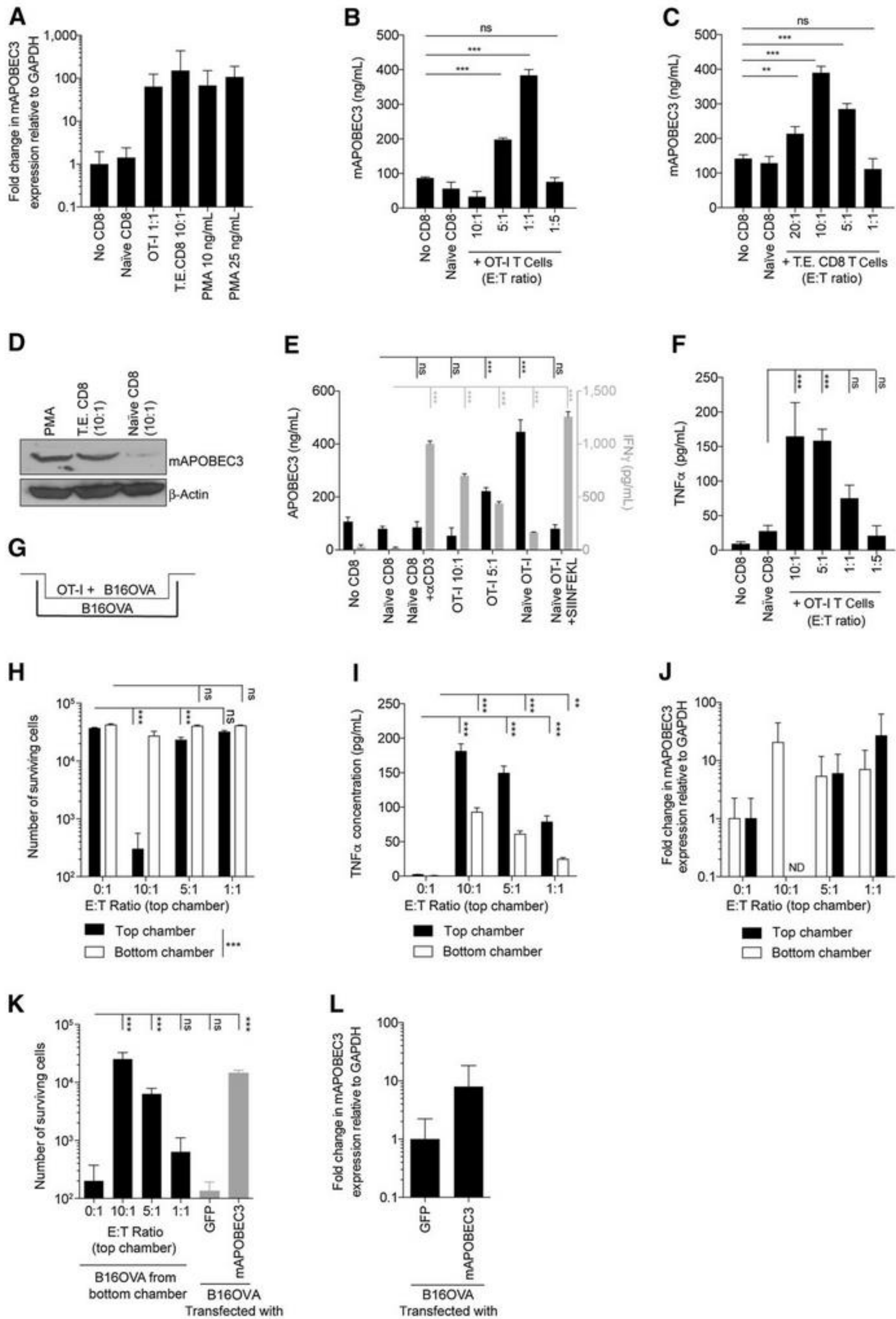
A
Fold change in mAPOBEC3 expression relative to GAPDH
No CD8
Naïve CD8
OT-I 1:1
T.E. CD8 10:1
PMA 10 ng/mL
PMA 25 ng/mL
B
mAPOBEC3 (ng/mL)
No CD8
Naïve CD8
+ OT-I T Cells (E:T ratio)
C
mAPOBEC3 (ng/mL)
+ T.E. CD8 T Cells (E:T ratio)
D
PMA
T.E. CD8 (10:1)
Naïve CD8 (10:1)
mAPOBEC3
β-Actin
E
APOBEC3 (ng/mL)
IFNγ (pg/mL)
Naïve CD8 +αCD3
OT-I 10:1
OT-I 5:1
Naïve OT-I
Naïve OT-I +SIINFEKL
F
TNFα (pg/mL)
+ OT-I T Cells (E:T ratio)
G
OT-I + B16OVA
B16OVA
H
Number of surviving cells
E:T Ratio (top chamber)
Top chamber
Bottom chamber
I
TNFα concentration (pg/mL)
E:T Ratio (top chamber)
J
Fold change in mAPOBEC3 expression relative to GAPDH
ND
E:T Ratio (top chamber)
K
Number of surviving cells
E:T Ratio (top chamber)
B16OVA from bottom chamber
GFP
mAPOBEC3
B16OVA Transfected with
L
Fold change in mAPOBEC3 expression relative to GAPDH
B16OVA Transfected with

Figure 3. Incomplete T cell killing of targets promotes mAPOBEC3 activation in bystander tumor cells

(**A**) B16TK cells were plated in the presence of no T cells, with CD8 T cells from naïve mice at an effector to target ratio of 10:1, *in vitro* activated OT-I CD8 T cells at an effector to target ratio of 1:1, with CD8 T cells from tumor experienced (T.E.) mice at an effector to target ratio of 10:1, or with PMA at 10 or 25ng/ml, for 12hrs. mAPOBEC3 expression in tumor cells was assessed by qRT-PCR. mAPOBEC3 expression levels were normalized to GAPDH and presented as fold change relative to untreated cells ± SD. (**B**) B16OVA cells were plated in the presence of *in vitro* activated OT-I CD8 T cells at various E:T ratios for 12 hours. (**C**) B16TK cells were plated in the presence of T.E. CD8 T cells at various E:T ratios for 12 hours. Tumor cells were lysed and the level of mAPOBEC3 was measured by ELISA. Mean ± SD of triplicate wells per treatment is shown. (**D**) Western blot for mAPOBEC3 in cells treated with PMA (25ng/ml), or naïve or T.E. CD8 T cells, as described in (**C**) is shown. (**E**) B16OVA cells were plated in the presence of no added CD8 T cells, naïve CD8 T cells (E:T ratio 10:1), naïve CD8 T cells activated *in vitro* with α CD3 antibody, *in vitro* activated OT-I CD8T cells at E:T ratios of 10:1 or 5:1, naïve OT-I CD8 T cells, or with naïve OT-I CD8 T cells in the presence of SIINFEKL peptide at 5μg/ml. The concentration of IFNγ in the supernatant at 12 hours was measured by ELISA (right y- axis). The levels of APOBEC3 in B16OVA cells were measured by ELISA (left y-axis). (**F**) B16OVA cells were plated in the presence of 4-day *in vitro* activated OT-I CD8 T cells at various E:T ratios for 12 hours. TNFα was measured in the supernatant by ELISA. **(G)** 24 hours following the plating of B16OVA cells in both upper and lower chambers of transwells, 2 day activated OT-I T cells were added to the upper chambers at E:T ratios of 0:1; 10:1; 5;1 or 1:1 **(H,I)** 24 hours post co-culture medium TNFα was measured by ELISA in the media from both chambers and

72 hours later, both upper and lower chambers were washed three times with PBS and the number of surviving cells in both upper or lower chambers were counted as shown. **(J)** In separate plates, B16OVA cells recovered from both upper and lower chambers at 12hrs post co-culture with OT-I in the upper chambers were recovered and levels of mAPOBEC3 expression measured by qrtPCR. **(K)** B16OVA cells transfected 48 hours previously with a plasmid expressing GFP or mAPOBEC3 (~60% transfection efficiency), or B16OVA cells recovered from the lower chambers of the experiment in **H** above, were co-cultured with 4 day activated OT-I T cells at an E:T ratio of 10:1 in triplicate. The number of surviving cells 48 hours after co-culture is shown. **(L)** qrtPCR for levels of mAPOBEC3 expression in the B16OVA cells used in **K** above (transfected 48hrs previously with a plasmid expressing GFP or APOBEC3. Means ± SD of triplicate wells are shown. ns $P > 0.05$; *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$.

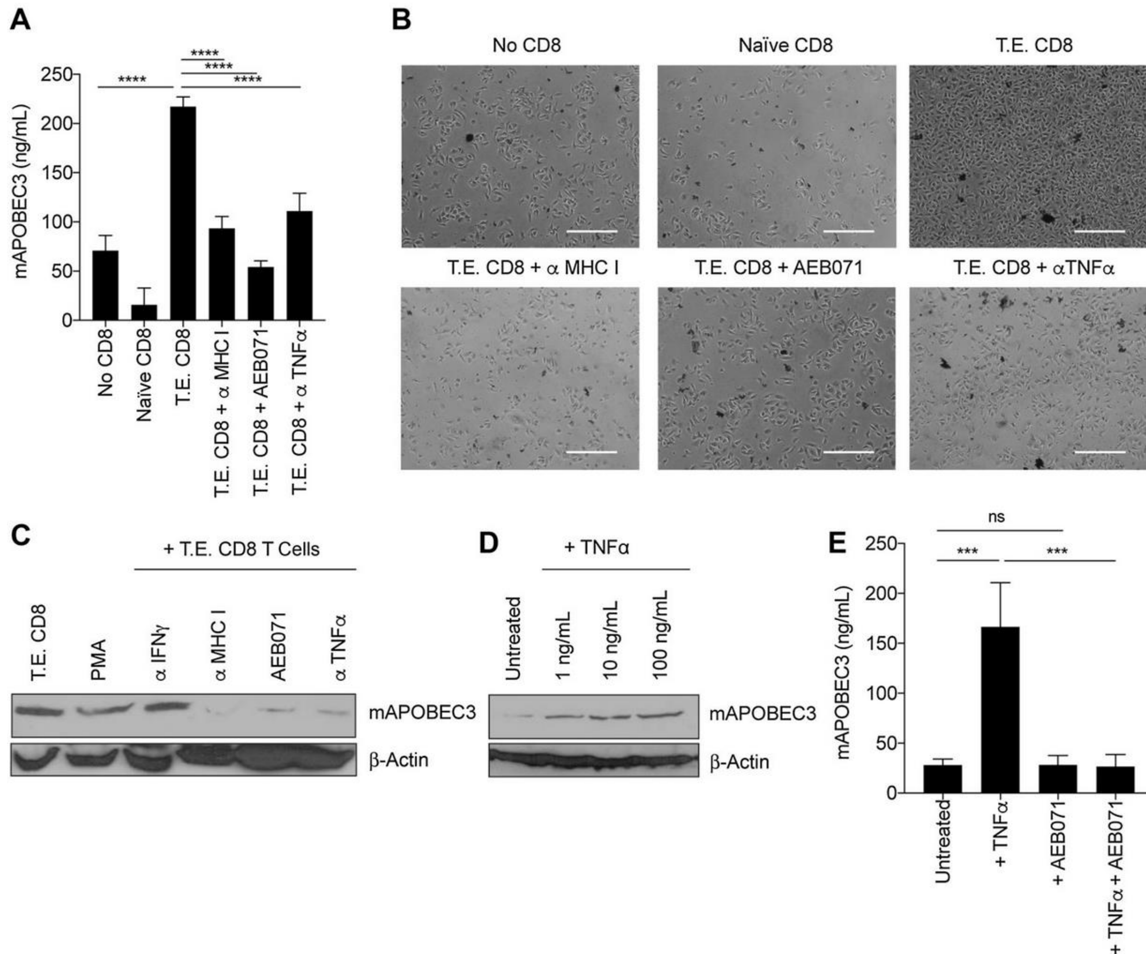

Figure 4. mAPOBEC3 is induced by T cells in an MHC class I, PKC and TNFα dependent manner.

(**A**) B16TK cells were co-cultured for 24hrs with GCV and purified naïve or T.E. CD8 T (E:T ratio 10:1) in the presence or absence of the anti-H-2K^{b} antibody (AF6-88.5; 0.5μg/ml), the inhibitor of PKC signaling (AEB071;10μM) or the anti-TNFα antibody (AF-410-NA; 0.5μg/ml). Levels of cell associated mAPOBEC3 were measured by ELISA. Means ± SD of triplicate wells are shown. (**B**) B16TK cells were cultured with the GCV, PBS, GCV regimen in **Fig 1C** for 21 days either with no added CD8 T cells or with naïve or T.E. CD8 T cells (E:T ratio 10:1). The blocking agents described in panel A were used between days 0-7 and 14-21. Micrographs were taken on day 15. Scale bar = 250 μm. (**C**) Expression of mAPOBEC3 was assessed by western blot in B16TK cells treated with

PMA (25ng/ml) or with purified T.E. CD8 T cells (E:T ratio 10:1) alone or with T.E. CD8 T cells in the presence of the anti-IFNγ antibody (MAB485; 0.5 µg/mL), anti-H-2K^b antibody AF6-88.5, the inhibitor of PKC signaling AEB071 or the anti-TNFα antibody (AF-410-NA). (**D**) B16TK cells were grown in the presence of various concentrations of TNFα for 12 hours and the expression of mAPOBEC3 was assessed by western blot (**E**) B16TK cells were grown in triplicate well for 24 hours with no added TNFα or with 10ng/ml TNFα and/or AB071 (10µM). mAPOBEC3 levels were assessed by ELISA. ns $P > 0.05$; *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$.

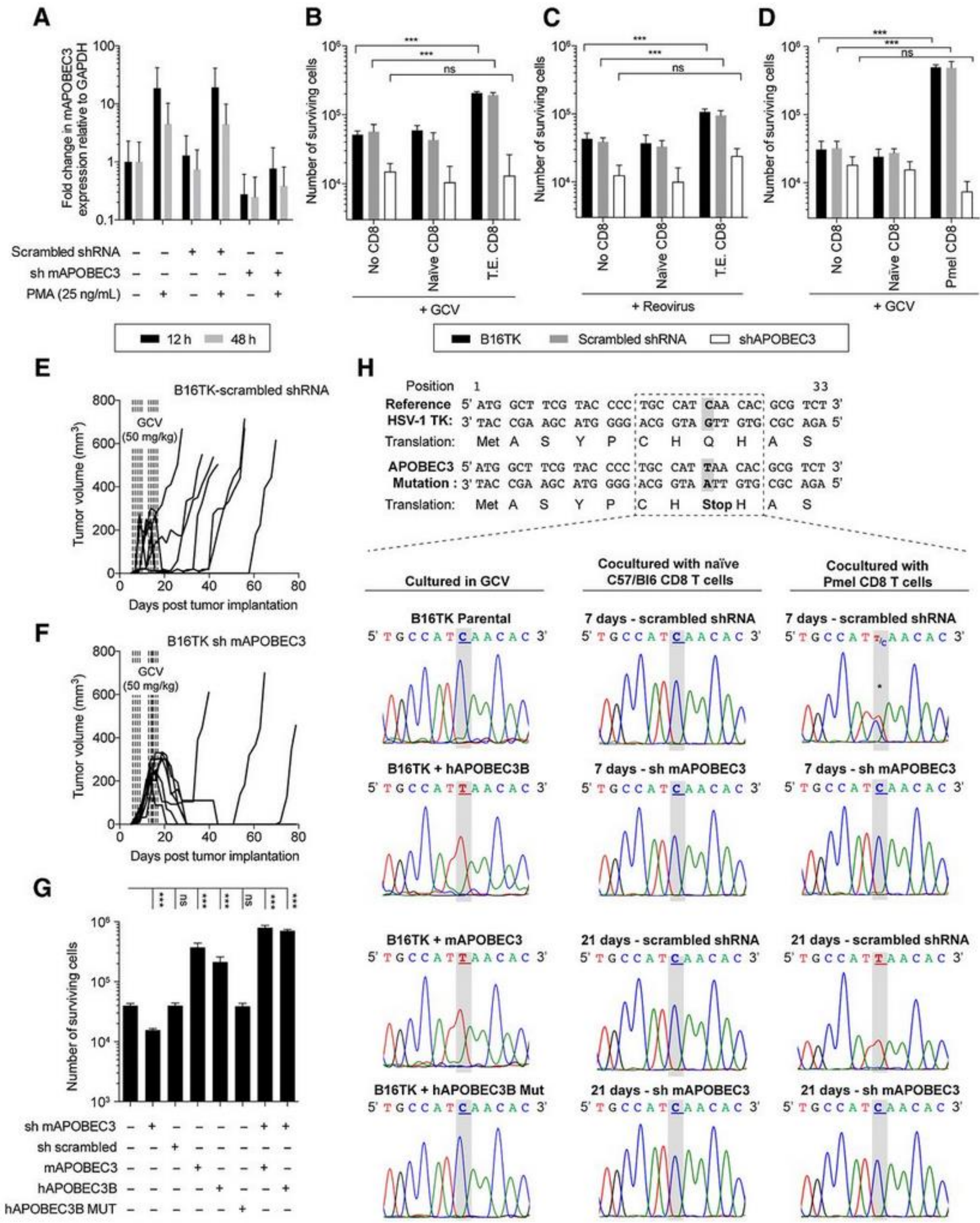

Figure 5. APOBEC3 Mediates T cell- driven mutator activity in tumor cells

(**A**) B16TK (shRNA mAPOBEC3) cells or B16TK (scrambled shRNA) were cultured for 12 or 48 hours in serum-free medium with, or without, the addition of PMA (25ng/ml) and APOBEC3 expression was assessed by qRT-PCR. mAPOBEC3 expression levels were

normalized to GAPDH and presented as fold change relative to untreated cells ± SD. Parental B16TK, B16TK (scrambled shRNA) or B16TK (shRNA mAPOBEC3) were cultured with the GCV regimen (**B**) or the reovirus regimen (**C**) described in **Fig 1C** for 21 days either with no added T cells, or with purified naïve or T.E. CD8 T cells at an E:T ratio of 10:1. All wells were washed with PBS to remove T cells and surviving cells were counted at the end of the 21 day culture period. Means ± SD of triplicate wells are shown. C57Bl/6 mice were seeded with $2x10^5$ B16TK (scrambled shRNA) cells (**D**) or B16TK (shRNA APOBEC3) cells (**E**). Mice were treated with GCV (50 mg/kg) on days 5,6,7,8,9,12,13,14,15,16. Tumor volume over time is shown for each mouse (7 mice/group). **(F)** B16TK cells, engineered to express shRNA against APOBEC3 or a scrambled shRNA, pCMV-APOBEC3, hAPOBEC3B or an enzymatically inactive hAPOBEC3B were plated in the presence of GCV (5µg/ml) for 7 days, washed 3 times in PBS, and then re-cultured in normal medium for a further 7 days. Following washing in PBS, cells were again cultured with GCV for a further 7 days, after which the number of surviving cells was counted as shown. Means ± SD of triplicate wells are shown. ns $P > 0.05$; $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$. **(G)** Sequences of the *HSV-TK* gene recovered from genomic DNA from pooled populations of the cell treatments of **F**.

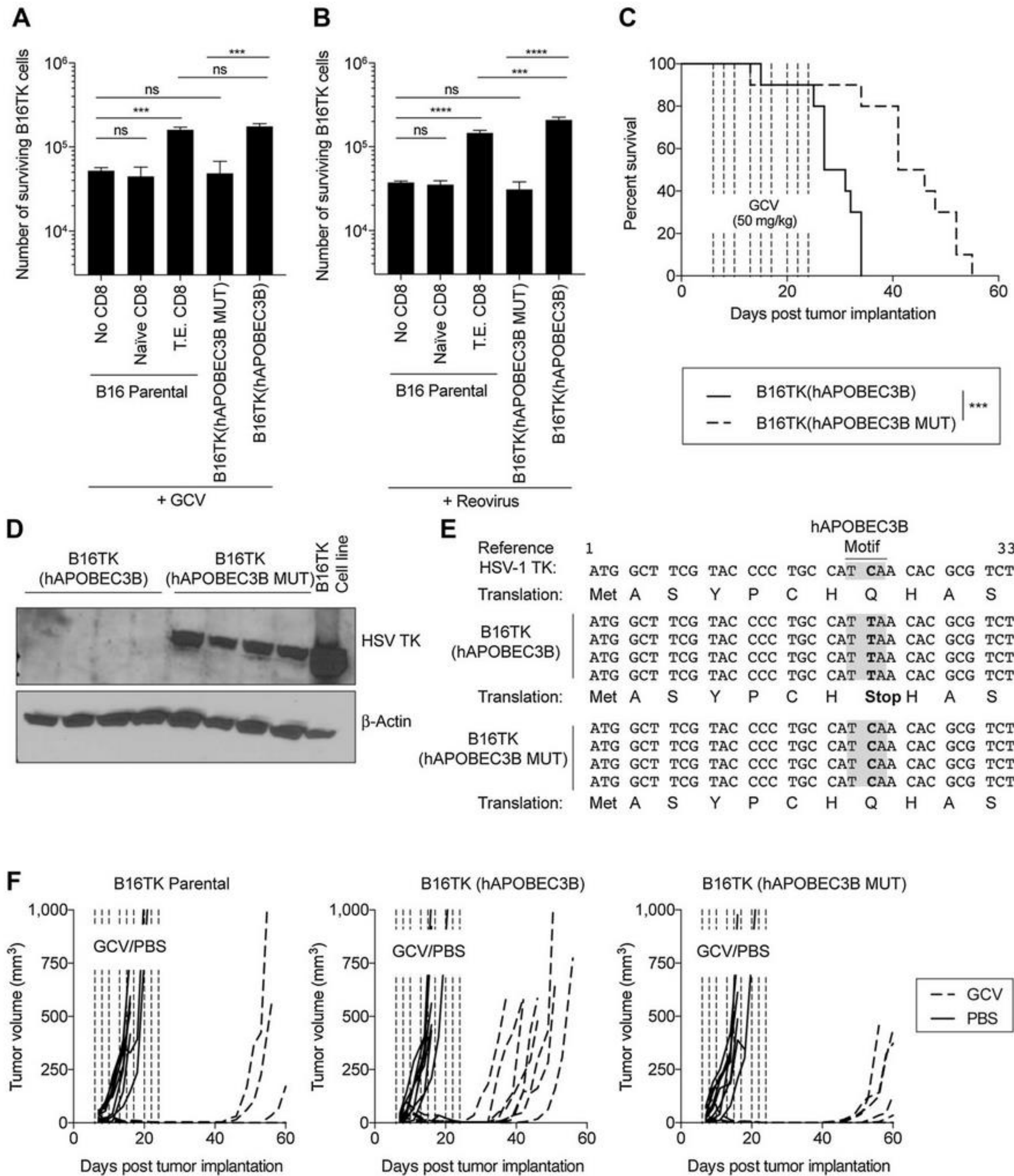

Figure 6. APOBEC3B over-expression drives tumor escape

B16TK cell lines were generated which stably overexpressed either hAPOBEC3B or a catalytically inactive form of hAPOBEC3B (MUT). Parental B16TK cells, B16TK (hAPOBE3B), and B16TK (hAPOBE3B MUT) were cultured with GCV (**A**) or reovirus (**B**) according to **Fig 1C**. Parental B16TK cells were also co-cultured with purified naïve or

T.E. CD8 T cells at an E:T ratio of 10:1. Surviving tumor cells were counted at the end of the 21 day culture period. Means ± SD of triplicate wells are shown. ns *P* > 0.05; **P* ≤ 0.05; ***P* ≤ 0.01; ****P* ≤ 0.001. (**C**) Intracranial B16TK, or B16TK (hAPOBEC3B), or B16TK (hAPOBEC3B MUT) tumors were established by injecting $1x10^4$ cells into the frontal lobe of C57BL/6 mice. Mice were treated with GCV treatment (50mg/kg) on days 6,8,10, 13, 15, 17, 20 ,22, 24 (n=10 mice/group) ****P* ≤ 0.001. (**D**) 4 tumors from each of the B16TK (hAPOBEC3B) and B16TK (hAPOBEC3B MUT) groups were recovered and screened for expression of the HSVTK protein by Western Blot and sequenced (**E**). (**F**) Subcutaneous B16TK, or B16TK (hAPOBEC3B), or B16TK (hAPOBEC3B MUT) tumors were established by injecting $2x10^5$ cells into the flank of C57BL/6 mice. Mice were treated with GCV (50mg/kg) or PBS on days 6,8,10, 13, 15, 17, 20 ,22, 24 (n=10 mice/group). Tumor size over time is shown.