

CRISPR-mediated TGFBR2 knockout renders human ovarian cancer tumor-infiltrating lymphocytes resistant to TGF- β signaling

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ABSTRACT

Background The correlation between elevated T-cell infiltration and improved survival of ovarian cancer (OvCa) patients suggests that endogenous tumor-infiltrating lymphocytes (TIL) possess some degree of antitumor activity that can be harnessed for OvCa immunotherapy. We previously optimized a protocol for ex vivo OvCa TIL expansion for adoptive cell therapy, which is now being tested in a clinical trial at our institution (NCT03610490). Building on this success, we embarked on genetic modification of OvCa TIL to overcome key immunosuppressive factors present in the tumor microenvironment. Here, we present the preclinical optimization of CRISPR/Cas9-mediated knockout of the TGF- β receptor 2 (*TGFBR2*) in patient-derived OvCa TIL. **Methods** OvCa TILs were generated from four patients' tumor samples obtained at surgical resection and subjected to CRISPR/Cas9-mediated knockout of *TGFBR2* before undergoing a rapid expansion protocol. *TGFBR2*-directed gRNAs were comprehensively evaluated for their *TGFBR2* knockout efficiency and off-target activity. Furthermore, the impact of *TGFBR2* knockout on TIL expansion, function, and downstream signaling was assayed.

Results *TGFBR2* knockout efficiencies ranging from 59 \pm 6% to 100 \pm 0% were achieved using 5 gRNAs tested in four independent OvCa TIL samples. *TGFBR2* knockout TIL were resistant to immunosuppressive TGF- β signaling as evidenced by a lack of SMAD phosphorylation, a lack of global transcriptional changes in response to TGF- β stimulation, equally strong secretion of proinflammatory cytokines in the presence and absence of TGF- β , and improved cytotoxicity in the presence of TGF- β . CRISPR-modification itself did not alter the *ex vivo* expansion efficiency, immunophenotype, nor the TCR clonal diversity of OvCa TIL. Importantly for clinical translation, comprehensive analysis of CRISPR off-target effects revealed no evidence of off-target activity for our top two *TGFBR2*-targeting gRNAs.

Conclusions CRISPR/Cas9-mediated gene knockout is feasible and efficient in patient-derived OvCa TIL using clinically-scalable methods. We achieved efficient and specific *TGFBR2* knockout, yielding an expanded OvCa TIL product that was resistant to the immunosuppressive

effects of TGF- β . This study lays the groundwork for clinical translation of CRISPR-modified TIL, providing opportunities for engineering more potent TIL therapies not only for OvCa treatment, but for the treatment of other solid cancers as well.

BACKGROUND

In ovarian cancer (OvCa), increased T-cell infiltration in the tumor correlates with improved survival,^{1 2} providing evidence of tumor control by endogenous T cells and hope for successful immunotherapeutic intervention. We hypothesize that boosting the activity and number of OvCa tumor-infiltrating lymphocytes (TIL) via *ex vivo* manipulation will be an attractive avenue for immunotherapy development for OvCa treatment.

Our group and others have demonstrated clinical efficacy of adoptive cell therapy (ACT) using *ex vivo* expanded TIL for metastatic melanoma treatment.³⁻⁷ To translate this success to OvCa, we developed an improved TIL culture method that produces clinically-relevant OvCa TIL expansion with a 95% success rate.⁸ Clinical testing of TIL immunotherapy in patients with recurrent, platinum-resistant OvCa is ongoing (NCT03610490, NCT03449108).

We hypothesize that protecting OvCa TIL from immunosuppression in the tumor microenvironment will maximize their therapeutic potential on adoptive transfer. Transforming growth factor beta (TGF- β) is upregulated in OvCa^{9 10} and has potent immunosuppressive effects. Thus, we set out to develop a translationally feasible method to render OvCa TIL resistant to TGF- β -signaling by eliminating their expression of TGF- β receptor 2 (*TGFBR2*). We hypothesized that using

clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9, we would be able to achieve efficient *TGFBR2* deletion in OvCa TIL while minimizing off-target editing elsewhere in the genome. Here, we present an optimized and clinically scalable method for CRISPR/Cas9-mediated deletion of the *TGFBR2* in OvCa TIL, which lays the foundation for clinical translation of CRISPR-enhanced TIL for OvCa treatment.

METHODS

CRISPR gene knockout in OvCa TIL

After IRB approval, patient-derived OvCa TIL were cultured from tumor fragments and viably frozen as described previously.^{8, 11} CRISPR-mediated *TGFBR2* knockout was achieved via electroporation-mediated delivery of Cas9 ribonucleoprotein (RNP) complexes (online supplemental table S1) into activated TIL as in figure 1A. Following CRISPR modification, TIL were either subjected to a rapid expansion protocol (REP) or were cultured for 3–5 days before pre-REP analyses.

Evaluation of gRNA efficiency and specificity

Genomic-level *TGFBR2* disruption was quantified via (1) duplexed probe-based quantitative PCR (qPCR) assays (online supplemental table S2) and (2) targeted next-generation sequencing (NGS) of amplified genomic cleavage sites for each guide RNA (gRNA) (Targeted Amplicon-Seq) (online supplemental table S3).

Off-target activity was assessed via two methods. First, Targeted Amplicon-Seq was used to evaluate cleavage at 79 bioinformatically-predicted off-target sites for each gRNA (online supplemental table S3). Off-target sites were predicted using Integrated DNA Technologies' gRNA design tool (https://www.idtdna.com/site/order/designtool/index/CRISPR_PREDESIGN).

Second, target-enriched GUIDE-seq (TEG-seq)¹² was used for genome-wide detection of off-target cleavage using gRNAs #3 and #4. For TEG-seq analysis, TIL were transfected with Cas9 RNP complexes with the addition of 20 pmol double stranded DNA tag to integrate into double strand break locations. On-target *TGFBR2* cleavage was confirmed by qPCR prior to TEG-seq analysis. Potential off-target sites identified by TEG-seq were validated by Targeted Amplicon-Seq (online supplemental table S4).

Bulk RNA sequencing

Pre-REP TIL samples were stimulated with 10 ng/mL human TGF- β 1 (R&D Systems) or an equivalent volume of vehicle (sterile water with 0.1% bovine serum albumin and 4 mM HCl) for 24 hours. Samples were preserved in RNeasy Lysis Buffer (Qiagen) and submitted to Avera Institute for Human Genetics for RNA isolation, quality control, and RNA sequencing using HiSeq2500 with 2 \times 101 paired-end reads, v2 chemistry and flow cells, and a coverage of 30–45 million reads per sample.

Proliferation assay

Pre-REP TIL were stained with CellTrace Violet (Invitrogen) and re-stimulated with 300 ng/mL plate-bound

anti-CD3 antibody (clone OKT3) in the presence of 10 ng/mL TGF- β 1 or vehicle. Cells were harvested after 96 hours, and proliferation was evaluated by flow cytometry to detect dye dilution.

Analysis of TGF- β signaling

Binding of TGF- β to its receptor on T cells results in phosphorylation of small mothers against decapentaplegic homolog (SMAD)-2 and -3. Intracellular staining and flow cytometry were used to evaluate the extent of SMAD-2/-3 phosphorylation in control and *TGFBR2*-knockout (*TGFBR2*-KO) TIL following TGF- β stimulation. Pre-REP or post-REP TIL were cultured overnight in TIL culture medium¹³ containing 0.1% human serum before 30 min stimulation with 10 ng/mL human TGF- β 1 or vehicle at 37°C. TIL were subsequently fixed and permeabilized using BD Phosflow Lyse/Fix Buffer and BD Phosflow Perm Buffer III (BD Biosciences), and intracellular staining was performed using 5 μ l of anti-Smad2 (pS465/pS467)/Smad3 (pS423/pS425) antibody (clone O72-670, BD Biosciences) per sample.

TCR repertoire analysis

Genomic DNA was isolated from *TGFBR2*-KO and control TIL on Day 14 of the REP and subjected to survey-resolution TCR- β sequencing (immunoSEQ, Adaptive Biotechnologies). Productive Simpson Clonality was compared among groups. Pair-wise scatter plots were generated to identify TCR clones with differential abundance in control vs *TGFBR2*-KO TIL samples, and clones with cumulative abundance less than 10 were excluded from differential abundance analysis.

Flow cytometry analysis

TGFBR2-KO and control TIL were immunophenotyped (1) after the 14-day REP and (2) after the 14-day REP with an additional 3 day culture in the presence of 10 ng/mL TGF- β 1 or vehicle. Samples were blocked using 5% goat serum and stained using fluorochrome-conjugated antibodies against CCR7 PerCP-Cy5.5 (G043H7) and TIM-3 BV605 (F38-2E2) (from Biolegend); LAG-3 PE (3DS223H) (from Invitrogen); and CD27 FITC (M-T271), PD-1 BV650 (EH12.1), CD45RA V450 (HI100), CTLA-4 BV786 (BNI3), CD3 BUV395 (SK7), CD4 BUV496 (L3T4), CD103 BUV661 (Ber-ACT8), CD8 Alexa Fluor 700 (RPA-T8), CD45RO APC-H7 (UCHL1), BTLA PE-CF594 (J168-540), CD28 PE-Cy7 (CD28.2) (from BD Biosciences). LIVE/DEAD Yellow (Life Technologies) was used to exclude dead cells. Data were acquired using the BD LSRFortessa X-20 Cell Analyzer (BD Biosciences) and analyzed using FlowJo Software (Tree Star). A representative gating strategy is presented in online supplemental figure S1.

Cytokine release assay

The function of *TGFBR2*-KO and control TIL was evaluated by analyzing cytokine release following TCR stimulation in the presence or absence of TGF- β . TILs were collected on day 14 of the REP and restimulated

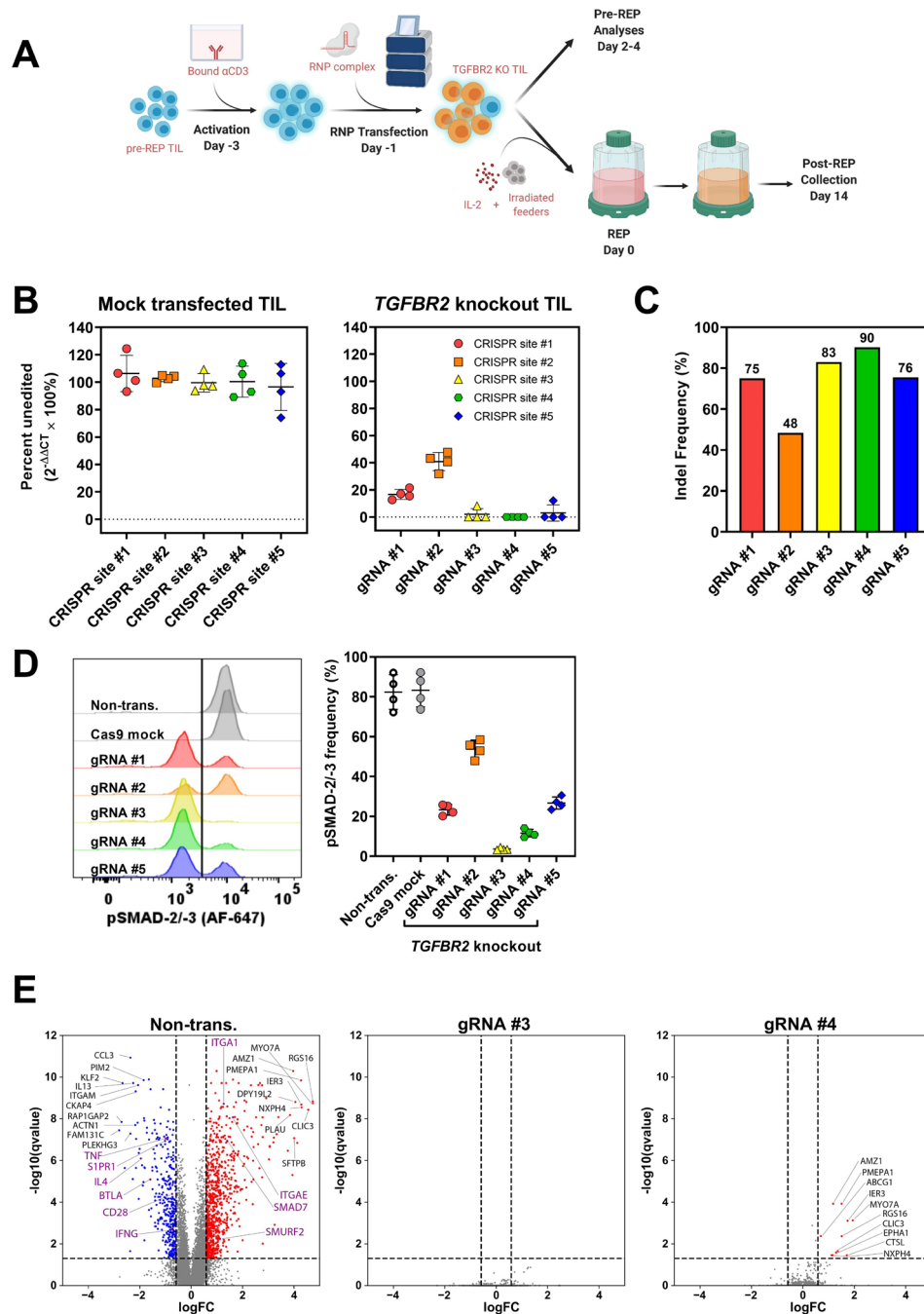


Figure 1 Efficient deletion of *TGFBR2* renders OvCa TIL resistant to TGF- β signaling. (A) Workflow for the CRISPR-modification of OvCa TIL prior to rapid expansion and analysis. Pre-REP TIL were activated with plate-bound anti-CD3 antibody (clone OKT3) for 2 days prior to electroporation-mediated delivery of Cas9 RNP complexes. Transfected TIL were either cultured for 1 day before initiating the REP or were cultured for 3–5 days before pre-REP analyses. (B) qPCR evaluation of *TGFBR2* disruption produced using Cas9 RNP complexes with five different gRNAs. The left panel shows evaluation of the respective cut-site for each gRNA in Cas9 mock transfected TIL, confirming a lack of genomic editing in the absence of functional Cas9 RNP complexes. The right panel shows the degree of *TGFBR2* disruption achieved using each gRNA delivered into TIL as an RNP complex ($n=4$ TIL donors). (C) Targeted Amplicon-Seq evaluation of indels produced using Cas9 RNP complexes with five different gRNAs ($n=1$ TIL donor). (D) Detection of SMAD-2 and SMAD-3 phosphorylation in *TGFBR2*-KO TIL vs control TIL after 30 min exposure to TGF- β . The left panel shows representative histograms of phospho-SMAD staining, and the right panel shows quantification of cells positive for phospho-SMAD-2/-3 after TGF- β exposure ($n=4$ TIL donors). (E) Volcano plots showing genes that were differentially expressed in TIL after exposure to TGF- β versus vehicle for 24 hours (left: non-transfected control TIL, middle: *TGFBR2*-KO TIL with gRNA #3, right: *TGFBR2*-KO TIL with gRNA #4) ($n=3$ technical replicates). Positive log fold change ($\log_{2}(\text{FC})$) indicates upregulation by TGF- β exposure, while negative $\log_{2}(\text{FC})$ indicates downregulation by TGF- β exposure. The top 10 significantly upregulated genes and top 10 significantly downregulated genes are highlighted with black text. Genes of interest that are discussed for control TIL in the main text are highlighted with purple text. OvCa, ovarian cancer; REP, rapid expansion protocol; TIL, tumor-infiltrating lymphocytes.

with 300 ng/mL plate-bound OKT3 in the presence of 3000 IU/mL IL2 and 10 ng/mL TGF- β 1 or vehicle. Cell culture supernatant was collected after 72 hours of stimulation. Cytokine concentration was quantified using the V-PLEX Proinflammatory Panel 1 Human Kit (IFN- γ , IL-1 β , IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, and TNF- α) and the MSD QuickPlex SQ120 instrument (Meso Scale Diagnostics).

xCELLigence cytotoxicity assay

Post-REP *TGFBR2*-KO and control TIL from patient #2 were cultured for 3 days in the presence of 10 ng/mL TGF- β 1 or vehicle. Meanwhile, HLA-matched SKOV3 OvCa tumor cells were plated at a density of 10 000 cells per well in 96-well RTCA E-Plates, and Cell Index was measured in real time using an xCELLigence Analyzer (Agilent). Approximately 24 hours after SKOV3 cells were plated, TGF- β -exposed and vehicle-exposed TIL were added to the wells at an effector: target ratio of 10:1, and the co-culture was supplemented with 10 ng/mL TGF- β 1 or vehicle, respectively. Tumor cell killing was monitored over the following 48 hours, and percent cytolysis was calculated as: $\% \text{Cytolysis}_{\text{condition}} = [\text{Normalized cell index}_{\text{control}} - \text{Normalized Cell Index}_{\text{condition}}] / \text{Normalized Cell Index}_{\text{control}} \times 100\%$, where the conditions were TIL:tumor cocultures supplemented with 10 ng/mL TGF- β or vehicle, and the controls were tumor cells alone supplemented with 10 ng/mL TGF- β or vehicle, respectively.

SKOV3 tumor cells were acquired from the American Type Culture Collection (ATCC) and maintained in DMEM/F12 media supplemented with 10% FBS. SKOV3 was confirmed to be negative for mycoplasma contamination before use in the cytotoxicity assay.

Statistical analyses

GraphPad Prism V.9 (GraphPad Software) was used for statistical analyses. A two-way, repeated-measures analysis of variance (ANOVA) with Sidak's multiple comparisons test was used to determine differences between pre-REP and post-REP samples and to determine differences in cytokine secretion or flow cytometry marker expression between TGF- β -exposed and vehicle-exposed samples. To compare REP efficiency and productive Simpson clonal diversity between groups, ordinary two-way ANOVAs were performed with Dunnett's multiple comparisons tests to compare the mean of each group to that of the control (non-transfected) TIL group. Ordinary two-way ANOVAs with Tukey multiple comparisons tests were used to compare mean percent cytolysis of each group with the mean percent cytolysis of every other group.

For additional details on TIL manipulation, on-target and off-target analyses, RNA-sequencing analysis, and proliferation analysis please refer to online supplemental methods.

RESULTS

Efficient *TGFBR2* knockout renders OvCa TIL resistant to TGF- β signaling without evidence of off-target cleavage elsewhere in the genome

Genomic-level *TGFBR2* knockout efficiencies ranging from 59 \pm 6%-100 \pm 0% were achieved using 5 gRNAs tested in four independent OvCa TIL samples (qPCR, [figure 1B](#)). These data were confirmed via Targeted Amplicon-Seq used to evaluate insertion/deletion (indel) formation at CRISPR cleavage locations ([figure 1C](#)). Greater than 80% of control TIL were positive for phospho-SMAD-2/-3 after TGF- β exposure, while *TGFBR2*-KO TIL displayed lower levels of SMAD phosphorylation that corresponded with *TGFBR2* knockout efficiency ([figure 1D](#)). The gRNAs #3 and #4 were chosen for further evaluation as they produced the highest *TGFBR2* knockout efficiency and greatest resistance to TGF- β -mediated SMAD phosphorylation.

Seventy-nine bioinformatically-predicted off-target sites for gRNA #1-#5 were evaluated by Targeted Amplicon-Seq, and no off-target activity was detected at any of the predicted sites for any of the gRNAs (online supplemental table S5). TEG-seq¹² was used to further evaluate off-target activity throughout the genome for gRNA #3 and gRNA #4. Three potential off-target sites were identified for gRNA #3, and five potential off-target sites were identified for gRNA #4 (online supplemental figure S2). All sites were classified as 'Low Probability' events according to criteria described in the Supplemental Methods, and subsequent Targeted Amplicon-Seq confirmed that there was *no* evidence of CRISPR activity at these potential off-target locations (online supplemental figure S2,S3 and online supplemental methods).

Bulk RNA sequencing was used to obtain a global view of the effects of TGF- β on control and *TGFBR2*-KO TIL. TGF- β exposure induced a strong transcriptional response in control TIL (vs vehicle), with a total of 1167 differentially expressed genes ([figure 1E](#), online supplemental table S6). As expected, TGF- β induced the expression of downstream regulators of the TGF- β pathway, *SMAD7* and *SMURF2*. Also expected were TGF- β -induced changes in gene expression associated with T cell tissue residence.¹⁴ This included upregulation of the adhesion molecules *ITGAI* (encodes CD49a) and *ITGAE* (encodes CD103) and downregulation of *SIPRI* (a receptor required for tissue egress). Additionally, TGF- β inhibited the expression of several pro-inflammatory cytokines, including *IENG*, *IL4*, and *TNF*. These data capture the pleiotropic effects of TGF- β on non-engineered T cells, including the promotion of tissue residence and inhibition of T-cell effector activity (cytokine production).

In contrast, TGF- β exposure had little to no effect on the gene expression of *TGFBR2*-KO TIL. Exposure to TGF- β versus vehicle resulted in zero differentially expressed genes for TIL engineered with gRNA #3 and 11 differentially expressed genes for TIL engineered with gRNA #4 ([figure 1E](#), online supplemental table S7), which is consistent with the relative level of protection from

TGF- β -mediated SMAD-phosphorylation afforded by *TGFBR2* knockout with these gRNAs. These data demonstrate that TGF- β has little to no effect on gene expression of *TGFBR2*-KO TIL, confirming that functional receptor elimination was achieved via CRISPR gene knockout with gRNA #3 and gRNA #4.

The proliferation of control and *TGFBR2*-KO TIL following TCR stimulation in the presence or absence of TGF- β was evaluated via dye dilution. TGF- β substantially reduced the proliferation of control TIL from patients #2 and #4, while the proliferation of control TIL from patient #3 was largely unaffected by TGF- β exposure

(online supplemental figure S4). Conversely, TGF- β -exposure did not impact the proliferation of *TGFBR2*-KO TIL from all three patient samples, which demonstrates a reversal of the suppressive effect of TGF- β on TIL from patients #2 and #4.

CRISPR-modified TIL expand to clinically relevant numbers while retaining *TGFBR2* deletion, resistance to TGF- β signaling, and TCR clonal diversity

On day 14 of the REP, TIL expansion reached 1000 \times –3400 \times (figure 2A), with no significant differences observed between control and *TGFBR2*-KO TIL. Thus,

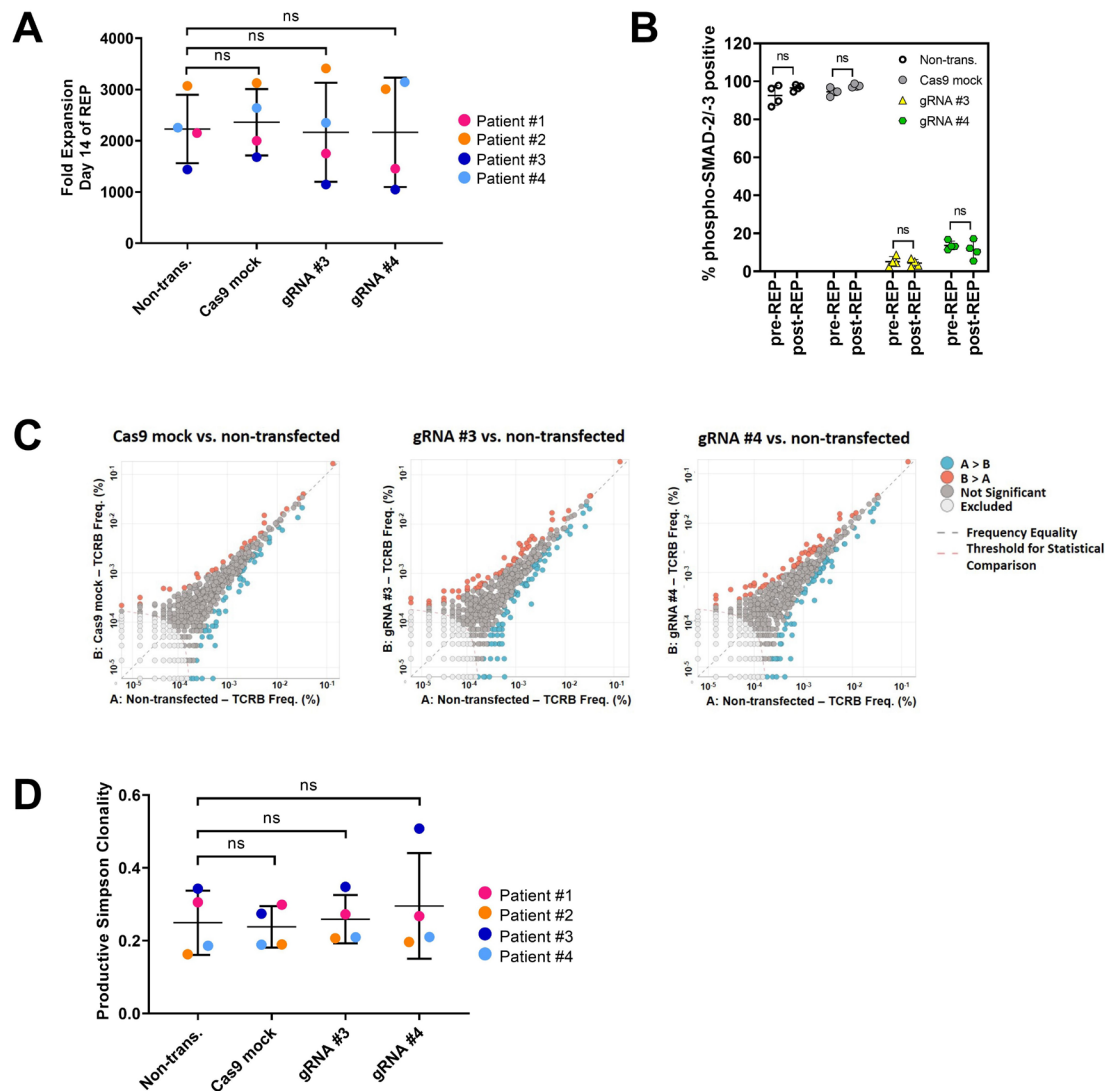


Figure 2 CRISPR-modified TIL expand to clinically relevant numbers while maintaining resistance to TGF- β signaling and TCR clonal diversity. (A) Control and *TGFBR2*-KO OvCa TIL expand with equal efficiency during the 14-day REP. Fold expansions ranging from 1000 \times to 3000 \times were observed across four independent patient samples. (B) Resistance of *TGFBR2*-KO TIL to TGF- β -mediated SMAD phosphorylation was maintained after the 14-day REP. (C) The majority of TCR clones are identified with equal abundance in control and CRISPR-modified TIL, with differentially abundant clones randomly distributed on either side of the line of frequency equity. These data indicate that CRISPR-modification does not skew the distribution of unique TCR clones in TIL samples. Data from patient #2 are presented and are representative of the results obtained from an additional three patient samples (shown in online supplemental figure S6). Left: Cas9 mock transfected TIL versus non-transfected TIL. Middle: *TGFBR2*-KO TIL with gRNA #3 versus non-transfected TIL. Right: *TGFBR2*-KO TIL with gRNA #4 versus non-transfected TIL. (D) Productive Simpson clonality is equivalent in *TGFBR2*-KO TIL compared with control TIL samples. ns, no significant; OvCa, ovarian cancer; REP, rapid expansion protocol; TIL, tumor-infiltrating lymphocytes.

electroporation and CRISPR engineering did not impact the proliferation of OvCa TIL.

TGFBR2-KO TIL samples retained strong genomic-level *TGFBR2* disruption after 14-days of expansion (online supplemental figure S5). Correspondingly, *TGFBR2*-KO TIL resisted TGF- β -mediated SMAD-2/-3 phosphorylation with equal efficiency before and after the REP (figure 2B).

We performed TCR repertoire analysis to evaluate if the cell loss caused by electroporation-mediated delivery of Cas9 RNP complexes impacted TIL clonal diversity. Survey-level TCR β chain sequencing was used to evaluate TCR clonal diversity in post-REP control versus *TGFBR2*-KO TIL. Representative scatter plots from patient #2 illustrate that many TCR clones are present with equal abundance in control and CRISPR-modified samples, and differentially abundant clones are randomly distributed on either side of the line of frequency equality (figure 2C; online supplemental figure S6 for patients #1, #3, and #4). Additionally, relatively few clones are found exclusively in either non-transfected TIL or CRISPR-modified TIL. We found no significant difference in Productive Simpson Clonality between control and *TGFBR2*-KO TIL (figure 2D). These data indicate that incorporating CRISPR engineering into the workflow for

TIL cultivation does not impact the TCR clonal diversity of the final TIL product.

***TGFBR2* knockout itself does not impact the immunophenotype of expanded TIL but prevents phenotypic shifts induced by TGF- β exposure**

We characterized the immunophenotype of post-REP TIL by analyzing markers related to differentiation and activation status (CD28, BTLA, CD45RO, CD45RA, CD27, CCR7, CTLA-4, PD-1, TIM-3, or LAG-3) and a marker known to be affected by TGF- β exposure (CD103). We observed no differences in the expression of these markers between control TIL and *TGFBR2*-KO TIL in the absence of exogenous TGF- β exposure (online supplemental figure S1, S7). Therefore, CRISPR engineering itself does not impact the expression of these markers in post-REP TIL.

The immunophenotype of post-REP TIL was also analyzed after 3 day exposure to TGF- β or vehicle. In control TIL, TGF- β induced CD103 expression (figure 3), which is consistent with upregulation of *ITGAE* (encodes CD103) observed via RNA sequencing of pre-REP TIL exposed to TGF- β (figure 1E). TGF- β is known to directly regulate CD103 downstream of SMAD3, thereby promoting a CD103⁺ tissue resident memory phenotype.¹⁴

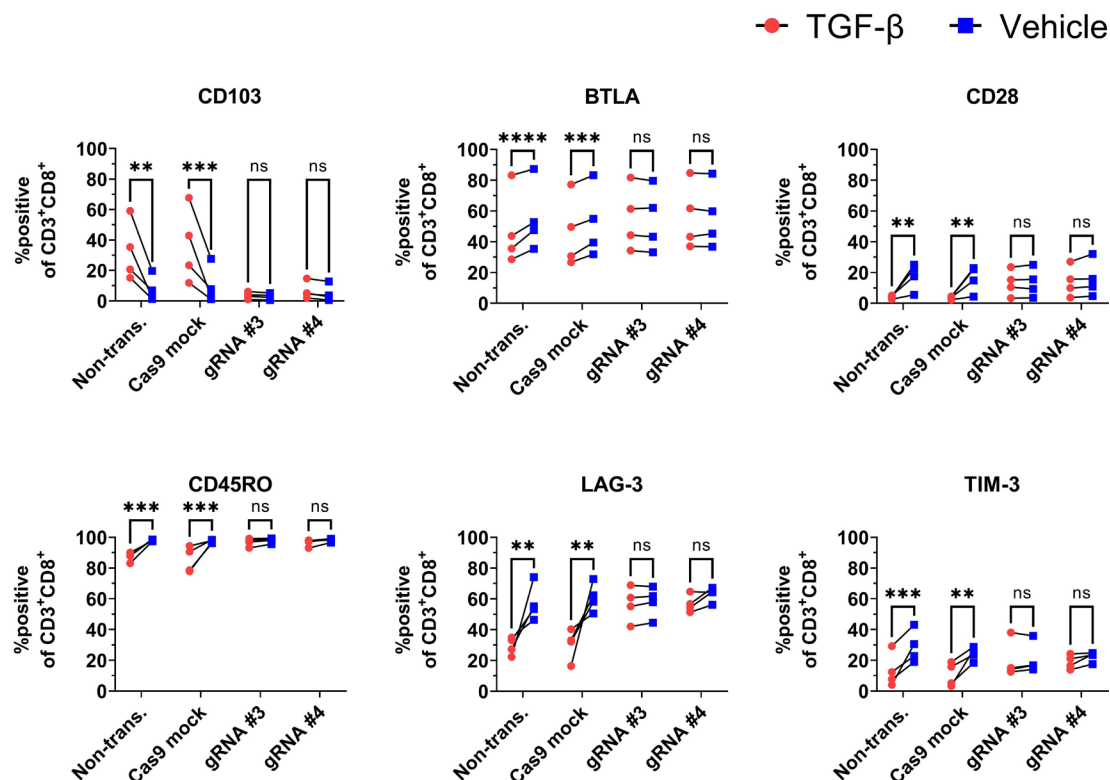


Figure 3 *TGFBR2* knockout prevents immunophenotypic shifts induced by TGF- β exposure. Post-REP TIL was exposed to 10 ng/mL TGF- β or vehicle for 3 days before staining and flow cytometry analysis (n=4 TIL donors). TGF- β exposure significantly induced the expression of CD103 in control TIL while repressing expression of BTLA, CD28, CD45RO, LAG-3, and TIM-3. Exposure of *TGFBR2*-KO TIL (generated using gRNA #3 or gRNA #4) to TGF- β resulted in no change in the expression of these markers, indicating resistance of *TGFBR2*-KO TIL to phenotypic shifts induced by TGF- β . Data are presented as percent marker positive of live, CD3⁺CD8⁺ TIL, and a representative gating hierarchy is presented in online supplemental figure S1. ns, not significant; **, P \leq 0.01; *** P \leq 0.001; REP, rapid expansion protocol; TIL, tumor-infiltrating lymphocytes.

Resistance of *TGFBR2*-KO TIL to TGF- β -mediated upregulation of CD103 is consistent with their resistance to TGF- β -mediated SMAD-phosphorylation and provides further evidence of functional *TGFBR2* elimination in our *TGFBR2*-KO TIL.

In control TIL, TGF- β -exposure decreased the expression of BTLA and CD28 (figure 3), markers that typically decrease as T cells differentiate, which is consistent with gene expression changes observed by RNA sequencing (figure 1E). We also observed a modest but significant decrease in CD45RO expression, and decreased expression of activation/exhaustion markers LAG-3 and TIM-3 in control TIL exposed to TGF- β (figure 3). The expression of all markers in *TGFBR2*-KO TIL was unaltered by TGF- β exposure, indicating that *TGFBR2*-KO TIL resist phenotypic shifts induced by TGF- β . The markers CCR7, CD27, CD45RA, CTLA-4, and PD-1 were unaltered by TGF- β exposure in control and *TGFBR2*-KO TIL (online supplemental figure S8).

***TGFBR2*-KO TIL retain functional capacity in the presence of TGF- β**

To evaluate the function of post-REP *TGFBR2*-KO TIL, we assayed cytokine release following TCR stimulation in the presence or absence of exogenous TGF- β . As expected, in absence of TGF- β , TCR stimulation triggered substantial production of IFN- γ , TNF- α , and IL-13, which decreased significantly in the presence of TGF- β for control TIL (figure 4A). Conversely, IFN- γ , TNF- α , and IL-13 production remained high in the presence of TGF- β for *TGFBR2*-KO TIL. A similar trend was observed for IL-2 despite cell culture supernatant being supplemented with 3000 IU/mL IL-2 at the start of the assay (72 hours before supernatant collection). Similar trends were observed for IL-4, IL-6, and IL-10 although with lower level secretion of these cytokines. IL-1 β and IL-8 were detected in our assay, but levels of these cytokines were unaffected by TGF- β exposure in control and *TGFBR2*-KO TIL.

TIL expanded from patient #2 and patient #3 were previously shown to exhibit HLA-restricted tumor reactivity against allogeneic, HLA-matched OvCa tumor cell lines,⁸ providing a model to evaluate the antitumor function of our post-REP TIL. TGF- β significantly decreased the cytolytic function of control TIL from patient #2 at all timepoints (figure 4B, online supplemental figure S9). After 4 hours of coculture, *TGFBR2*-KO TIL controlled the tumor equally well in the presence and absence of TGF- β (figure 4C), however, TGF- β negatively impacted cytotoxicity at later timepoints (figure 4B, online supplemental figure S9). The seemingly transient protection afforded by *TGFBR2* knockout could be due to effects of TGF- β on the tumor cells (rather than TIL) making the tumor cells more resistant to T-cell killing.

Control TIL from patient #3 were not sensitive to TGF- β -mediated inhibition of tumor cell killing and therefore could not be used as a model to evaluate benefit afforded by *TGFBR2*-KO in this context.

DISCUSSION

Our group recently developed a protocol to generate OvCa TIL with robust expansion and antitumor activity *in vitro*,⁸ and we have initiated a clinical trial at our institution to test TIL therapy in OvCa patients who have relapsed on conventional treatments. However, we hypothesize that TGF- β , a predominant immunosuppressive cytokine in the OvCa tumor microenvironment,^{9,10} may limit TIL function on adoptive transfer. Here, we developed a CRISPR-based method to delete *TGFBR2* in OvCa TIL, producing TGF- β -resistant TIL without altering TIL expansion efficiency, post-REP immunophenotype, nor TCR clonal diversity.

TGF- β is a pleiotropic cytokine, playing diverse and paradoxical roles in many cell types, making it a challenging therapeutic target. Throughout this report, we show that *TGFBR2*-KO TIL are protected from the immunosuppressive effects of TGF- β on effector cytokine production (figure 1E, figure 4A), proliferation (online supplemental figure S4), and cytotoxicity (figure 4B, figure 4C). However, it is well established that TGF- β also promotes tissue residency by modulating genes associated with the accumulation and retention of T cells in tissue. We observe this effect of TGF- β on control TIL as evidenced by the induction of adhesion molecules (*ITGAE* (CD103) and *ITGA1*) and repression of *SIPRI*, a receptor involved in tissue egress. Therefore, blocking TGF- β in TIL may be a double-edged sword where TIL are protected from the immunosuppressive effects of TGF- β (enhancing TIL function within the hostile tumor microenvironment) while also resisting TGF- β -enhanced tissue residency (possibly hindering TIL retention within tumors). It remains to be seen how this dichotomy will impact overall efficacy of *TGFBR2*-KO TIL in the context of intravenous administration of *ex vivo* expanded TIL, however, we hypothesize that the benefit afforded by *TGFBR2* knockout will outweigh potential negative effects.

A safety concern regarding clinical use of CRISPR-modified cells is the risk of off-target mutations causing detrimental changes in adoptively transferred cells. Here, we took several steps to minimize the risk of off-target cleavage. First, we introduced CRISPR/Cas9 machinery (RNP complexes) into TIL transiently via electroporation, which limits off-target activity compared with plasmid DNA transfection.^{12,15} Second, we used a high-fidelity variant of Cas9 that has dramatically reduced off-target cleavage potential compared with wildtype Cas9.¹⁶ Encouragingly, we did not observe off-target cleavage at predicted off-target sites nor throughout the genome via TEG-seq, which supports the safety profile of our CRISPR-modified TIL product.

Our TIL cultivation protocol was optimized with clinical scale up in mind as inspired by our protocol for TIL engineering that is implemented in two clinical trials (NCT01740557, NCT01955460).¹⁷ We genetically engineered relatively few TIL prior to the REP that in turn yielded >1000 \times TIL expansion. This enables convenient manipulation of relatively few cells and conservation of

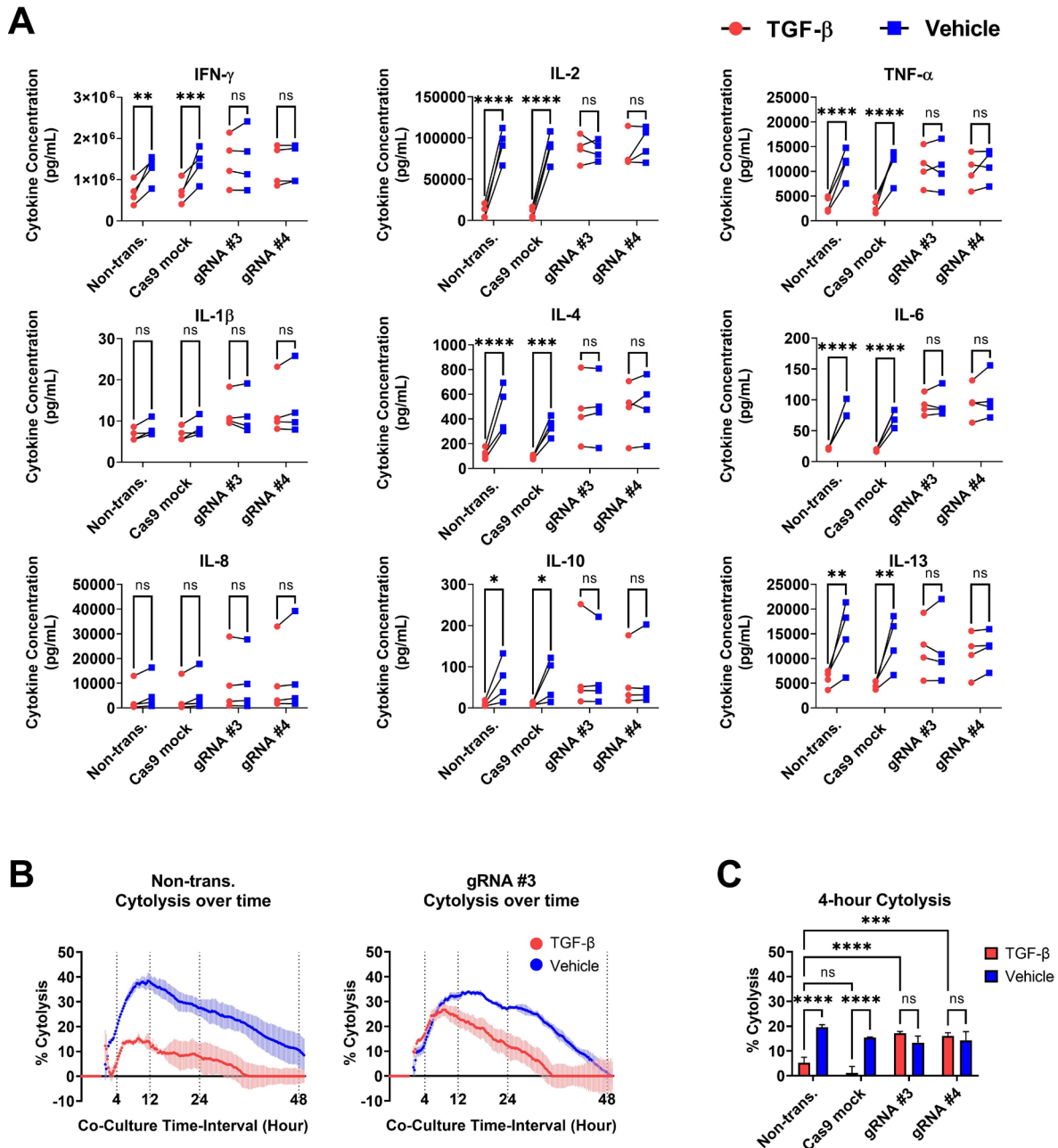


Figure 4 *TGFBR2*-KO TIL retain functional capacity in the presence of TGF- β . (A) TIL were collected on day 14 of the REP and restimulated with 300 ng/mL plate-bound anti-CD3 antibody (clone OKT3) in the presence of 3000 IU/mL IL-2 and 10 ng/mL human TGF- β or vehicle ($n=4$ TIL donors). Cell culture supernatant was collected after 72 hours of stimulation and assayed for the presence of 10 proinflammatory cytokines (IFN- γ , IL-1 β , IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, and TNF- α). For TIL with intact *TGFBR2* (non-transfected and Cas9 mock transfected TIL), the production of many proinflammatory cytokines decreased significantly in the presence of TGF- β . Conversely, *TGFBR2*-KO TIL (generated using gRNA #3 or gRNA #4) retained cytokine secretion in the presence of TGF- β . IL-12p70 was below the limit of detection in this assay and is therefore not presented. (B) Post-REP control and *TGFBR2*-KO TIL from patient #2 were cultured in the presence of 10 ng/mL TGF- β or vehicle for 3 days and then were tested for antitumor activity against SKOV3 target cells with TGF- β or vehicle being added to the coculture media as well. Percent cytotoxicity was captured in real time during a 48-hour coculture via xCELLigence real-time cell analysis. Curves show percent SKOV3 cytotoxicity overtime when cocultured with control TIL (non-transfected, left) and *TGFBR2*-KO TIL (gRNA #3, right) in the presence of TGF- β or vehicle ($n=3$ technical replicates). TGF- β substantially reduced the anti-tumor activity of control TIL at all timepoints. *TGFBR2*-KO TIL performed equally well in the presence and absence of TGF- β at early timepoints (e.g., at 4 hours), but TGF- β hindered cytotoxicity at later timepoints. Refer to online supplemental figure S9A for cytotoxicity curves using control TIL (Cas9 mock transfected) and *TGFBR2*-KO TIL (gRNA #4). (C) At the 4-hour coculture timepoint, the antitumor activity of control TIL was significantly inhibited by TGF- β exposure compared with vehicle. At the same time point, *TGFBR2*-KO TIL demonstrated equal cytolytic capacity in the presence and absence of TGF- β . Refer to online supplemental figure S9B for additional timepoints. ns, not significant; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$; TIL, tumor-infiltrating lymphocytes.

good manufacturing practice-grade CRISPR reagents. The 4D Nucleofector (Lonza) used for electroporation can accommodate a wide range of cell numbers (2.5×10^5 – 1×10^9) for transfection; therefore, the electroporation conditions optimized here can be seamlessly scaled up for transfection of pre-REP TIL at clinical scale.

Other approaches to block TGF- β signaling to improve ACT include concurrent use of small molecule inhibitors of TGF- β signaling and transduction of adoptively transferred cells with a dominant negative version of TGFBR2 (TGF β DNR2). Stüber *et al* recently demonstrated that inhibiting TGF- β signaling with the kinase inhibitor SD-208 improves the function of ROR1-specific CAR T-cells against breast cancer *in vitro*.¹⁸ However, due to the pleiotropic roles of TGF- β in normal physiology, there is concern that prolonged use of TGF- β inhibitors will result in systemic toxicities. Therefore, Stüber *et al* proposed short term administration of SD-208 on CAR T-cell administration to provide a limited window of TGF- β resistance.¹⁸ In practice, this approach may suffer from eventual TGF- β -mediated inhibition of the cellular therapy on clearance of the inhibitor.

Conversely, virally transducing T cells with a TGF β DNR2 provides selective and long-term inhibition of TGF- β signaling only within the adoptively transferred cells, limiting concerns of systemic toxicity. This approach has been shown to increase the anti-tumor efficacy of antigen specific T cells and CAR T cells in mouse models of melanoma¹⁹ and prostate cancer,²⁰ respectively, and we have a clinical trial ongoing at our institution to evaluate TGF β DNR2-transduced TIL for the treatment of metastatic melanoma (NCT01955460). Our approach of CRISPR-mediated *TGFBR2* knockout builds on this prior experience by providing a non-viral alternative to eliminate *TGFBR2* expression and downstream TGF- β signaling without the need for stable TGF β DNR2 transgene expression and efficient competition between the TGF β DNR2 and the native receptor. Thus, we expect our new approach of *TGFBR2* knockout to provide superior protection of TIL from TGF- β -mediated immunosuppression.

CONCLUSION

In summary, we developed an efficient and clinically scalable method to knockout *TGFBR2* in OvCa TIL using CRISPR/Cas9 technology, resulting in a TIL product that resisted the immunosuppressive effects of TGF- β exposure. These data provide a foundation for clinical translation of *TGFBR2*-KO TIL, which has the potential to provide a novel immunotherapy for OvCa patients who have exhausted conventional treatments. Furthermore, our method lays the groundwork for CRISPR-engineering of *ex vivo*-expanded human TIL in general, opening opportunities to engineer more potent TIL therapies across solid cancer types.

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Contributors SMF, M-AF and DS-T performed experiments and analyzed data. SMF drafted the manuscript. YW performed RNA sequencing data analysis. TMG maintained tumor cell lines and assisted with experiments. ML performed immunophenotyping panel validation, staining, and acquisition. CLH supervised and guided immunophenotyping panel validation and flow cytometry data analysis. ALD provided laboratory support and assistance with experiments. RB provided technical support and training regarding CRISPR modification of lymphocytes. CR and SK performed sequencing for off-target detection (Targeted Amplicon-Seq and TEG-Seq) and the associated bioinformatics analysis. SMF, AAJ and PH conceived the project. LAM, PH, CB and AAJ supervised and guided project development. All authors were involved in the design of experiments and interpretation of results. All authors read and approved the final manuscript.

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Competing interests SMF and PH have a patent for Engineered T Cells and Tumor-Infiltrating Lymphocytes to Overcome Immunosuppression in the Tumor Microenvironment pending. PH reports personal fees from Dragonfly and Imantics outside the submitted work. CB reports being on the scientific advisory board for Myst Therapeutics now Turnstone Biologics, and having received research support from Iovance Biotherapeutics and Obsidian Therapeutics. LAM reports research support from AstraZeneca, being on a scientific advisory board for GSK, and stocks in Crispr Therapeutics, Johnson & Johnson, Invitae, and Bristol-Myer Squibb. CR reports being an employee of Thermo Fisher Scientific and being involved in Cell Line Generation using Genome Editing. SK reports having been an employee of Thermo Fisher Scientific with involvement in Cell Line Generation using Genome Editing at the time this work was performed. AAJ reports no relevant financial or conflicts of interest related to the current research. Unrelated disclosures for AAJ include clinical trial funding to institution from: AstraZeneca, BMS, Iovance, Aravive, Merck, Pfizer, Eli Lilly, and Imantics; Advisory Board honoraria from: Agenus, Instil Bio, AstraZeneca, Esai, Alkermes, GlaxoSmithKline, MacroGenics, EMD-Serono; Consulting: Guidepoint and Gerson Lehrman Group; Scientific Advisor Board Payments: AvengeBio and Nuprobe. CLH reports speaker's fees from the Society for Immunotherapy of Cancer, serves as an advisory board member for Briacell and the Mesothelioma Applied Research Foundation, has received personal fees from Nanobiotix and receives funding to the MD Anderson Cancer Center from Iovance, Sanofi, Dragonfly Therapeutics, and BTG outside the submitted work.

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