Published in final edited form as:

Cancer Immunol Res. 2018 February ; 6(2): 163–177. doi:10.1158/2326-6066.CIR-17-0273.

***TUSC2* immunogene therapy synergizes with anti–PD-1 through enhanced proliferation and infiltration of natural killer cells in syngeneic *Kras*-mutant mouse lung cancer models**

Ismail M Meraz^{1,*}, Mourad Majidi¹, Xiaobo Cao¹, Heather Lin², Lerong Li³, Jing wang³, Veera Baladandayuthapani², David Rice¹, Boris Sepesi¹, Lin Ji¹, and Jack A Roth¹

¹Section of Thoracic Molecular Oncology, Department of Thoracic and Cardiovascular Surgery, The University of Texas MD Anderson Cancer Center, Houston, Texas

²Department of Biostatistics, The University of Texas MD Anderson Cancer Center, Houston, Texas

³Department of Bioinformatics and Computational Biology, The University of Texas MD Anderson Cancer Center, Houston, Texas

Abstract

Expression of the multikinase inhibitor encoded by tumor suppressor gene *TUSC2* (also known as *FUS1*) is lost or decreased in non-small cell lung carcinoma (NSCLC). *TUSC2* delivered systemically by nanovesicles has mediated tumor regression in clinical trials. Because of the role of *TUSC2* in regulating immune cells, we assessed *TUSC2* efficacy on antitumor immune responses alone and in combination with anti–PD-1 in two *Kras*-mutant syngeneic mouse lung cancer models. *TUSC2* alone significantly reduced tumor growth and prolonged survival compared with anti–PD-1. When combined, this effect was significantly enhanced, and correlated with a pronounced increases in circulating and splenic natural killer (NK) cells and CD8⁺ T cells, and a decrease in Tregs, myeloid-derived suppressor cells (MDSCs), and T-cell checkpoint receptors PD-1, CTLA-4, and TIM-3. *TUSC2* combined with anti–PD-1 induced tumor infiltrating more NK and CD8⁺ T cells and fewer MDSCs and Tregs than each agent alone, both in subcutaneous tumor and in lung metastases. NK-cell depletion abrogated the antitumor effect and Th1-mediated immune response of this combination, indicating that NK cells mediate *TUSC2*/anti–PD-1 synergy. Release of IL15 and IL18 cytokines and expression of the IL15R α chain and IL18R1 were associated with NK-cell activation by *TUSC2*. Immune response-related gene expression in the tumor microenvironment was altered by combination treatment. These data provide a rationale for immunogene therapy combined with immune checkpoint blockade in the treatment of lung NSCLC.

*Corresponding author: Ismail M Meraz, Section of Thoracic Molecular Oncology, Department of Thoracic and Cardiovascular Surgery, Division of Surgery, The University of Texas MD Anderson Cancer Center, Houston, TX, 1515 Holcombe Boulevard, Houston, TX 77030, Room Number: T4.3986A, Phone: 713-794-1960, imeraz@mdanderson.org.

Disclosure of Potential Conflicts of Interest

Jack A. Roth is a consultant, stock owner (including pending patent) in Genprex, Inc. All other authors have declared that no competing interests exist. This does not alter the authors' adherence to all policies of the Cancer Immunology Research-AACR on sharing data and materials.

Keywords

TUSC2 immunogene; checkpoint blockades; *Kras*-mutant; syngeneic mouse model; lung cancer

INTRODUCTION

Expression of tumor suppressor candidate 2 (*TUSC2*), a tumor suppressor gene, is reduced or absent in more than 80% of all lung cancers. Researchers reported that NSCLC patients with loss of *TUSC2* expression had significantly worse overall survival (OS) than did those with normal *TUSC2* expression (1). *TUSC2*, a mitochondrial protein, is multifactorial in its mechanism of action and is involved in a wide array of cellular processes, including induction of apoptosis, inhibition of many kinases, mitochondrial calcium homeostasis, inhibition of reactive oxygen species (ROS) response, and increase of immune stimulation (2–6). In a previous study, we developed nanovesicles encapsulating an optimized *TUSC2* expression plasmid. The *TUSC2* nanovesicles, injected intravenously, are selectively taken up by cancer cells to a greater extent than normal cells. This is due to the selective endocytosis of the nanovesicle lipid formulation and the enhanced permeability and retention (EPR) characteristics of tumor vasculature. The nanoparticles then mediate tumor regression in orthotopic mouse xenograft models of lung cancer (7–9). *TUSC2* delivery to normal cells is not toxic. A phase I clinical trial showed safety for nanoparticle mediated delivery of *TUSC2*, uptake of the particles by human tumors, and antitumor efficacy (10). The prolonged disease control observed in patients suggests a role for activation of the anticancer immune response by *TUSC2*, which is supported by observations that *TUSC2* positively regulates innate immunity by augmenting IL15 expression (4).

TUSC2 gene therapy is different than cytokine gene therapy (e. g. IL2, IL15), due to the multifunctional activity of *TUSC2*, which has the potential to block subsequent bypass pathways mediating drug resistance. *TUSC2* regulates expression of critically important cytokines in the homeostasis and activation of the adaptive and innate immune system (e.g., IL2 and IL15) (4, 11). Natural Killer (NK) cell-mediated innate immune activation is an important component of *TUSC2* gene therapy. Other gene therapies such as adenovirus mediated p53 also showed durable NK cell-mediated antitumor responses (12). NK cells are important innate effectors being tested in the clinic for various cancer treatments (13). NK cells can also regulate T-cell responses through direct and indirect mechanisms, through release of IFN γ or other modulators (14). These effector cells are regulated by immune checkpoints such as PD-1 or CTLA-4. Immunotherapy with antibodies to CTLA-4, PD-1, and PD-L1 can inhibit tumor growth and extend patient survival (15). Anti-PD-1 treatment enhances NK function through modulating the PD-1/PD-L1 axis (16). However, anti-PD-1 immunotherapy produces a response in only a small subset of patients with advanced NSCLC (17% to 21%) (17). Resistance to anti-PD-1 therapy is associated with activation of other immune checkpoint-related pathways (18), which can be overcome through combination treatment strategies. Anti-PD-1 combinations with other agents showed tumor regression through innate (NK cell) and adaptive (CD8⁺ T cell) immune responses (19).

TUSC2 nanovesicle-based immunogene therapy is distinct from other currently approved cancer therapeutics, including targeted drugs (e.g., erlotinib) and checkpoint inhibitors (e.g., antibodies to PD-1, such as nivolumab), which target only single molecules. Resistance to targeted drugs and checkpoint inhibitors develops through activation of alternate bypass pathways. Therefore, treatment agents modulating multiple distinct pathways, including the innate immune response, may be more effective than agents targeting single molecules. In this study, we hypothesized that TUSC2 nanovesicles immunotherapy may synergize with anti-PD-1 treatment, facilitating anti-PD-1 function and generating a cytotoxic effector cell response that is superior to that resulting from either treatment alone. Other potential effects on the immune system include induction of tumor cell apoptosis by TUSC2, which could increase antigen release and presentation, thus promoting an enhanced antitumor response in the presence of anti-PD-1. To test this hypothesis, we treated two *Kras*-mutant syngeneic mouse models of lung cancer with combination TUSC2 and anti-PD-1 therapy and showed that combination treatment significantly enhanced antitumor activity and immune responses through activation of NK cells.

MATERIALS and METHODS

Tumor cell lines and mice

The CMT167 cell line is a metastatic subclone of the murine alveogenic lung carcinoma cell line CMT64, which was originally isolated from a primary tumor in a C57BL/6 mouse. CMT167 was isolated by subcloning and *in vivo* screening for high metastatic potential. Injection of CMT167 cells into the lungs of syngeneic C57BL/6 mice results in a primary tumor that progresses to form secondary pulmonary tumors and then metastasizes to the lymph nodes and distant organs (20). Stable clones of CMT167 cells expressing firefly luciferase at high levels constitutively driven by an SV40 promoter (CMT167-luc), were kindly provided by the Mayo Clinic.

344SQ cells are metastatic clones derived from *p53R172H* *g/+ K-rasLA1/+* mice on a 129/Sv background (21). The cells were stably transfected with the dual-reporter pEGFP-Luc2 vector. A stable luciferase-positive 344SQ clone (344SQ-luc) was kindly provided by Dr. Frank R. Jirik (University of Calgary, Calgary, Alberta, Canada). Both CMT167-luc and 344SQ-luc cells contain a *KrasG12V* mutation. 344SQ cells have a *KrasG12D* allele and knock-in of a *Trp53R172H* *G* allele. Authenticated cells have been received and were tested for mycoplasma upon arrival.

Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Atlanta Biological, GA, USA) and 1% penicillin and streptomycin (life science technologies, US). Female 129/Sv and C57BL/6-Elite mice (6 to 8 weeks old) were purchased from Charles River Laboratories (Houston, TX). All animal procedures were reviewed and approved by the Animal Care and Use Committee of The University of Texas MD Anderson Cancer Center. All mouse experiments were conducted using the procedures recommended by Landis et al. (22).

TUSC2 nanovesicle formulation and checkpoint antibodies for immunotherapy

TUSC2, previously known as FUS1, was encapsulated in nanovesicles composed of 1, 2-bis(oleoyloxy)-3-(trimethylammonio)propane (DOTAP):cholesterol nanovesicles and a DNA plasmid expressing TUSC2. The plasmid (PLJ 143/pKGB2/FUS 1) was 3968-bp **long** and contained a kanamycin resistance gene, an origin of replication, and the human wild-type *TUSC2* gene driven by a cytomegalovirus promoter. The formulation is routinely manufactured in a GMP facility following standard protocols (7, 10). GMP-grade TUSC2 nanovesicles were used as study agents in a syngeneic lung cancer mouse model.

Anti-PD-1 and anti-CTLA-4 monoclonal antibodies (mAbs) were purchased from Bio X Cell (West Lebanon, NH; catalog no. BE0146 [clone RMP1-14] and catalog no. BE0164 [clone 9D9], respectively). The mAbs were administered with TUSC2 nanovesicles as indicated in Results. InVivoPlus isotype Abs (Bio X Cell; catalog no. BE0089 [clone 2A3]) and GMP-grade nanovesicles containing the plasmid without the *TUSC2* gene were used as a control.

Study design

The objective of this study was to evaluate the therapeutic efficacy of TUSC2 immunogene therapy in combination with checkpoint-blockade immunotherapy and to determine whether TUSC2 and checkpoint-blockade treatments could synergize in 2 syngeneic *Kras*-mutant lung cancer mouse models. Mice were given TUSC2 followed by anti-PD-1 [clone RMP1-14] and/or anti-CTLA-4 [clone 9D9] treatment in a sequential manner to obtain maximum efficacy. In the subcutaneous tumor model, mice were imaged, and tumor volumes were measured to assess tumor progression. In the lung metastasis model, survival curves were generated, and lung metastasis was monitored via imaging. The mice were killed, and the phenotype of the peripheral immune system and the tumor microenvironment in each mouse of every treatment groups was characterized at various time points. Mice were randomized to different treatment groups on the basis of bioluminescent imaging to make sure that all treatment groups started with similar tumor sizes. All experiments were run at least in triplicate except if specified otherwise, and in all therapeutic experiments, at least 10 mice were used for every treatment.

Tumor model

In the CMT167-luc syngeneic model, 6- to 8-week-old female C57BL/6-Elite mice were injected (on day 0) with CMT167-luc cells (1×10^6 cells/100 μ L phosphate-buffered saline) subcutaneously in the right flank. On day 5, the mice were imaged using an IVIS 200 imaging platform (Caliper Life Sciences, Hopkinton, MA) and randomized into treatment groups based on tumor luminescence intensity. For therapeutic experiments, each treatment group consisted of 10 mice. The treatment groups were as follows: control (empty-vector nanovesicles, isotype antibody), anti-PD-1, *TUSC2* nanovesicles, and combination (*TUSC2+anti*-PD-1). Treatment schedules and dosages are shown schematically in Fig. 1. Briefly, 25 μ g of *TUSC2* per mouse was injected intravenously every 48 h for 3 cycles, and 0.25 mg of anti-PD-1 antibody was injected intraperitoneally (i.p.) every 4 days for 3 cycles. Mice were monitored daily for side effects. Two perpendicular tumor diameters were measured twice per week, and tumor surface area was calculated according to a formula

$1/2(\text{Length} \times \text{Width}^2)$. *In vivo* imaging system (IVIS) images of the mice were obtained once a week to monitor tumor progression based on bioluminescence intensity. The mice were euthanized at 3 to 4 weeks after tumor-cell injection and the tumors were harvested for immune histopathological analysis. For immune phenotyping experiments, mice were killed 2 weeks after tumor-cell injection, and peripheral blood was collected from them via cardiac puncture. PBLs were prepared using red blood cell lysis. Serum from the treated mice was collected at the indicated times for cytokine analysis.

In the 344SQ experimental metastasis model, 6- to 8-week-old female 129/Sv mice were intravenously injected with 1×10^5 344SQ-luc cells; lung-specific tumor nodules subsequently developed in them. The tumor model (21) and optimized in our laboratory to get organ specific (lung) colonization through intravenous injection of tumor cells. On day 5, all mice were imaged and randomized into treatment groups based on tumor luminescence. Mice were treated with TUSC2, anti-PD-1, and anti-CTLA-4 (protocol in Fig. 5). The timing of the administration of the agents was synchronized to obtain the maximum therapeutic benefit. In this metastasis model, 15 μg of TUSC2 was administered per mouse 3 times. The treatment groups for the metastasis model were as follows: control (empty-vector nanovesicles, isotype antibody), anti-PD-1, anti-PD-1 and anti-CTLA-4, TUSC2, TUSC2 and anti-PD-1, and triple combination (TUSC2+anti-PD-1 + anti-CTLA-4). The mice were monitored routinely, and their deaths were recorded to create a survival curve. Tumor-bearing mice were imaged using the IVIS platform once a week. For analysis of intratumoral lymphocytes, mice from each treatment groups were killed at week 2, and lungs containing metastases were harvested from them for single-cell analysis. All measurements quantifying experimental outcomes were blinded to the intervention.

Antibody-mediated NK-cell and CD8⁺ T-cell depletion

InVivoPlus mAbs to mouse NK1.1 (BioXcell; catalog no. BP0036 [clone PK136]), to mouse CD8 α (BioXcell; clone YTS 169.4), or an IgG control were injected into the mice (100 μg , i.p.) every 3 days for 4 cycles beginning on day 0 after subcutaneous injection of cancer cells. NK-cell CD8⁺ T-cell depletion status was monitored via flow cytometry analysis of splenocytes at different times to confirm the degree of NK-cell depletion during treatment. NK-cell and CD8⁺ T-cell depletion was studied in the CMT167-luc mouse model. The tumor growth in NK cell and CD8⁺ T cell-depleted mice given TUSC2 or TUSC2 and anti-PD-1 treatment was monitored. Tumor volume was measured every other day, and tumor bioluminescence intensity was quantitated using mouse images taken once a week. Mouse serum was collected from NK cell-depleted mice at week 2 to measure serum cytokine levels.

Multicolor flow cytometry

PBLs were isolated from whole blood after red blood cell lysis. The cells were stained according to standard protocols for flow cytometry. Single-cell suspensions from the spleen and tumor-nodule-bearing lungs were prepared as described elsewhere (23) and stained for flow cytometry analysis. Multi-color panels were developed and optimized for use with a Gallios Flow Cytometer Research System (Beckman Coulter, Brea, CA). Mouse mAbs were purchased from BioLegend (San Diego, CA) unless otherwise mentioned. Single-cell

suspensions were washed with fluorescence-activated cell sorting staining buffer, incubated with a mouse Fc receptor-binding inhibitor for 10 min, and stained with mAbs to CD45-Alexa Fluor 700 (clone 30-F11), CD3PerCP/Cy5.5 (clone 17A2), CD4-Brilliant Violet 510 (clone GK1.5), CD8-FITC (clone 53-6.7), CD19-PE/Cy7 (clone 6D5), CD25-Brilliant Violet 421 (clone PC61), CD49b-PE (clone DX5), and CD62L-APC (clone MEL-14) in 1 panel to analyze major immune populations. The following checkpoint markers were analyzed in a separate panel: FITC anti-mouse CD279 (PD-1) (clone 29F.1A12), Brilliant Violet 421 CD152 (clone UC10-4B9), PerCP/Cy5.5 CD366 (Tim-3) (clone RMT3-23), APC CD223 (LAG-3) (clone C9B7W), PE/Cy7 CD357 (GITR) (clone DTA-1), and CD45, CD3, and CD4 antibodies. A myeloid panel was designed to analyze MDSCs using Brilliant Violet 510 CD11b (clone M1/70), APC/Cy7 I-A/I-E antibody (clone M5/114.15.2), FITC Ly-6G/Ly-6C (Gr-1) (clone RB6-8C5), PE/Cy7 CD68 (clone FA-11), Alexa Fluor 700 CD11c (N418), and Brilliant Violet 421 CD274 (PD-L1) (clone 10F.9G2). The data were acquired using a Gallios Flow Cytometer (Beckman Coulter) and analyzed using FlowJo software version 10 (FlowJo, Ashland, OR), and the leukocyte population was selected by gating CD45+ cells.

Immunohistochemistry and analysis using the Vectra imaging system

CMT167-luc-bearing mice were killed 3 weeks after tumor-cell implantation, and their tumors were fixed in 10% paraformaldehyde. Sections (8 μ m) of formalin-fixed paraffin-embedded tissue were cut and placed on Superfrost Plus microscope slides (Fisher Scientific, Waltham, MA). Immunohistochemical staining of the sections was performed using anti-CD8, anti-Foxp3, anti-Gr-1 and anti-NKp46 antibodies. All immunohistochemical analyses were performed at the MD Anderson Histology Core Laboratory (Smithville, TX), and all the listed antibodies were previously validated and tested in that core laboratory. At least 5 tumor samples from each group were stained for each antibody. All stained slides were imaged and analyzed using a 200-slide Vectra 3.0 Automated Quantitative Pathology Imaging System (PerkinElmer, Waltham, MA) at the Imaging Core Facility at MD Anderson (Houston, TX). Stained slides were loaded into the Vectra slide scanner, and a scanning protocol was produced. The automated imaging platform was set to collect the number of images that covered 25% of each entire section, and acquisition protocols were run in an automated mode according to the manufacturer's instructions. Hundreds of 20 $\times$ multispectral images (around 100 images per treatment group) were acquired by the Vectra system. Images were imported using inForm software version 1.4(PerkinElmer). H-scores ranging from 0 to +3 were generated via per-cell intensity quantitation. The data were analyzed by the Biostatistics Resource Group at MD Anderson.

Quantitative PCR

Collected tumors and sorted NK cells were stored in RNAlater stabilization solution (Sigma-Aldrich, St. Louis, MO) at -20°C . Total RNA was extracted from tumor tissue and isolated NK cells using an RNeasy Mini Kit (Qiagen, Hilden, Germany). cDNA from these samples were synthesized by using a SuperScript III kit (Invitrogen, Carlsbad, CA), and quantitative PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). Primers were designed using National Center for Biotechnology Information primer design software. Relative expression levels were normalized according to $\beta 2m/\beta$ -actin and

calculated using the 2^{-Ct} method. cDNA from sorted NK cells was used to measure the mRNA levels for IL12R, IL15R, and IL18R using quantitative real-time PCR with an ABI Vii7 Real-Time PCR System (Applied Biosystems). The relative quantification was performed using the comparative CT method described by the manufacturer.

Luminex assay

Affymetrix (eBioscience) ProcartaPlex 36-plex immunoassays (Affymetrix, Santa Clara, CA); catalog no. EPX360-26092-901) were performed according to the manufacturer's instructions to identify cytokines and chemokines in mouse serum. Serum from all treated and untreated mice (3 to 5 mice per treatment group) was tested in duplicates to ensure the reliability of the statistics. Briefly, frozen serum samples were thawed on ice and mixed well by vortexing followed by centrifugation at $10,000\times g$ for 10 min to remove particulates. Seven standards were prepared according to the manufacturer's protocol, and 25 μ L of serum samples was mixed with antibody-coated beads. The samples were then incubated for 2 h at room temperature with shaking at 500 rpm. ProcartaPlex Multiplex Immunoassays used Luminex xMAP (multi-analyte profiling) technology. At the end of the assay, the plates were read using a Luminex 200 system (Luminex, Austin, TX) with a 5-parameter logistic regression curve to plot the standard curve. The Luminex system allows for calibration of low and high RP1 target values. Samples and controls were read at both a low RP1 target setting (used to maximize assay sensitivity when the expected concentrations were below 3200 pg/ml) and a high RP1 target setting (used for broad range concentrations). ProcartaPlex Analyst software version 1.0 was used to analyze the data to obtain the concentration of each analyte.

NanoString gene expression analyses

CMT167-luc tumors were implanted via injection of 1×10^6 cells into the right flanks of mice on day 0. The treatments were performed according to the protocol described above. At week 3, the mice were euthanized and their harvested tumors were preserved in RNAlater solution. Total RNA was extracted from each tumor tissue using a Qiagen RNeasy Mini Kit. The RNA samples were submitted to the Genomic Core Facility at Baylor College of Medicine (Houston, TX) to run the NanoString panel. The NanoString PanCancer Immune Profiling panel (NanoString Technologies, Seattle, WA) which profiles 776 genes related to specific immune-cell types and immune-cell functions was used for 12 tumor samples subjected to anti-PD-1, *TUSC2*, combination (*TUSC2*+anti-PD-1), or empty-vector control treatment (3 tumors per treatment group). RNA samples were subjected to quality control first to meet the standards for the NanoString experiment. Quality control-verified RNA was hybridized with the NanoString nCounter PanCancer Immune Profiling mouse panel codeset and quantified using an nCounter Digital Analyzer at the Baylor College of Medicine Genomics Core Facility. The data were analyzed at the Bioinformatics Core Facility at MD Anderson.

Statistical analyses

All experiments were designed and planned with biostatistician Dr. Wang and Dr. Veera from the bioinformatics department at MD Anderson Cancer Center. For the CMT167 model, generalized linear regression models were used to study the tumor growth over time.

A heterogeneous autoregressive covariance structure was used to account for inter-mouse variability and the longitudinal nature of the data. An interaction between treatment and time was assessed to test the heterogeneity of slopes, i.e., the tumor growth rate when time was included in the model as a continuous variable. CONTRAST statement in PROC MIXED procedure in SAS was used to compare the imaging intensity between each pair of the treatment groups with treatment indicator, time, and the interaction between treatment and time in the models where time was included as a discrete variable. The transformation of logarithm to the base 2 of the imaging intensity was used in the analyses to satisfy the normality assumption of the models. Both nominal *P* values and multiple testing adjusted *P* values were reported. SAS version 9.4 and S-Plus version 8.04 are used for the computations for all analyses.

For 344SQ lung metastasis model, the distribution of overall survival (OS) was estimated by the Kaplan-Meier method. Log-rank test was performed to test the difference in survival between groups. Regression analyses of survival data based on the Cox proportional hazards model was conducted on OS defined as from the time of treatment onset to the time of death or to the time of last contact. Both nominal *P* values and multiple testing adjusted *P* values were reported. SAS version 9.4 and S-Plus version 8.04 are used for the computations for all analyses.

Statistical analysis of flow cytometry data and cytokine chemokine luminex data were done by general linear regression models to compare the data among the different treatment groups CONTRAST statement in PROC GENMOD procedure in SAS was used to compare the data between each pair of the treatment groups with treatment indicator in the models. Both nominal *P* values and multiple testing adjusted *P* values were reported. SAS version 9.2 and S-Plus version 8.04 are used for the computations for all analyses.

For analysis of Vectra data generalized linear regression models were used for statistical analysis of H scores among treatment groups. A compound symmetry covariance structure was used to account for inter-mouse variability and the repeated measure nature of the data. ESTIMATE statement in PROC MIXED procedure in SAS was used to compare the H scores between each pair of the treatment groups. The transformation of logarithm to the base 2 of the H score was used in the analyses to satisfy the normality assumption of the models. SAS version 9.4 and S-Plus version 8.04 are used to carry out the computations for all analyses.

For NanoString data analysis, data normalization and statistical analysis were performed. Data generated by nCounter system is normalized prior to being used to quantify the gene profile and statistical analysis. The positive controls, housekeeping genes and negative controls are used to adjust for sample preparation variation, background noise and RNA content variation. The package *NanoStringNorm* in the R statistical language is used to pre-process the raw expression data. Linear model is used to evaluate the overall treatment effect and contrast is used to make pairwise comparisons of interest. The resulting *p* values are modeled using the beta-uniform mixture (BUM) model to determine a false discovery rate (FDR) cutoff and identify significantly differentially expressed genes. All statistical analyses are performed using R statistical software.

RESULTS

TUSC2+anti-PD-1 inhibited tumor growth

In an effort to improve upon the efficacy of single agent checkpoint blockade immunotherapy, we evaluated combination therapy with TUSC2 immunogene therapy and anti-PD-1. The murine lung carcinoma cell line CMT167-luc with a *Kras* G12V mutation and little TUSC2 expression was implanted subcutaneously in C57BL/6 mice. Treatment (empty vesicles, anti-PD-1, TUSC2, and TUSC2+anti-PD-1) ($n = 10$ mice/group) was begun 5 days after tumor cell inoculation when tumor volumes were approximately 50 mm^3 , and mice were randomized before treatment based on tumor intensity measured by IVIS imaging (Fig. 1). The mice received sequential treatments of TUSC2 (i.v.) and anti-PD-1 (i.p.) (Fig. 1A). The combination treatment was not toxic. PD-L1 was expressed in 23.7% of the CMT167 tumor cells (Fig. 1B). Monotherapy with anti-PD-1 had little effect on the rate of tumor growth ($P > 0.24$), as measured by bioluminescence (Fig. 1C), whereas treatment with TUSC2 exerted a significant tumor growth inhibitory effect ($P < 0.05$) as compared to empty nanoparticles/isotype control treated animals. The TUSC2+anti-PD-1 combination showed the greatest reduction in tumor growth versus either monotherapy ($P < 0.0001$ vs anti-PD-1, $P < 0.006$ vs TUSC2). Similar antitumor efficacy was observed using tumor volume measurement (Fig. 1D). The mean volumes for isotype control, anti-PD-1, TUSC2, and TUSC2+anti-PD-1 were 800 mm^3 , 600 mm^3 , 300 mm^3 , and 180 mm^3 , respectively (* $P < 0.05$; ** $P < 0.01$; and *** $P < 0.001$). Representative IVIS images of tumor bearing mice are shown in Fig. 1E. These results suggest that in this *Kras* model, TUSC2 immunogene synergizes with anti-PD-1 in reducing CMT167 tumor growth.

Combination upregulates NK and CD8⁺ T cells, downregulates regulatory cells

To delineate the immune impact of TUSC2 combined with anti-PD-1 we profiled major immune populations in peripheral blood leukocytes (PBLs) and splenocytes using ten color panel flow cytometry (gating strategy in Supplementary Fig. S1). Intravenous TUSC2 nanovesicle treatment increased NK cells and decreased B cells, whereas treatment did not affect the number of CD3⁺ T lymphocytes (Fig. 2A). Effects of TUSC2 and TUSC2+anti-PD-1 treatments in tumor-free mice were not different. Exogenous expression of TUSC2 was evaluated in sorted immune cells (Fig. 2A).

In tumor bearing mice, combination treatment significantly increased the number of NK cells in peripheral blood (PBL) ($P < 0.001$) and spleen ($P < 0.05$), as compared with control groups (Fig. 2B). This induction appears to be mostly a function of TUSC2, as TUSC2 monotherapy showed a slightly higher increase in splenic and circulatory NK frequency than the combination. TUSC2 increased the frequency of NK cells 2.5 fold ($P < 0.001$), whereas anti-PD-1 monotherapy had no effect. CD8⁺ T-cell frequency was increased in PBL with TUSC2 or the combination treatment, which was statistically significant compared with control ($P < 0.05$; $P < 0.0001$, respectively) (Fig. 2B). The combination treatment had a slightly better but insignificant effect compared to TUSC2 alone on CD8⁺ T density. TUSC2 mono- and combination therapy reduced B cell frequency by 50% ($P < 0.001$) and 35% ($P < 0.001$), respectively, whereas anti-PD-1 treatment alone had no effect (Fig. 2B).

TUSC2 monotherapy decreased the frequency of monocytic (M-MDSC) and granulocytic MDSCs (G-MDSC) by 50% in peripheral blood ($P = 0.005$ and $P = 0.0005$, respectively) (Fig. 2C). This effect was significantly enhanced when TUSC2 was combined with anti-PD-1 ($P < 0.001$). Similarly, the combination treatment decreased the number of Treg (CD4+CD25+) cells by 70% in PBLs ($P < 0.005$) and 40% in splenocytes ($P < 0.005$) as compared to controls. Anti-PD-1 monotherapy also reduced the frequency of Treg cells, although not to the same extent as TUSC2 alone or the combination. The number of circulating T cells expressing PD-1, CTLA-4, and Tim3 were reduced by the combination ($P < 0.0001$, $P = 0.003$, and $P = 0.03$, respectively) (Fig. 2E). The greatest difference in combination treatment was in enhancing the ratio of NK cells to MDSCs and CD8⁺ T cells to Tregs ($P < 0.001$) (Fig. 2F). Taken together, these results indicate TUSC2+anti-PD-1 synergy is likely associated with increased expansion of effector cells.

TUSC2+anti-PD-1 enhanced infiltration of cytotoxic effector cells

To determine whether TUSC2+anti-PD-1 treatment is associated with denser tumor immune-cell infiltration, we used the Vectra high-throughput system to analyze immune infiltrates in tumor tissue, covering 25% of each tumor's area ($n = 5$ tumors per treatment group). TUSC2 increased CD8⁺ T-cell infiltration by several fold over compared to empty vector control mice ($P < 0.0001$; Fig. 3A). This effect was enhanced in the combination group ($P < 0.0001$ vs control) and significantly greater than anti-PD-1 ($P = 0.0021$). Infiltration of activated NK cells was highest in tumors treated with TUSC2 ($P < 0.0001$), followed by the combination ($P < 0.0001$) (Fig. 3A). Anti-PD-1 increased NK infiltrates slightly, as compared to TUSC2 or the combination.

This increased infiltration of effector T cells and NK cells was inversely related to frequencies of infiltrated Treg cells and MDSCs. TUSC2 and TUSC2+anti-PD-1 significantly suppressed tumor-infiltrated Foxp3⁺ T cells ($P < 0.0001$), a marker expressed by Tregs (Fig. 3A). Anti-PD-1 monotherapy had an insignificant effect on Foxp3⁺ T-cell infiltration ($P = 0.18$). The number of MDSCs was significantly lower after TUSC2 and combination treatments (combination vs anti-PD-1 and control; $P = 0.0002$ and $P < 0.0001$, respectively; TUSC2 vs anti-PD-1 and control; $p = 0.0001$ and $P < 0.0001$, respectively), whereas anti-PD-1 had no apparent effect in reducing MDSC infiltration ($P = 0.8$) (Fig. 3A). This suggests that TUSC2 and the combination altered the immune microenvironment to make it more favorable for an antitumor immune response possibly mediated by CD8⁺ T lymphocytes and NK cells.

TUSC2+anti-PD-1 enhanced cytotoxic cell-attracting chemokine expression

Chemokines are involved in the recruitment of immune cells to tumors. NanoString analysis of the tumor microenvironment showed that a set of chemokine genes, including those associated with migration of T lymphocytes and NK cells was upregulated after exposure to TUSC2 and the combination (Fig. 3B). Expression of *Ccl3* and *Ccl4*, involved in NK cell migration via CCR5 recognition, more than doubled, whereas *Ccl21a* and *Ccl19*, which interact with CCR7 receptors and recruit T cells and dendritic cells to tumors, increased by more than four fold compared to untreated controls. *Ccl4* and *Ccl5* serum chemokine levels

were also increased by TUSC2 and TUSC2+anti-PD-1 treatment as compared with the control group (Fig. 3C).

TUSC2+anti-PD-1 antitumor activity regulated by NK and cytotoxic T cells

The finding that the densities of both NK cells and CD8⁺ T cells were upregulated after combination therapy strongly suggested that these cells regulate TUSC2+anti-PD-1-induced tumor regression. To confirm this hypothesis, NK or CD8⁺ T cells were depleted in CMT167 tumor-bearing mice *via* intraperitoneal injection of anti-NK1.1 or anti-CD8⁺ T-cell antibodies (Supplementary Figs. S2 and S3). NK1.1 and anti-CD8 depleted approximately 80% of NK and CD8⁺ T cells throughout the experiment, without affecting other major subsets. The antitumor effects of both TUSC2 and the combination were completely abrogated when NK cells were depleted, demonstrating a regulatory role for NK cells in TUSC2+anti-PD-1 synergy, which remained statistically significant under non-NK cell-depleted conditions ($P = 0.002$), as before (Fig. 4A). Antitumor activity of TUSC2 and TUSC2+anti-PD-1 in CD8⁺ T cell-depleted mice remained significant, although the effectiveness of combination treatment was significantly impaired in CD8⁺ T cell-depleted as compared with non-depleted mice ($p < 0.01$) (Fig. 4B). These findings suggest that although CD8⁺ T cells may contribute to TUSC2-enhanced sensitivity to anti-PD-1, NK cells are indispensable for this synergy.

Analysis of serum cytokines using a Luminex assay showed that both TUSC2 and the combination induced a strong Th1-mediated immune response (control vs TUSC2: $P < 0.0001$; control vs combination: $P = 0.007$) where the IFN γ /IL4 ratio was at least 10-fold higher as compared with that of control. The effect was abrogated when NK cells were depleted (TUSC2 vs TUSC2/NK1.1: $p = 0.008$; combination vs combination/NK1.1: $P = 0.0009$) (Fig. 4C). IFN γ secretion was higher but insignificant (TUSC2 vs combination: $P = 0.08$) in the TUSC2 group compared to the combination. Anti-PD-1 treatment released more IL4 (Th2 cytokine) than any other treatment groups (P value 0.001 vs control). This suggests that NK cells played a functional role in inducing a Th1-mediated immune response. Significantly higher expression of IL15 ($P = 0.0001$) and IL18 ($P < 0.0001$) serum cytokines were found in TUSC2 and its combination as compared to untreated control and anti-PD-1 groups (Fig. 4D). As expected, expression of IL15 (control vs TUSC2: $P = 0.03$) and IL18 (control vs TUSC2: $P = 0.0005$) cytokines decreased significantly when mice were depleted of NK cells. IL15 was induced to the same extent in the TUSC2 and combination groups, whereas IL18 concentrations were significantly higher in TUSC2 than the combination. With or without NK depletion, the difference in concentration of IL18 between TUSC2 and the combination was significant, but not for IL15. Release of IL15 and IL18 was not significant, following anti-PD-1 alone treatment ($P = 0.53$, $P = 0.10$ respectively). Finally, an expression profile of IL15 and IL18 in sorted NK cells using qPCR showed significantly higher expression of *IL15R* and *IL18R* in TUSC2-treated than their untreated counterparts ($P = 0.01$ and $P = 0.001$ respectively; Fig. 4E). Higher expression of these two cytokines were also found in the tumor microenvironment following TUSC2 treatment (Fig. 4F), which suggests that these cytokines are involved in NK cell proliferation and activation.

Improved survival in a *Kras*-mutant lung metastasis model with combination

To further validate TUSC2+anti-PD-1 therapeutic synergy, observed in the subcutaneous CMT167 *Kras*-mutant model (Fig. 1), we evaluated efficacy in a *Kras* metastatic lung model. We optimized an aggressive metastatic lung cancer model comprising 129SvE mice injected with 344SQ lung cancer cells. 344SQ cells contain a *KrasG12D* allele and a knock-in *Tip53R172H* G allele. Intravenous injection of 344SQ-luc cells specifically colonized the lung and emitted detectable bioluminescence signals within 5 days of tumor cell injection. PD-L1 was expressed in only 4.5% of 344SQ cells, significantly less than the percentage in the CMT167 mouse tumor model ($P < 0.0001$) (Fig. 5A). The treatment strategy was sequential (Fig. 5B). Two treatment groups were added: anti-PD-1 combined with anti-CTLA-4 and TUSC2 combined with anti-PD-1 and anti-CTLA-4. The anti-PD-1 and anti-CTLA-4 combination was used in this experiment because of reports of enhanced clinical efficacy compared with each drug alone (24). Mice were treated with TUSC2 with or without either anti-PD-1, anti-CTLA-4, or both, 5 days after tumor injection. TUSC2 significantly improved survival in mice with *Kras*-mutant PD-1-insensitive lung metastases (TUSC2 vs control: $P < 0.0001$). TUSC2 combined with anti-PD-1 treatment significantly improved survival in combination-treated mice (combination vs control: $P < 0.0001$) (Fig. 5C). The combination treatment was significantly more effective than TUSC2 alone ($P = 0.024$). We also tested whether the addition of anti-CTLA-4 treatment would increase the effectiveness of the combination treatment. Anti-CTLA-4 and anti-PD-1 treatment improved survival over that in untreated control mice ($P = 0.02$). Combining TUSC2 with the two checkpoint-blockade treatments achieved the longest survival (TUSC2+anti-PD-1 + anti-CTLA-4 vs anti-PD-1 + anti-CTLA-4: $P < 0.0001$; TUSC2+anti-PD-1 + anti-CTLA-4 vs TUSC2: $P = 0.0005$). Bioluminescent imaging showed that lung-specific metastases developed in mice after injection with 344SQ cells and that the signal intensity was reduced by TUSC2 and combination treatments (Fig. 5D). Lungs of mice given TUSC2 had significantly fewer or no visible tumor nodules, compared to untreated animals, at week 2 (Fig. 5E). Thus, TUSC2-sensitization to anti-PD-1 could be produced in both *Kras*-mutant lung cancer mouse models.

Analysis of immune-cell infiltration using single-cell analysis showed significantly higher NK-cell infiltration by TUSC2 compared to control or anti-PD-1+anti-CTLA-4 groups ($P < 0.001$) (Fig. 5F). The effects of TUSC2+anti-PD-1 or TUSC2+anti-PD-1+anti-CTLA-4 were slightly higher than that of TUSC2. In contrast, Treg and MDSC cell infiltration were significantly suppressed by TUSC2 ($P = 0.004$; $P = 0.0003$), an effect that was further enhanced by TUSC2 with checkpoint blockades (Figs. 5G and H). PD-L1⁺ and PD-L2⁺ CD11b⁺ cells in lung metastasis area were significantly inhibited by TUSC2 and the effect remained the same or greater when TUSC2 combined with anti-checkpoint treatment (Fig. 5I). Thus, TUSC2 in combination with checkpoint blockade exerts synergistic therapeutic efficacy against *Kras*-mutant PD-1-insensitive aggressive metastatic lung cancer in a mouse model. This efficacy is associated with high infiltration of NK cells and low abundance of immune suppressor cells in tumors.

TUSC2+anti-PD-1 combination treatment altered immune gene expression

To identify specific immune genes differentially expressed in the TUSC2+anti-PD-1 combination, RNA from tumor samples was subjected to digital multiplexed profiling using a mouse pan-cancer panel consisting of 770 mouse immune genes (NanoString Technologies Inc.). Welch's *t* test with false discovery rate ($q < 0.05$) correction was applied to derive statistically significant gene expression differences between treatment groups. A *P* value of < 0.05 was considered significant. The expression of 33 genes was significantly altered after treatment (overall F test; $P < 0.05$) (Fig. 6A). Because TUSC2 addition enhanced response to anti-PD-1 treatment, we focused on pairwise comparison between anti-PD-1 and TUSC2+anti-PD-1 groups where 13 genes were significantly altered (Fig. 6B). We did pairwise comparisons between all other groups (data not shown). First, a six gene cluster was found to be significantly upregulated in the combination group. This includes *Cd1d2*, *Ltf*, *Klra21*, *H60a*, *Tnfrsf18*, and *Bcl6*. Another cluster was found to be significantly downregulated and consists of *Egr3*, *Cd46*, *Ncr1*, *Klra5*, *Ccl1*, *Il12rb2*, and *Cd59b* (Fig. 6B, Supplementary Fig. S4). All these genes are important for NK cell and CD8⁺ T-cell regulation (25–27). Briefly, *H60a*, an NKG2D ligand, is a marker of NK-cell activation. *Cd1d1* encodes for a MHC class I-like molecule involved in the presentation of lipid antigens to NKT cells.

The combination treatment also upregulated expression of genes associated with T cell-mediated antitumor functions in the tumor microenvironment (Fig. 6C–F). Expression of *Cd8a*, *Cd8b1*, *Gzmk*, *Gzmm*, *Cd27*, *Cd86*, *FasL*, *Cd1d2*, *Btla*, and *Ltk* were at least twice as high in the combination treatment group as in either single-agent treatment group, whereas expression of *IL10* and *Foxp3* was downregulated (Fig. 6C–D). Expression of type I interferons, such as *Ifna1*, *Ifna2*, and *Ifna4*, was also highly increased in the combination treatment group. Higher interferon- γ expression was found with the combination treatment (Fig. 6E). TUSC2+anti-PD-1 treatment also increased *Tbet* (Th1 cells) and decreased *Gata3* transcription factor expression (Th2) (Fig. 6F) which lead to a Th1 mediated immune response. These results support NK and CD8⁺T immunoprofiling and tumor infiltration data (Figs. 2 and 3).

DISCUSSION

Checkpoint blockade immunotherapy has produced durable responses in a subpopulation of lung cancers, suggesting that combination approaches may help overcome tumor-cell immune evasion in patients with metastatic disease (28). G12V and G12D activating *Kras* mutations are the most common driver mutations in lung adenocarcinomas, accounting for about 21% to 17% of lung cancers with a poor prognosis, and are often resistant to conventional therapy (29, 30). No consistent associations between the presence of *Kras* mutations and benefit derived from chemotherapy, immunotherapy, or targeted therapy have been established (30). PD-1/PD-L1 expression is highly associated or elevated with the presence of *Kras* mutations, suggesting that *Kras* mutation affects the function of the PD-1/PD-L1 immune checkpoint pathway (29, 31). The observation that the TUSC2 and anti-PD-1 treatment is effective in *Kras*-mutant tumors is therefore encouraging. Simultaneous or sequential therapies with distinct but potentially complementary

mechanisms of action may have synergistic potential (28). In this study, we found that TUSC2 significantly inhibited tumor growth and extended survival in two G12V and G12D *Kras*-mutant syngeneic mouse models with high and low PD-L1 expression, respectively. Anti-PD-1 showed limited efficacy to restrain tumor growth and enhance survival, but when combined with TUSC2 gene restoration, the impact on tumor regression and survival was far superior.

We examined the antitumor immune response produced by TUSC2 treatment in syngeneic tumor models. Lung cancer cells transfected with TUSC2 express several cytokine genes differently than wild-type cells. In this study, we asked whether the pro-inflammatory cytokines whose expression was induced by TUSC2 altered the innate and adaptive immune-cell populations in peripheral blood and *in vivo* lung tumor model. TUSC2 affects the IL15 cytokine pathway (4), and consistent with that, NK cells significantly increased after TUSC2 treatment.

TUSC2+anti-PD-1 treatment produced a synergistic immune response associated with more NKp46⁺ NK and CD8⁺ T cells both in circulation and in infiltrating the tumor. Cancer patients who have high numbers of cytotoxic T lymphocytes (CTLs) in their tumors tend to have better survival (32), whereas a low density of T cells is associated with a poor prognosis (33). Depletion of NK and CD8⁺ T cells impacted the antitumor activity by combination treatment suggesting the involvement of both NK and CD8⁺ T cells. NK depletion completely abrogated the TUSC2 antitumor effect supporting the role of TUSC2-mediated NK-cell activation in tumor regression. CD8⁺ T-cell depletion significantly reduced the antitumor efficacy by TUSC2+anti-PD-1 suggesting a role for cytotoxic T cells in the combination treatment. This is consistent with others that anti-PD-1 therapy augments T-cell function (18).

NKp46-positive cells are activated NK cells that produce high levels of Th1 cytokines (IFN γ) and low levels of Th2 cytokines (IL4 and IL13). The combination treatment elicited a Th1-mediated immune response by inducing high IFN γ expression, which was TUSC2-mediated and NK cell-dependent. We suggest that the synergistic immune response induced by the combination treatment increases NK-cell population via TUSC2. TUSC2 also enabled NK cells to infiltrate the tumor bed and induced production of higher levels of IFN γ , which facilitated the proliferation of CD8⁺ T cells. Anti-PD-1 treatment followed by TUSC2 treatment may protect the activated CD8⁺ T cells from exhaustion, producing a sustained cytotoxic effect in the tumor microenvironment as we have seen increased infiltration of CD8⁺ T cells in the tumor and a significantly fewer Foxp3 positive cells.

Regulatory immune cells provide a more favorable immune environment for tumor growth and escape. The *Kras*-driven lung tumor microenvironment consists of diverse immune cells (34). *Kras*-driven 344SQ mouse tumors are metastatic and associated with fewer infiltrating CD8⁺ T cells in the tumor (21). TUSC2+anti-PD-1 treatment inhibited MDSC proliferation both in PBLs and tumor. TUSC2 alone, but not anti-PD-1, inhibited MDSC proliferation by an unknown mechanism, but our results suggest that TUSC2 may specifically control CD11b⁺Gr-1⁺ MDSC proliferation without having any noticeable effect on innate CD11b⁺ cells both in tumors and in circulation. Low numbers of myeloid cells were in areas of lung

metastases, inversely correlating with the number of NK cells. Anti-PD-1 treatment had no significant effect on MDSCs, but the combination treatment did decrease MDSCs more effectively, suggesting that TUSC2 and anti-PD-1 synergize to induce a strong antitumor response.

Tregs maintain immunologic self-tolerance, tend to correlate with advancing tumor stage and are accompanied by concomitant Teff and CTL impairment. Anti-PD-1 and anti-CTLA-4 treatment has had encouraging success in NSCLC patients, through mechanisms that may include depleting or functionally impairing Tregs (35). Treg cells constitutively express CTLA-4 and PD-1, and the combination treatment downregulated Treg cells as well as checkpoint markers.

Transplanted tumor cells rapidly cause a Th2 response with increased CD19⁺ B cells. Successful therapy shifts this response to the Th1 phenotype with decreased CD19⁺ B cells and increases numbers of long-term memory CD8⁺ effector T cells (36). Combination with anti-CD19 along with checkpoint blockade treatment eradicates established tumors in a melanoma preclinical model (36). In our study, the number of CD19⁺ B cells was significantly lowered by treatment with TUSC2 and the combination, which was associated with Th1-mediated cytokine secretion.

Gene expression profile analysis, using the Nanostring Technology, revealed a thirteen gene signature associated with NK and CD8⁺ T-cell regulation differentially regulated between anti-PD-1 and TUSC2+anti-PD-1 combination treatment. One cluster was significantly upregulated (*Cd1d2*, *Ltf*, *Klra21*, *H60a*, *Tnfrsf18*, and *Bcl6*), whereas the other was significantly downregulated (*Egr3*, *Cd46*, *Ncr1*, *Klra5*, *Ccl1*, *Il12rb2*, and *Cd59b*). All these genes are important for NK and CD8⁺ T-cell regulation (25–27).

The combination treatment with TUSC2 and anti-PD-1 significantly increased antitumor activity and increased survival of mice with lung metastases with concomitant upregulation of NK cells and CD8⁺ T cells in the blood and tumor microenvironment. Our data suggests NK cell-mediated IFN γ secretion by treatment with TUSC2 and the combination of TUSC2 and anti-PD-1 might polarize T cells or boost CTL response through CD8⁺ T cells proliferation and tumor infiltration, although we do not yet have direct evidence of interaction between NK-cell activation and CTL response. Activated NK cells could interact with CD8⁺ T cells by two possible mechanisms: (I) direct NK cell-T cell interaction in which cytotoxic NK cells kill the target cells through MHC class I-mediated interaction (14); (II) indirect NK cell-T cell interaction through production of IFN γ that polarizes helper T cells (CD4⁺ T cells), increasing CTL function.

The precise mechanism of TUSC2 immunostimulatory potency in combination with anti-PD-1 is not known. Possible mechanism for the TUSC2-enhanced response to anti-PD-1, is that TUSC2 regulates the fate of NK-cell proliferation and cytotoxicity as well as T-cell activation through the PI3K/AKT/mTOR pathway. Several reports have revealed a critical role for this pathway in a broad range of CD8⁺ T cells and IL15-induced NK cell proliferation and cytotoxicity. This is compatible with our previous reports that TUSC2 inhibits mTOR signaling in EGFR wildtype NSCLC (2). TUSC2 enhanced IL15/ IL15R,

IL18, and Th1, all of which are regulated by mTOR, and perhaps mTOR inhibition by TUSC2 promotes constitutive release of these cytokines and others, therefore promoting enhanced proliferation and activation of NK and CT8⁺ T cells.

TUSC2+anti-PD-1 treatment had significant therapeutic effects on two types of *Kras*-mutant tumors including both subcutaneous tumors and lung metastases. The combined treatment exerted synergistic antitumor effects irrespective of PD-L1 status and was observed with both anti-PD-1 and the anti-PD-1 plus anti-CTLA combined checkpoint blockade. In conclusion, the TUSC2 gene successfully alters both innate and adaptive antitumor responses and increases the effectiveness of checkpoint blockade therapy in *Kras*-mutant mouse models of lung cancer, indicating that clinical trials of this therapeutic strategy are warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Financial support: This work was supported in part by the National Institutes of Health/National Cancer Institute through The University of Texas MD Anderson Cancer Center's Cancer Center Support Grant (CCSG) CA-016672 - Lung Program and Shared Core Facilities (J.A. Roth); Specialized Program of Research Excellence (SPORE) Grant CA-070907 (J. A. Roth); Lung Cancer Moon Shot Program (J.A. Roth), The University of Texas MD Anderson Cancer Center, sponsored research agreement from Genprex, Inc; Philanthropist Lois Chiles; and Petrin Foundation.

We acknowledge MD Anderson Cancer Center Flow cytometry and imaging core laboratory; RuPing Shao & Meng Feng for their help in *in vitro* and *in vivo* experiments and Dara S Keener for her assistance in manuscript formatting.

References

1. Prudkin L, Behrens C, Liu DD, Zhou X, Ozburn NC, Bekele BN, et al. Loss and reduction of FUS1 protein expression is a frequent phenomenon in the pathogenesis of lung cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research*. 2008; 14:41–7. [PubMed: 18172250]
2. Meng J, Majidi M, Fang B, Ji L, Bekele BN, Minna JD, et al. The tumor suppressor gene TUSC2 (FUS1) sensitizes NSCLC to the AKT inhibitor MK2206 in LKB1-dependent manner. *PloS one*. 2013; 8:e77067. [PubMed: 24146957]
3. Dai B, Yan S, Lara-Guerra H, Kawashima H, Sakai R, Jayachandran G, et al. Exogenous Restoration of TUSC2 Expression Induces Responsiveness to Erlotinib in Wildtype Epidermal Growth Factor Receptor (EGFR) Lung Cancer Cells through Context Specific Pathways Resulting in Enhanced Therapeutic Efficacy. *PloS one*. 2015; 10:e0123967. [PubMed: 26053020]
4. Ivanova AV, Ivanov SV, Pascal V, Lumsden JM, Ward JM, Morris N, et al. Autoimmunity, spontaneous tumorigenesis, and IL-15 insufficiency in mice with a targeted disruption of the tumour suppressor gene *Fus1*. *The Journal of pathology*. 2007; 211:591–601. [PubMed: 17318811]
5. Ji L, Roth JA. Tumor suppressor FUS1 signaling pathway. *Journal of thoracic oncology : official publication of the International Association for the Study of Lung Cancer*. 2008; 3:327–30.
6. Rimkus T, Sirkisoon S, Harrison A, Lo HW. Tumor suppressor candidate 2 (TUSC2, FUS-1) and human cancers. *Discovery medicine*. 2017; 23:325–30. [PubMed: 28715648]
7. Ito I, Ji L, Tanaka F, Saito Y, Gopalan B, Branch CD, et al. Liposomal vector mediated delivery of the 3p FUS1 gene demonstrates potent antitumor activity against human lung cancer in vivo. *Cancer gene therapy*. 2004; 11:733–9. [PubMed: 15486560]

8. Ito I, Began G, Mohiuddin I, Saeki T, Saito Y, Branch CD, et al. Increased uptake of liposomal-DNA complexes by lung metastases following intravenous administration. *Molecular therapy : the journal of the American Society of Gene Therapy*. 2003; 7:409–18. [PubMed: 12668137]
9. Ramesh R, Saeki T, Templeton NS, Ji L, Stephens LC, Ito I, et al. Successful treatment of primary and disseminated human lung cancers by systemic delivery of tumor suppressor genes using an improved liposome vector. *Molecular therapy : the journal of the American Society of Gene Therapy*. 2001; 3:337–50. [PubMed: 11273776]
10. Lu C, Stewart DJ, Lee JJ, Ji L, Ramesh R, Jayachandran G, et al. Phase I clinical trial of systemically administered TUSC2(FUS1)-nanoparticles mediating functional gene transfer in humans. *PloS one*. 2012; 7:e34833. [PubMed: 22558101]
11. Ren J, Yu C, Wu S, Peng F, Jiang Q, Zhang X, et al. Cationic liposome mediated delivery of FUS1 and hIL-12 coexpression plasmid demonstrates enhanced activity against human lung cancer. *Current cancer drug targets*. 2014; 14:167–80. [PubMed: 24410728]
12. Carroll JL, Nielsen LL, Pruett SB, Mathis JM. The role of natural killer cells in adenovirus-mediated p53 gene therapy. *Molecular cancer therapeutics*. 2001; 1:49–60. [PubMed: 12467238]
13. Childs RW, Carlsten M. Therapeutic approaches to enhance natural killer cell cytotoxicity against cancer: the force awakens. *Nature reviews Drug discovery*. 2015; 14:487–98. [PubMed: 26000725]
14. Crouse J, Xu HC, Lang PA, Oxenius A. NK cells regulating T cell responses: mechanisms and outcome. *Trends in immunology*. 2015; 36:49–58. [PubMed: 25432489]
15. Teng MW, Khanna R, Smyth MJ. Checkpoint Immunotherapy: Picking a Winner. *Cancer discovery*. 2016; 6:818–20. [PubMed: 27485001]
16. Benson DM Jr, Bakan CE, Mishra A, Hofmeister CC, Efebera Y, Becknell B, et al. The PD-1/PD-L1 axis modulates the natural killer cell versus multiple myeloma effect: a therapeutic target for CT-011, a novel monoclonal anti-PD-1 antibody. *Blood*. 2010; 116:2286–94. [PubMed: 20460501]
17. Rizvi NA, Hellmann MD, Snyder A, Kvistborg P, Makarov V, Havel JJ, et al. Cancer immunology. Mutational landscape determines sensitivity to PD-1 blockade in non-small cell lung cancer. *Science*. 2015; 348:124–8. [PubMed: 25765070]
18. Koyama S, Akbay EA, Li YY, Herter-Sprie GS, Buczkowski KA, Richards WG, et al. Adaptive resistance to therapeutic PD-1 blockade is associated with upregulation of alternative immune checkpoints. *Nature communications*. 2016; 7:10501.
19. Rajani K, Parrish C, Kottke T, Thompson J, Zaidi S, Ilett L, et al. Combination Therapy With Reovirus and Anti-PD-1 Blockade Controls Tumor Growth Through Innate and Adaptive Immune Responses. *Molecular therapy : the journal of the American Society of Gene Therapy*. 2016; 24:166–74. [PubMed: 26310630]
20. Weiser-Evans MC, Wang XQ, Amin J, Van Putten V, Choudhary R, Winn RA, et al. Depletion of cytosolic phospholipase A2 in bone marrow-derived macrophages protects against lung cancer progression and metastasis. *Cancer research*. 2009; 69:1733–8. [PubMed: 19208832]
21. Chen L, Gibbons DL, Goswami S, Cortez MA, Ahn YH, Byers LA, et al. Metastasis is regulated via microRNA-200/ZEB1 axis control of tumour cell PD-L1 expression and intratumoral immunosuppression. *Nature communications*. 2014; 5:5241.
22. Landis SC, Amara SG, Asadullah K, Austin CP, Blumenstein R, Bradley EW, et al. A call for transparent reporting to optimize the predictive value of preclinical research. *Nature*. 2012; 490:187–91. [PubMed: 23060188]
23. Cho KW, Morris DL, Lumeng CN. Flow cytometry analyses of adipose tissue macrophages. *Methods in enzymology*. 2014; 537:297–314. [PubMed: 24480353]
24. Larkin J, Chiarion-Sileni V, Gonzalez R, Grob JJ, Cowey CL, Lao CD, et al. Combined Nivolumab and Ipilimumab or Monotherapy in Untreated Melanoma. *The New England journal of medicine*. 2015; 373:23–34. [PubMed: 26027431]
25. Deng M, Zhang W, Tang H, Ye Q, Liao Q, Zhou Y, et al. Lactotransferrin acts as a tumor suppressor in nasopharyngeal carcinoma by repressing AKT through multiple mechanisms. *Oncogene*. 2013; 32:4273–83. [PubMed: 23069661]
26. Shevach EM, Stephens GL. The GITR-GITRL interaction: co-stimulation or contrasuppression of regulatory activity? *Nature reviews Immunology*. 2006; 6:613–8.

27. Orr MT, Sun JC, Hesslein DG, Arase H, Phillips JH, Takai T, et al. Ly49H signaling through DAP10 is essential for optimal natural killer cell responses to mouse cytomegalovirus infection. *The Journal of experimental medicine*. 2009; 206:807–17. [PubMed: 19332875]
28. Melero I, Berman DM, Aznar MA, Korman AJ, Perez Gracia JL, Haanen J. Evolving synergistic combinations of targeted immunotherapies to combat cancer. *Nature reviews Cancer*. 2015; 15:457–72. [PubMed: 26205340]
29. Wood K, Hensing T, Malik R, Salgia R. Prognostic and Predictive Value in KRAS in Non-Small-Cell Lung Cancer: A Review. *JAMA oncology*. 2016; 2:805–12. [PubMed: 27100819]
30. Matikas A, Mistriotis D, Georgoulas V, Kotsakis A. Targeting KRAS mutated non-small cell lung cancer: A history of failures and a future of hope for a diverse entity. *Critical reviews in oncology/hematology*. 2017; 110:1–12. [PubMed: 28109399]
31. Song Z, Yu X, Cheng G, Zhang Y. Programmed death-ligand 1 expression associated with molecular characteristics in surgically resected lung adenocarcinoma. *Journal of translational medicine*. 2016; 14:188. [PubMed: 27342566]
32. Bindea G, Mlecnik B, Tosolini M, Kirilovsky A, Waldner M, Obenauf AC, et al. Spatiotemporal dynamics of intratumoral immune cells reveal the immune landscape in human cancer. *Immunity*. 2013; 39:782–95. [PubMed: 24138885]
33. Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, Lagorce-Pages C, et al. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science*. 2006; 313:1960–4. [PubMed: 17008531]
34. Choi H, Sheng J, Gao D, Li F, Durrans A, Ryu S, et al. Transcriptome analysis of individual stromal cell populations identifies stroma-tumor crosstalk in mouse lung cancer model. *Cell reports*. 2015; 10:1187–201. [PubMed: 25704820]
35. Tanaka A, Sakaguchi S. Regulatory T cells in cancer immunotherapy. *Cell research*. 2017; 27:109–18. [PubMed: 27995907]
36. Dai M, Yip YY, Hellstrom I, Hellstrom KE. Curing mice with large tumors by locally delivering combinations of immunomodulatory antibodies. *Clinical cancer research : an official journal of the American Association for Cancer Research*. 2015; 21:1127–38. [PubMed: 25142145]

Figure 1. TUSC2+anti-PD-1 treatment enhanced antitumor activity

A) Sequential treatment strategy. B) Surface expression PD-L1 on CMT167-luc cells determined by flow cytometry. C, D) Tumor growth curves for four different treatment groups ($n = 10$ mice/group) were determined based on tumor volume and bioluminescence intensity generated by IVIS 200. Control group was treated with nanovesicles loaded with empty vector. E) Representative IVIS images of tumor bearing mice. The data is representative of four independent experiments. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$

Figure 2. Combined TUSC2 and anti-PD-1 upregulates natural killer and cytotoxic T cells and downregulates regulatory cells

A) Effect of TUSC2 on peripheral NK, T cells, and B cells in tumor-free mice. Pooled samples of $n = 3$ mice/group. *In vivo* uptake of TUSC2 nanovesicles determined based on exogenous TUSC2 expression by RT-PCR in sorted T, B, NK, and lineage-negative cells. B) Effect of treatments on NK, T, and B cells at week 2 of tumor implantation. C) TUSC2 treatment altered MDSC status. Gating strategy for MDSC; CD45⁺CD3⁻>MHCII^{low}>CD11b⁺>Gr-1⁺. Granulocytic MDSC: CD11b⁺Gr-1^{high} and monocytic MDSC: CD11b⁺Gr-1^{low}. D) Effect of treatment on Treg (CD4⁺CD25⁺) cells. E) Surface expression of PD-1, CTLA-4 and Tim-3 on T cells. F) Effect on ratios of NK/MDSC and CD8⁺ T/Treg cells. Data shown as mean $\pm$ SD, $n=5$, *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$.

Figure 3. TUSC2 combination increased infiltration of NK and CD8 T cells and impeded MDSC and Treg

A) Immunohistochemistry images for formalin fixed tumor. Vectra automated imaging system was employed to take high resolution images (20X) with imaging of 25% of the tumor area. $n = 5$ tumor sections/groups were imaged. Approximately 100 images per treatment group were analyzed by InForm software for H-Scoring. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$. B) Chemokine gene expression analysis in tumor samples ($n=3$ /treatment) shown and determined by NanoString technology. C) Concentration of CCL4 and CCL5 chemokines in serum induced by TUSC2 treatment are shown. Mean $\pm$ SD; $n = 3$; ***, $P < 0.001$.

Figure 4. Dependence of the antitumor activity of TUSC2 on natural killer cells

Anti-tumor activity of TUSC2+anti-PD-1 treatment was affected by depletion of (A) NK cells and (B) CD8⁺ T cells, shown in tumor intensity graphs ($n = 5$ mice/group). C) Ratio of IFN-γ (Th1) and IL4 (Th2) serum cytokines determined by Luminex assay. D) Concentration of IL18 and IL15 cytokine in NK cell-depleted and non-depleted mice. Mean $\pm$ SD, $n = 3$. E) Fold expression of *IL15Ra* and *IL18R1* in sorted NK cells from mice treated with TUSC2. Mean $\pm$ SD, $n = 3$. F) NanoString analysis of mRNA expression of *IL15Ra* and *IL18R1* in tumors treated with TUSC2. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Figure 5. TUSC2+anti-PD-1 treatment significantly improved survival in a *KRAS*-mutant lung metastasis model

A) PD-L1 expression determined by flow cytometry in 344SQ-luc and CMT167-luc cells. B) Sequential treatment shown schematically. C) Survival is shown in Kaplan-Meier curves after treatment ($n = 10$ mice/group). D) Bioluminescence showed lung specific colonization of tumor cells. A representative of 3 independent experiments shown. E) Dissected lung images show tumor nodule status 2 wk after tumor implantation. (F) NK, (G) Treg, (H) MDSC, and (I) PD-L1⁺ and PD-L2⁺ leukocyte infiltration determined by flow cytometry of single cells prepared from metastasized lungs. Data were normalized based on per gram of tumor tissue (23). PD+CT indicate anti-PD-1+anti-CTLA-4 treatment. Mean $\pm$ SD, $n = 5$. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Figure 6. TUSC2+anti-PD-1 treatment altered expression of immune genes in tumor microenvironment

Gene expression analysis of a NanoString pan-cancer immune panel of 776 genes were performed for 12 samples ($n = 3/\text{treatment}$) from four treatment groups. A) Heat map shows the overall significant genes among treatment groups. B) Pair-wise comparison between TUSC2+anti-PD-1 and anti-PD-1 statistically significant genes are shown in colors. C) Selected genes known for antitumor immune response were highly upregulated at least 2-fold in the combination treatment. D), E) & F) show the fold changes in expression of CD8, IFN γ , and transcription factors Tbx21 and Gata3, respectively.