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. Meraz1, Mourad Majidi1, Xiaobo Cao1, Heather Lin2, Lerong Li3, Jing Wang3, Veera Baladandayuthapani2, David Rice1, Boris Sepesi1, Lin Ji1, and Jack A. Roth1

Abstract

Expression of the multikinase inhibitor encoded by the 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 regulatory T cells (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 than 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 IL15Rα 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 NSCLC. Cancer Immunol Res; 6(2); 163–77. ©2018 AACR.

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 and 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; refs. 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 IFNγ 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%–21%; ref. 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 immuno gene 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 immuno gene therapy 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 p53R172HAg/+ K-rasLA1/+ mice on a 129/Sv background (21). The cells were stably transduced 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 Trp53R172HAg 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) and 1% penicillin and streptomycin (Life Science Technologies). Female 129/Sv and C57BL/6-Elite mice (6 to 8 weeks old) were purchased from Charles River Laboratories. 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 and colleagues (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 (pLI 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. In vivo, 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 (mAb) were purchased from Bio X Cell [catalog no. BE0146 (clone RMPI-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 two syngeneic Kras-mutant lung cancer mouse models. Mice were given TUSC2 followed by anti–PD-1 (clone RMPI-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 group 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 × 10⁶ cells/100 μl phosphate-buffered saline) subcutaneously in the right flank. On day 5, the mice were imaged using an IVIS 200 imaging platform (Caliper Life Sciences) 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 μg of TUSC2 per mouse was injected.
intravenously every 48 hours 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 $\frac{1}{2} (\text{length} \times \text{width}^2)$. In vivo imaging system (IVIS) images of the mice were obtained once a week to monitor tumor progression based on bioluminescence intensity generated by IVIS 200. Control group was treated with nanovesicles loaded with empty vector. Representative IVIS images of tumor-bearing mice. The data are representative of four independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

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 and 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 TUSC2+PD1. Control group was treated with nanovesicles loaded with empty vector. F, Surface expression PD-L1 on CMT167-luc cells determined by flow cytometry. G, 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. In vivo imaging system (IVIS) images of tumor-bearing mice. The data are representative of four independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

TUSC2 Immunogene Combination with Anti–PD-1 Therapy

www.aacrjournals.org Cancer Immunol Res; 6(2) February 2018

Published OnlineFirst January 16, 2018; DOI: 10.1158/2326-6066.CIR-17-0273

www.aacrjournals.org Cancer Immunol Res; 6(2) February 2018

165

Downloaded from cancerimmunolres.aacrjournals.org on November 9, 2021. © 2018 American Association for Cancer Research.
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 nanoparticles, 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 group 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 CD8α (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 CD8α+ 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 CD8α+ T-cell depletion was studied in the CMT167-luc mouse model. The tumor growth in NK cell and CD8α+ 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. Multicolor panels were developed and optimized for use with a Gallios Flow Cytometer Research System (Beckman Coulter). Mouse mAbs were purchased from BioLegend 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 minutes, 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-Briliant 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 29E.1A12), Brilliant Violet 421 CD152 (clone 2B4/10-BB), 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-6C/Ly-6G (Gr-1; clone RB6-8C5), PE/Cy7 CD68 (clone FA-11), Alexa Fluor 700 CD11c (N418), and Brilliant Violet 421 CD274 (PD-L1; clone 10E.9G2).

The data were acquired using a Gallios Flow Cytometer (Beckman Coulter) and analyzed using FlowJo software version 10 (FlowJo), and the leukocyte population was selected by gating CD45+ cells. The leukocytes were sorted and stained according to standard protocols for flow cytometry analysis. Multicolor panels were collected from NK cell and CD8α+ T-cell depletion status was studied in the CMT167-luc mouse model. The tumor growth in NK cell and CD8α+ 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.

**Quantitative PCR**

Collected tumors and sorted NK cells were stored in RNA later stabilization solution (Sigma-Aldrich) at −20°C. Total RNA was extracted from tumor tissue and isolated NK cells using an RNeasy Mini Kit (Qiagen). cDNA from these samples was synthesized by using a SuperScript III kit (Invitrogen), and quantitative PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems). Primers were designed using National Center for Biotechnology Information primer design software. Relative expression levels were normalized according to β2m/β–actin and calculated using the 2−ΔΔCt 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 Viia7 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; 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 (3–5 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 × g for 10 minutes to remove particulates. Seven standards were prepared according to the manufacturer's protocol, and 25 μL of serum samples was mixed with antibody-coated beads. The samples were then incubated for

Meraz et al.
NanoString gene expression analyses

CMT167-luc tumors were implanted via injection of $1 \times 10^6$ 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 RNA later 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) 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 coverage 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 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 preprocess 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 $50 \, \text{mm}^3$, 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 with 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 mm³, 600 mm³, 300 mm³, and 180 mm³, 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 PBLs and splenocytes using 10-color panel flow cytometry (gating strategy in Supplementary Fig. S1). Intravenous TUSC2 nano-vesicle 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 circulating 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 with 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 monocyte (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 regulatory T cells (Treg; CD4⁺CD25⁺) cells by 70% in PBLs (P < 0.005) and 40% in splenocytes (P < 0.005) as compared with controls. Anti–PD-1 monotherapy also reduced the frequency of Tregs, 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 with 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 with TUSC2 or the combination.

This increased infiltration of effector T cells and NK cells was inversely related to frequencies of infiltrated Tregs 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 4-fold compared with 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 nondepleted mice (P = 0.01; Fig. 4B). These findings suggest that although CD8⁺ T cells may contribute to TUSC2-enhanced...
Combined TUSC2 and anti-PD-1 upregulates NK 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⁻MHCIIlowCD11b⁺Gr-1⁺. Granulocytic MDSC: CD11b⁺Gr-1high and monocytic MDSC: CD11b⁺Gr-1low. D, Effect of treatment on Tregs (CD4⁺CD25⁺). E, Surface expression of PD-1, CTLA-4, and Tim-3 on T cells. F, Effect on ratios of NK/MDSC and CD8⁺ T cells/Tregs. Data shown as mean ± SD; n = 5; *P < 0.05; **P < 0.01; ***P < 0.001.
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\( \gamma \)/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\( \gamma \) secretion was higher but insignificant (TUSC2 vs. combination: \( P = 0.08 \)) in the TUSC2 group compared with the combination. Anti–PD-1 treatment released more IL4 (Th2 cytokine) than any other treatment groups (\( P = 0.001 \) 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 with 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

Figure 3.
TUSC2 combination increased infiltration of NK and CD8 T cells and impeded MDSCs and Tregs. A, Immunohistochemistry images for formalin fixed tumor. Vectra automated imaging system was used to take high-resolution images (20×) 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 (\( n = 5/treatment \)) shown and determined by NanoString technology. C, Concentration of CCL4 and CCL5 chemokines in serum induced by TUSC2 treatment are shown. Mean ± SD; \( n = 3; ***, P < 0.001 \).
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.

Figure 4.
Dependence of the antitumor activity of TUSC2 on NK cells. Antitumor 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 IFNγ (Th1) and IL4 (Th2) serum cytokines determined by Luminex assay. D, Concentration of IL18 and IL15 cytokine in NK cell-depleted and nondepleted mice. Mean ± SD, n = 3. E, Fold expression of IL15Ra and IL18R1 in sorted NK cells from mice treated with TUSC2. Mean ± 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 CMT67-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 weeks 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 ± SD; n = 5, *P < 0.05; **P < 0.01; ***P < 0.001.
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 Trp53R172H/ΔC 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 with 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 with 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 (Fig. 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 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, Kfra21, H60a, Trnf1s18, and Bcl6. Another cluster was found to be significantly downregulated and consists of Egr3, Cd46, Ncr1, Kfra5, Ccl1, Il2rb2, 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 NGK2D ligand, is a marker of NK-cell activation. Cd1d1 encodes for an MHC class I–like molecule involved in the presentation of lipid antigens to NK 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, Cd5b1, Gzmk, Gzmnm, Cd27, Cdb6, Fas1, Cd1d2, Bila, and Ltb were at least as high in the combination treatment group as in either single-agent treatment group, whereas expression of Il10 and Foxp3 was downregulated (Fig. 6C and D). Expression of type 1 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 (Fig. 7H). This lead to a Th1-mediated immune response. These results support NK and CD8+ T-cell immunomodulating 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...
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 was performed for 12 samples (n = 3/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–F, Fold changes in expression of CD8, IFNγ, and transcription factors Tbx21 and Gata3, respectively.
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 proinflammatory 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 (CTL) 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 (IFNγ) and low levels of Th2 cytokines (IL4 and IL13). The combination treatment elicited a Th1-mediated immune response by inducing high IFNγ expression, which was more 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 IFNγ, 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). Tregs 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 13-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, Kfra21, H60a, Tnfsf18, and Bcl6), whereas the other was significantly downregulated (Egr3, Cd46, Ncr1, Kfra5, Ccl1, Il12b2, 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 suggest NK cell–mediated IFNγ 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-cell 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 IFNγ 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 mechanisms 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 consistent with our previous reports that TUSC2 inhibits mTOR signaling in EGFR wild-type 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 CD8+ 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.

Disclosure of Potential Conflicts of Interest

J.A. Roth has ownership interest in and is a consultant/advisory board member for Genprex, Inc. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: I.M. Meraz, M. Majidi, J.A. Roth
Development of methodology: I.M. Meraz, M. Majidi, X. Cao, L. Li, J.A. Roth
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): I.M. Meraz, M. Majidi, X. Cao, L. Li, D. Rice
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Majidi, X. Cao, H. Lin, L. Li, J. Wang, V. Baladandayuthapani, B. Sepesi, J.A. Roth
Writing, review, and/or revision of the manuscript: I.M. Meraz, M. Majidi, H. Lin, L. Li, B. Sepesi, J.A. Roth

References

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, Mourad Majidi, Xiaobo Cao, et al.


Updated version
Access the most recent version of this article at: doi:10.1158/2326-6066.CIR-17-0273

Supplementary Material
Access the most recent supplemental material at: http://cancerimmunolres.aacrjournals.org/content/suppl/2018/01/13/2326-6066.CIR-17-0273.DC1

Cited articles
This article cites 36 articles, 9 of which you can access for free at: http://cancerimmunolres.aacrjournals.org/content/6/2/163.full#ref-list-1

Citing articles
This article has been cited by 1 HighWire-hosted articles. Access the articles at: http://cancerimmunolres.aacrjournals.org/content/6/2/163.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://cancerimmunolres.aacrjournals.org/content/6/2/163. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.