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Engineering T cells to express tumoricidal MDA-7/IL-24 enhances cancer immunotherapy

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Abstract

Antigen-specific immunotherapy can be limited by induced tumor immunoediting (e.g., antigen loss) or through failure to recognize antigen-negative tumor clones. Melanoma differentiation associated gene-7/interleukin-24 (MDA-7/IL-24) has profound tumor-specific cytotoxic effects in a broad spectrum of cancers. Here we report the enhanced therapeutic impact of genetically engineering mouse tumor-reactive or antigen-specific T cells to produce human MDA-7/IL-24. While mock-transduced T cells only killed antigen-expressing tumor cells, MDA-7/IL-24-producing T cells destroyed both antigen-positive and negative cancer targets. MDA-7/IL-24-expressing T cells were superior to their mock-engineered counterparts in suppressing mouse prostate cancer and melanoma growth as well as metastasis. This enhanced antitumor potency correlated with increased tumor infiltration and expansion of antigen-specific T cells as well as

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Conception and design: XYW, PBF; Development of methodology: ZL, CG, XY, JJW, MM; Acquisition of data: ZL, CG, SKD, XY, AKP, XL, YN, SC, WL; Analysis and interpretation of data: ZL, CG, HB, MM, PBF, XYW; Writing, review, and/or revision of the manuscript: ZL, CG, HB, MM, PBF, XYW; Study supervision: XYW

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induction of a Th1-skewed immunostimulatory tumor environment. MDA-7/IL-24-potentiated T cell expansion was dependent on T cell-intrinsic STAT3 signaling. Lastly, MDA-7/IL-24-modified T cell therapy significantly inhibited progression of spontaneous prostate cancers in Hi-Myc transgenic mice. Taken together, arming T cells with tumoricidal and immune-potentiating MDA-7/IL-24 confers new capabilities of eradicating antigen-negative cancer cell clones and improving T cell expansion within tumors. This promising approach may be used to optimize cellular immunotherapy for treating heterogeneous solid cancers and provides a mechanism for inhibiting tumor escape.

Keywords

MDA-7/IL-24; T cells; cytotoxicity; cancer; immunotherapy

Introduction

Melanoma differentiation associated gene-7/Interleukin-24 (*mda-7/IL-24*) is a unique IL-10 family gene member that demonstrates profound anti-cancer, anti-proliferative and cytotoxic effects in a broad spectrum of histologically distinct human cancers without harming normal cells (1,2). Other attributes of this therapeutic gene product include ‘bystander’ antitumor activity (3), anti-angiogenesis (4), immune augmentation (5), inhibition of cancer-initiating/stem cells (6), and synergy with other cancer treatment modalities, e.g., radiotherapy or chemotherapy (7,8). A phase I clinical trial in advanced cancers has established the safety and therapeutic efficacy of MDA-7/IL-24, when administered intratumorally by a non-replication competent adenovirus (9), further supporting the potential for translation of MDA-7/IL-24 into the clinic for therapy and its rational integration with other treatment regimens.

Immunotherapy based on the adoptive transfer of naturally occurring (e.g., tumor-infiltrating lymphocytes or TILs) or genetically-engineered T cells (e.g., chimeric antigen receptor or CAR) can mediate cancer regression in patients with advanced disease (10,11). While CAR T-cell therapies have shown remarkable success in the treatment of certain hematologic malignancies, such as relapsed or refractory lymphoblastic leukemia and non-Hodgkin lymphoma, their therapeutic effect in solid tumors has been limited. Accumulating clinical and pre-clinical evidence suggests that this partially results from heterogeneous expression of tumor antigens, immune evasion caused by immuno-editing, e.g., selection of non-immunogenic or antigen loss cancer cell variants (12,13), a lack of sufficient specificity or numbers of TILs (14), and the impact of an immunosuppressive tumor microenvironment (TME) that limit T cell function and persistence (15,16). In fact, immunotherapeutic targeting of tumor-associated antigens can promote the recurrence of the antigen negative tumor variants (17,18). Given the well-documented highly selective tumoricidal activity of MDA-7/IL-24 that is independent of antigen recognition, strategically equipping T lymphocytes with MDA7/IL-24-mediated multivalent cancer-killing features may help overcome the barriers to T cell-based immunotherapy.

In this study, we rigorously evaluated the therapeutic activity of tumor-reactive or antigen-specific T cells that were genetically engineered to carry human MDA-7/IL-24 using multiple clinically relevant mouse syngenic tumor models, including a spontaneous prostate cancer in a transgenic strain of mice. We show that T lymphocytes with a high specificity of tumor recognition can serve as an excellent platform to deliver MDA-7/IL-24 to the tumor sites. More importantly, MDA-7/IL-24 engineering conferred T cells with superior anticancer activity in eradicating established tumors and metastases, which is attributed to synergistic tumor destruction by tumor-reactive or antigen-specific T cells and therapeutic MDA-7/IL-24 as well as a previously undocumented role of MDA-7/IL-24 of promoting expansion and persistence of T cells in the TME.

Materials and Methods

Mice and cell lines

OT-I mice, Pmel mice, B6.PL-Thy1a/CyJ (Thy1.1) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Male C57BL/6 mice were purchased from Envigo (Somerset, NJ). The Hi-Myc transgenic strain mice (19) were kindly provided by Dr. Charles Sawyers (Memorial Sloan Kettering Cancer Center, NY). All experimental procedures were conducted according to protocols approved by the VCU Institutional Animal Care and Use Committee (IACUC).

Mouse prostate tumor RM1 cells, mouse melanoma B16 cells, and 293T cells were purchased from ATCC (Manassas, VA). OVA or luciferase expressing RM1 cells were generated in our laboratory (20,21). Cells were maintained in Dulbecco's Modified Eagles' Medium (DMEM) supplemented with 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin (Hyclone, Logan, UT). All cell lines were routinely tested for mycoplasma contamination using a PCR-based mycoplasma detection kit (ATCC).

Reagents and antibodies

OVA₂₅₇₋₂₆₄ (SIINFEKL) and gp100₂₅₋₃₃ (KVPRNQDWL) peptides were purchased from AnaSpec Inc (Fremont, CA). Recombinant human IL-2, IL-7 and IL-15 cytokines were kindly provided by the National Cancer Institute (NCI, MD). For fluorescence-activated cell sorting (FACS) analysis, mouse monoclonal antibodies to CD4 (GK1.5), CD8a (53-6.7), CD3 (HL3), IFN- γ (XMG1.2), CD25 (PC61), CD90.1 (OX-7), isotype control rat IgG2b (RTK4530), and IgG1 (RTK2071) were purchased from BioLegend (San Diego, CA); FoxP3 antibody (FJK-16s) was purchased from eBioscience (San Diego, CA). For Western blotting analysis, anti-human MDA-7/IL-24 antibody was purchased from GenHunter (Nashville, TN, USA); anti-mouse MDA-7/IL-24 antibodies were purchased from R&D Systems (Minneapolis, MN); phosphor-STAT1 (Y701), phosphor-STAT3 (Y705), total STAT1 and total STAT3 antibodies were purchased from Cell Signaling Technology (Davers, MA). Cucurbitacin I was purchased from Sigma Aldrich (St. Louis, MO). BrdU Flow Kit was purchased from BD Biosciences (San Jose, CA). Polybrene was purchased from Sigma Aldrich (St. Louis, MO). CytoTox 96[®] Non-Radioactive Cytotoxicity Assay kits were purchased from Promega (Madison, WI). Cell Counting Kits 8 (CCK8) were purchased from Abcam (Cambridge, MA).

Preparation and expansion of T cells

Ex vivo expansion of tumor-sensitized T cells was performed as previously described with modifications (22,23). Briefly, spleen and lymph nodes were collected from tumor-bearing mice and dispersed into a single cell suspension in complete RPMI 1640 media at 2×10^6 cells/mL. Cells were then stimulated with Bryostatin 1 (5 nM) and Ionomycin (1 μ M) plus IL-2 (80 IU/mL) for 16–18 hours followed by culture in RPMI 1640 media containing IL-7 and IL-15 (10 ng/mL each) at $1\text{--}2 \times 10^6$ cells/mL for 5 days. For *ex vivo* expansion of antigen-specific OT-I or Pmel T cells, cells were stimulated with OVA_{257–264} peptide or gp100_{25–33} peptide (1 μ g/mL), respectively, for 16–18 hours, followed by culture in RPMI 1640 media containing IL-7 and IL-15 (10 ng/mL each) at $1\text{--}2 \times 10^6$ cells/mL for 5 days.

Lentivirus packaging and cell transduction

The pLenti CMV Puro DEST (w118-1) vector (Addgene plasmid # 17452) encoding human MDA-7/IL-24 or the empty vector together with pMD2.G (Addgene plasmid # 12259) and psPAX2 plasmid (Addgene plasmid # 12260) were co-transfected into phoenix cells using Eugene HD Promega (Madison, WI). Lentiviral supernatants containing control lentivirus (LV-vec) or lentivirus expressing MDA-7/IL-24 (LV-mda-7) were collected 48 and 72 h after transfection. Day 5 expanded T cells were transduced with LV-vec or LV-mda-7 ($2\text{--}3 \times 10^6$ cells/mL in 6-well plate at MOI of 5 in the presence of 6 μ g/mL polybrene) under centrifugation at 2,000 g for 90 mins at 32 °C. Transduced T cells were cultured in the presence of IL-7 and IL-15 overnight before collection for studies.

Tumor studies

Tumors were established by subcutaneous (s.c.) injection of 2×10^5 tumor cells into the right dorsal flank. Mice were randomly assigned to control and treatment groups and received whole body irradiation (WBI, 4.5 Gy) followed by intravenous (i.v.) injection of tumor-sensitized or antigen-specific T cells (10^7 in 200 μ L PBS) at the indicated time points. Tumor growth was monitored in a blinded manner by measuring the perpendicular diameters of tumors. Animals were euthanized when tumors reached the size of approximately 16-mm in diameter or when mice had lost 10% of their total body weight. To establish experimental lung metastases, mice were i.v. injected with 1×10^5 RM1-Luc cells on day 0. Mice were treated with T cells on days 2 and 8. Lung metastases were monitored using IVIS Spectrum imaging system (PerkinElmer, USA). For analysis of leukocyte infiltration into the tumor microenvironment, tumors or lungs were digested with collagenase D (1 mg/mL) and DNase I (20 μ g/mL) to prepare single cell suspensions as previously described (24). Tumor draining lymph nodes (TDL) and spleens (SP) were collected at indicated time points for immune analyses.

For treatment of Hi-Myc transgenic mice, 4-month-old male mice received WBI and engineered tumor-sensitized T cells (1×10^7) that were derived from prostate cancer-bearing 6-month-old Hi-Myc mice. Treatments continued weekly for a total of 4 times. Animals were euthanized at 6 months old and prostate tissues were collected for analyses.

In vivo Bromodeoxyuridine / 5-bromo-2'-deoxyuridine (BrdU) incorporation assay

BrdU incorporation into T cells was assessed by following intraperitoneal (i.p.) injection of 1 mg BrdU 12 hours before collection of lung tissues. Cell suspensions were fixed, permeabilized, and stained with FITC-conjugated anti-BrdU antibody according to the manufacturer's protocol. Frequency of BrdU⁺ cells was determined by flow cytometric analysis using a FACSCalibur flow cytometer (BD Biosciences).

Quantitative Real-time PCR

Total RNA was extracted using TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA). Reverse transcription and real-time reverse transcription-PCR were conducted using primers and carboxy fluorescein (FAM)-labeled probe sets (Thermo Fisher Scientific, Waltham, MA) (25). Gene expression was quantified relative to the expression of β -actin. An analysis of relative gene expression data was performed using the 2^{-CT} method.

Statistical analysis

Statistical analysis was performed using Sigmaplot 14.0 or GraphPad Prism 5. Data are expressed as mean $\pm$ S.D. values. Statistical differences between groups within experiments were determined by the two-way repeated measures analysis of variance (ANOVA) test or the Student *t* test. Survival mice in the experimental groups were compared using the Log-rank test. Values of $p < 0.05$ were considered to be statistically significant.

Results

MDA-7/IL-24-engineering confers compacity for T cells to kill both antigen-positive and antigen-negative cancer cells

Tumor-reactive T cells derived from RM1-OVA prostate tumor-bearing mice were expanded *ex vivo* using a modified protocol involving bryostatin/ionomycin/IL-2 and common γ -chain cytokines (IL-7/IL-15) (23,26). Consistent with our previous reports, use of γ -chain cytokines resulted in a marked increase in the numbers of CD8⁺ and CD4⁺ T cells (23- and 9-fold, respectively), which preferentially exhibited a central memory phenotype (CD44⁺CD62L^{high}) (Supplementary Fig. S1 A–D). ELISPOT analysis showed that the frequency of OVA-specific CD8⁺ T cells was also increased during T cell expansion (Supplementary Fig. S1E). To genetically engineer T cells to produce human MDA-7/IL-24, we constructed a human *mda-7/IL-24*-encoding lentivirus, i.e., LV-*mda-7* (Supplementary Fig. S2 A–B). Expression and secretion of MDA-7/IL-24 protein was confirmed by immunoblotting analysis of media of T cell cultures (Fig. 1A and Supplementary Fig. S2C). The concentration of MDA-7/IL-24 in the culture media 72 h post transduction was estimated to be 300 ng/mL. The ratio of CD8⁺ to CD4⁺ T cells and the central memory phenotype of T cells are comparable in T cells infected with LV-*mda-7* or an empty virus, i.e., LV-vec (Fig. 1B). Infection with LV-*mda-7* did not significantly affect T cell viability (Supplementary Fig. S2D). Transduction of T cells with either LV-vec or LV-*mda-7* did not trigger the activation of T cells (Supplementary Fig. S2E).

We next examined the impact of cellular production of human MDA-7/IL-24 on effector function of T cells. Cytolytic assays showed that RM1 prostate tumor or B16 melanoma-

sensitized T cells, upon engineering to produce human MDA-7/IL-24 (referred to T-mda-7 unless specifically indicated), were more effective than mock-transduced T cell counterparts (T-vec) in killing corresponding cancer cell targets (Fig. 1C and Supplementary Fig. S3A). Immunoblotting analysis showed that MDA-7/IL-24 receptors (i.e., IL-20/22R) were present on RM1 and B16 tumor cells (Supplementary Fig. S3B). Recombinant human MDA-7/IL-24 protein also induced cytotoxicity in RM1 and B16 tumor cells (Supplementary Fig. S3C), which is consistent with our recent reports of killing of human cancer cells by MDA-7/IL-24 protein following binding to its cognate receptors. (27,28)

To examine whether genetic engineering with *mda-7/IL-24* enabled antigen-specific T cells to more efficiently destroy antigen-negative tumor cells, we compared the tumoricidal activity of OVA_{257–264}-specific OT-I cells that had been engineered to produce MDA-7/IL-24 against antigen-negative parental RM1 cells or antigen-positive RM1-OVA cells. While mock transduced OT-I cells only recognized and killed RM1-OVA cells, MDA-7/IL-24-secreting OT-I cells were capable of killing both RM1-OVA and RM1 tumor cells (Fig. 1D). Similar results indicated that OT-I cells carrying MDA-7/IL-24 showed increased cytotoxicity against antigen-positive targets (i.e., RM1-OVA) as compared to mock-transduced OT-I cells (Fig. 1D).

MDA-7/IL-24 production enhances adoptive T cell therapy against established mouse prostate cancer

We first evaluated the therapeutic activity of MDA-7/IL-24-producing OT-I cells in the treatment of mouse prostate cancer. Administration LV-vec-modified OT-I cells to mice bearing RM1-OVA tumors resulted in a modest growth inhibition of this aggressive tumor. However, treatment with MDA-7/IL-24-producing OT-I cells caused profound tumor suppression compared to mock-transduced OT-I cells, which was evident by significantly reduced tumor sizes as well as prolonged life-span (Fig. 2A and B, Supplementary Fig. S4A) and correlated with tumor-associated expression of human MDA-7/IL-24 protein following transfer of MDA-7/IL-24-producing OT-I cells. Additionally, there was a significant elevation of *ifng*, *tnfa*, and *tbet* gene transcription in the tumor upon treatment with MDA-7/IL-24-producing OT-I cells (Fig. 2C), suggesting a Th1-skewed tumor immune environment.

We showed that engineering T cells to produce MDA-7/IL-24 enhanced their *in vivo* persistence or expansion after adoptive transfer, which was supported by a higher frequency of tumor-infiltrating V α 2⁺V β 5⁺CD8⁺ OT-I cells in mice treated with T-mda-7 cells compared to those treated with T-vec cells (Fig. 2D). Treatment with T-mda-7 cells also promoted tumor infiltration by endogenous T cells and their activation based on intracellular IFN- γ staining and flow cytometry analyses (Fig. 2E and F). In addition, immune activation enhanced by MDA-7/IL-24 modification appeared to be systemic, because there was a higher frequency of OVA-specific T cells in the lymphoid tissues such as spleen following T-mda-7 therapy (Supplementary Fig. S4B).

MDA-7/IL-24 engineering enhances the anti-melanoma activity of adoptively transferred T cells

We next used mouse B16 melanoma as an additional independent model to test the therapeutic effect of MDA-7/IL-24-producing T cells. To evaluate the robustness of T cell therapy, mice received tumor-sensitized T-vec or T-mda-7 cells beginning at different times (day 5 or day 9) after inoculation of B16 melanoma (Fig. 3A). In the setting of low tumor burden, MDA-7/IL-24-producing T cells were more efficient than mock-transduced cells in suppressing tumor growth (Fig. 3A, left). In the setting of high tumor burden, MDA-7/IL-24-producing T cells continued to display therapeutic activity whereas mock-transduced T cells failed to control progression of B16 tumors (Fig. 3A, right).

Consistent with our observations in the RM1 prostate cancer model, MDA-7/IL-24 modified T cells induced upregulation of tumor-suppressive immune genes (i.e., *ifn γ* , *tnfa*, *inos*) and downregulation of *arginase 1* that is known to facilitate tumor growth (Fig. 3B and Supplementary Fig. S5A). Similarly, MDA-7/IL-24 expression was associated with enhanced tumor infiltration by adoptively transferred CD90.1⁺CD8⁺ T cells with an activated phenotype (Fig. 3C, D) as well as endogenous CD8⁺ or CD4⁺ T cells (Supplementary Fig. S5B). Furthermore, the ratio between CD4 effector T cells (CD3⁺CD4⁺CD25⁺Foxp3⁻) and regulatory T cells (Treg, CD3⁺CD4⁺CD25⁺Foxp3⁺) or the ratio between CD8 T cells (CD3⁺CD8⁺) and Treg (CD3⁺CD4⁺CD25⁺Foxp3⁺) significantly increased in the tumors after treatment with MDA-7/IL-24-producing T cells (Fig. 3E). However, these changes were not observed in spleens or tumor-draining lymph nodes (Supplementary Fig. S5C).

An independent experiment was performed using T cells from Pmel-1 transgenic mice (Pmel cells) that express a TCR specific for a MHC Class I-restricted T-cell epitope of the melanoma antigen gp100, i.e., gp100₂₅₋₃₃ that is recognized by CD8⁺ T cells (29). Administration of gp100₂₅₋₃₃-specific T cells secreting MDA-7/IL-24 resulted in a profound tumor inhibition compared to mock-transduced T cells (Fig. 3F), which was associated with enhanced T cell activation in the tumor sites, indicated by intracellular IFN- γ staining of tumor-infiltrating CD8⁺CD90.1⁺ Pmel cells (Fig. 3G) and the frequency of IFN- γ -producing T cells in the tumors (Fig. 3H).

MDA-7/IL-24 engineering enhances T cell therapy of cancer metastases

We next studied whether MDA-7/IL-24 engineering improves the efficacy of T cells for eradicating tumor metastases. Experimental lung metastases were established by i.v. injection of luciferase-expressing RM1 cells, followed by treatment with tumor-sensitized T cells that had been engineered with LV-vec or LV-mda-7. Bioluminescence imaging analysis showed that MDA-7/IL-24-producing T cells were the most efficient in reducing lung metastases among all treatment groups (Fig. 4A). This superior antitumor activity of MDA-7/IL-24-producing T cells was also validated by colony-forming assays using lung cell suspensions prepared from treated mice, as indicated by significantly reduced numbers of colony-forming cells (Fig. 4B). Although MDA-7/IL-24 engineering did not alter the memory phenotype of T cells (Supplementary Fig. S6A), it increased the frequency and activation of adoptively transferred as well as endogenous CD8⁺ T cells (Fig. 4C–D) or

CD4⁺ T cells with activated phenotypes (Supplementary Fig. S6B) in the tumor sites. This increased presence of T cells in the tumor sites correlated with elevated gene transcription of *ifng* and *tnfa* that are known to mediate Th1 immunity (Fig. 4E). In addition to the lungs with tumor lesions, MDA-7/IL-24 engineering-enhanced immune activation was also evident in lymphoid tissues (Supplementary Fig. S6C and S6D).

Considering tumor-sensitized T cells contained both CD8⁺ and CD4⁺ T cells after *ex vivo* expansion (Fig. 1B), we sought to define the relative contribution of CD8⁺ and CD4⁺ T cells to the enhanced antitumor activity of MDA-7/IL-24-producing T cells. Mice with established RM1 tumor metastases were treated with MDA-7/IL-24 engineered T cells, sorted CD8⁺ T cells or CD4⁺ T cells, followed by colony forming assays to assess metastatic burden in the lungs. We showed that while CD8⁺ were more effective than CD4⁺ T cells in reducing colony formation, possibly due to their direct cytolytic activity, T cells containing both subsets exhibited the highest therapeutic potency (Fig. 4F).

STAT3 is involved in MDA-7/IL-24-enhanced proliferation of T cells

Given the increased tumor infiltration by T cells following engineering to produce MDA-7/IL-24, we tested the possibility of MDA-7/IL-24 to promote expansion of adoptively transferred T cells using *in vivo* BrdU incorporation assays. Indeed, MDA-7/IL-24-engineered CD8⁺ T cells in lung metastases showed significantly increased BrdU incorporation indicating active proliferation when compared to mock-transduced RM1 tumor-sensitized T cells (Fig. 5A, left), which correlated with a much higher frequency of tumor-infiltrating CD90.1⁺CD8⁺ T cells (Fig. 5A, right). MDA-7/IL-24 engineering also enhanced the proliferation of CD90.1⁺CD4⁺ T cells in transferred tumor-sensitized T cells or endogenous CD90.1⁺CD4⁺ T cells (Supplementary Fig. S7A). However, the MDA-7/IL-24-enhanced T cell incorporation of BrdU proliferation was not evident in the lymphoid tissues (Supplementary Fig. S7B). Similar results were obtained in mice with RM1-OVA tumor metastases that were treated with OVA-specific OT-I cells producing MDA-7/IL-24 (Fig. 5B).

To demonstrate the direct effect of MDA-7/IL-24 production on T cell proliferation, *ex vivo* expanded OT-I cells engineered with LV-vec or LV-mda-7 were stimulated with OVA_{257–264} peptide, followed by ³H-thymidine incorporation assays to examine T cell proliferation. MDA-7/IL-24-producing OT-I cells proliferated more efficiently than mock-transduced counterparts upon antigen stimulation (Fig. 5C). Exogenous addition of MDA-7/IL-24 protein to T cell culture had the same proliferation-promoting effect (Fig. 5D). Considering that MDA-7/IL-24 has been reported to induce cell proliferation through the STAT1 and STAT3 pathways (30,31), we next examined the phosphorylation of STAT1 or STAT3 in MDA-7/IL-24-engineered T cells upon activation. We showed that production of MDA-7/IL-24 increased the phosphorylation of STAT3, not STAT1, when compared to that in mock-transduced T cells (Fig. 5E). Further, pharmacological inhibition of the STAT3 pathway with Cucurbitacin I abrogated MDA-7/IL-24-facilitated T cell proliferation (Fig. 5F).

Activated T cells induce mouse MDA-7/IL-24 in cancer cells

qRT-PCR analyses revealed that treatment with human MDA-7/IL-24-producing murine T cells led to elevation of mRNA levels of human *mda-7/IL-24* in tumor tissues and tumor-draining lymph nodes (DLNs), which was expected as a result of T cell trafficking to the tumor sites and homing to lymphoid organs (Fig. 6A). Intriguingly, we found that in mice receiving human MDA-7/IL-24-producing T cells high levels of endogenous mouse MDA-7/IL-24 (FISP) were induced in the tumors (Fig. 6B), but not in the DLNs (Fig. 6B), suggesting that tumor cells could be the source of the mouse MDA-7/IL-24 (FISP).

Human MDA-7/IL-24 and mouse MDA-7/IL-24 share 61% similarity based on sequence alignment. Previous studies support the antitumor effects of both versions of MDA-7/IL-24. (32) To understand the underlying mechanism of induction of endogenous mouse MDA-7/IL-24 (FISP), we co-cultured RM1 tumor cells with RM1 tumor-sensitized T cells with or without human MDA-7/IL-24 engineering. While the presence of unstimulated T cells only slightly upregulated mouse *mda-7/IL-24 (fisp)* gene expression, activation of T cells with anti-CD3/CD28 antibodies induced a sharp increase of *mouse mda-7/IL-24 (fisp)* mRNA, regardless of T cells producing human MDA-7/IL-24 or not (Fig. 6C). When co-culturing RM1 tumor cells with activated T cells at various ratios, a dose-dependent induction of mouse MDA-7/IL-24 (FISP) was evident. However, this induction was similar when co-culturing with activated MDA-7/IL-24-producing T cells and mock-transduced T cells (Supplementary Fig. S8A). We further validated these results using conditioned media from T-vec or T-mda-7 cells with or without stimulation (Fig. 6D). Lastly, treatment of mouse tumor cells with human MDA-7/IL-24 protein or co-culturing mouse tumor cells with conditioned media derived from gamma-chain cytokines expanded T cells in the presence of exogenous human MDA-7/IL-24 failed to induce tumor-intrinsic mouse MDA-7/IL-24 (FISP) (Supplementary Fig. S8B), suggesting that activation of T cells rather than human MDA-7/IL-24 contributes to elevation of tumor-associated mouse MDA-7/IL-24 (FISP). A similar observation was made when co-culturing B16 tumor cells with activated Gp100-specific Pmel T cells, suggesting that it was not a tumor type-specific effect (Supplementary Fig. S8C). To test the functional significance of induced mouse MDA-7/IL-24 (FISP) in cancer cells, we infected mouse B16 melanoma or RM1 prostate cancer cells with a lentivirus expressing mouse MDA-7/IL-24 (FISP). We showed that overexpression of mouse MDA-7/IL-24 (FISP) significantly reduced viability of mouse tumor cells (Fig. 6E), supporting its tumor-suppressive activity.

Human MDA-7/IL-24-producing T cells exhibit superior antitumor activity in a prostate cancer transgenic mouse model

Lastly, we evaluated the therapeutic potency of human MDA-7/IL-24-producing T cells in treatment of Hi-Myc transgenic mice that spontaneously develop prostate cancer due to prostate-specific expression of c-Myc driven by two Androgen Responsive Elements and the prostate-specific probasin promoter (19). These mice develop prostatic intraepithelial neoplasia (PIN) as early as 6 to 8 weeks of age and progress to develop invasive prostatic adenocarcinoma by 3 to 6 months of age (19,33). Different cohorts of 4-month-old Hi-Myc mice with presumed prostate cancer were treated with tumor-sensitized T cells that had been engineered to produce human MDA-7/IL-24 (Fig. 7A). Examination of the prostates

after euthanizing animals at 6 months showed that T-mda-7 therapy was more effective than T-vec at inhibiting prostate cancer progression, indicated by reduced weights of prostates (Fig. 7B). Administration of MDA-7/IL-24-producing T cells was associated with elevation of mRNA levels of human *mda-7/IL-24*, *tnfa*, and *ifng* in prostate tissues, indicating trafficking of human MDA-7/IL-24-producing T cells to the tumor lesions as well as their activated status (Fig. 7C). H&E staining of prostate tissue sections showed progressing adenocarcinoma in the control group, which was modestly delayed by T-vec treatment. In contrast to the pathology in these two groups, sharply reduced lesions were observed in the prostates of T-mda-7-treated mice (Fig. 7D).

Discussion

It has been suggested that inadequate immune infiltration and T cell persistence, impaired antigen processing or presentation, and immunosuppression in the TME may contribute to the limited therapeutic outcomes of adoptive cell immunotherapies, e.g., CAR-T therapy, in the treatment of solid cancers. In fact, the recurrence of CD19-negative leukemia has been reported following CAR-T cell therapy (17). In this study, we scrutinized a molecular-targeted therapy based on the distinct anticancer activity of the cytokine MDA-7/IL-24 delivered by adoptive immunotherapy through tumor/antigen-specific T cells. Using multiple preclinical cancer models including transgenic mice that spontaneously develop prostate cancer, we demonstrate that MDA-7/IL-24-engineered T cells exhibit superior antitumor activity by counteracting multiple immune limiting factors in the TME and targeting cancer cells beyond an antigen-specific fashion (Fig. 7E).

MDA-7/IL-24 is well documented as a novel therapeutic agent that is active against a broad spectrum of cancers without harming normal cells (1,2). In the current study, we show that tumor-sensitized T cells or antigen-specific T cells engineered to carry human MDA-7/IL-24 have acquired new killing features in addition to T cell-inherent tumoricidal activity (Fig. 1). Considering that MDA-7/IL-24-induced tumor toxicity does not rely entirely on T-cell receptor-mediated antigen recognition, we postulate that MDA-7/IL-24-equipped T cells would have an advantage of eliminating both antigen-positive as well as antigen-negative cancer targets. Indeed, our study shows that MDA-7/IL-24-producing, OVA_{257–264}-specific OT-I cells are capable of destroying both parental RM1 tumor cells and RM1-OVA tumor cells, whereas mock-transduced OT-I cells can only kill RM1-OVA cells (Fig. 1). Our findings support the idea that targeted delivery of MDA-7/IL-24 to tumor sites by antigen-specific T cells (e.g., TILs, CAR-T) can be utilized to facilitate elimination of antigen-loss or low-expressing cancer cells within the heterogeneous tumor cell populations and potentially could overcome the acquired tumor resistance to T cells. Therefore, our molecular-targeted adoptive immunotherapy synergistically augments both immune and non-immune mediated cancer cell toxicity, which can potentially reduce tumor escape from immune-mediated attack during adoptive T cell therapy and help achieve better tumor eradication. In support of this notion, we have demonstrated that tumor-reactive or antigen-specific T cells producing MDA-7/IL-24 display significantly improved therapeutic activity in the treatment of prostate cancer and melanoma primary tumors as well as metastases (Fig. 2–4). Furthermore, treatment of the Hi-Myc transgenic mice with MDA-7/IL-24-engineered T cell therapy profoundly inhibits the progression of spontaneously developed prostate

cancer compared to untreated control animals or those receiving mock-modified T cells (Fig. 7). While there is a limitation of the current study relative to testing human cancer models, our findings strongly support the further scrutiny of this approach in the treatment of human cancers using MDA-7/IL-24-engineered CAR/TCR-T cells.

We also demonstrated that delivery of MDA-7/IL-24 by tumor-reactive or antigen-specific T cells can efficiently reprogram the TME, indicated by substantial elevation of tumor-inhibitory cytokines IFN- γ and TNF- α . This was associated with increased tumor infiltration by not only adoptively transferred T cells but also endogenous CD8⁺ and CD4⁺ T cells. These findings suggest that MDA-7/IL-24-engineered T cell therapy is able to skew an immunosuppressive TME toward an immunostimulatory one with a Th1-polarized phenotype. Our data are consistent with a previously proposed role of MDA-7/IL-24 as a pro-Th1 cytokine (34). It is also possible that tumoricidal MDA-7/IL-24 can trigger immunogenic cancer cell death, further amplifying antitumor immune responses (35). Indeed, we previously showed that MDA-7/IL-24 induced cancer cell death by potentiating endoplasmic reticulum (ER) stress signals in a cancer cell-specific manner (36). Induction of an ER stress response has been identified as one common feature of immunogenic cell death (37). Nonetheless, confirming a potential role of MDA-7/IL-24 for driving immunogenic tumor cell death requires more studies.

We have made an intriguing observation that engineering T cells to produce MDA-7/IL-24 results in increased levels of TILs following adoptive T cell therapy, suggesting that MDA-7/IL-24 produced by therapeutic T cells may support the survival and expansion of effector T-cells after their trafficking to the tumor sites (Fig. 5). The *in vivo* BrdU incorporation assay reveals that those TILs derived from adoptively transferred MDA-7/IL-24-producing T cells proliferate more actively than mock-transduced T cells. Surprisingly, this is not seen in those engineered T cells that have homed to the peripheral lymphoid tissues, suggesting that proliferation of TILs may require sustained engagement by antigenic signals from cancer cells or possibly tumor-associated antigen-presenting cells. Our mechanistic studies using a pharmacologic inhibitor of STAT3 reveal that MDA-7/IL-24-facilitated T cell proliferation depends on the STAT3 signaling pathway. Given the distinct features of MDA-7/IL-24 in selective destruction of cancer cells and in promoting survival, proliferation and persistence of effector T cells, future studies are warranted to investigate MDA-7/IL-24-engineered TILs or CAR-T cells for potentially improved treatment of human solid cancers.

Another major finding that we have made is that treatment with MDA-7/IL-24-carrying T cells induces the elevation of endogenous murine homolog of MDA-7/IL-24 or FISP (Fig. 6). Although autocrine induction of MDA-7/IL-24 in human cancer cells by extracellular MDA-7/IL-24 protein was previously reported (38), our extensive studies using cancer-T cell co-culture, conditioned media from unstimulated or stimulated T cells, and recombinant human MDA-7/IL-24 uncover that T cell activation instead of MDA-7/IL-24 from engineered T cells is responsible for inducing MDA-7/IL-24 (FISP) in mouse tumor cells. Indeed, overexpression of mouse MDA-7/IL-24 (FISP) causes significant inhibition of mouse cancer cells, which is supported by previous reports of mouse MDA-7/IL-24 (FISP)-mediated antitumor activity (39,40). While induction of tumor-endogenous MDA-7/IL-24

(FISP) by activated T cells may also contribute to the overall antitumor response, additional studies are necessary to define its exact role in the setting of cancer immunotherapy.

In summary, we have established a novel targeted adoptive cell immunotherapy using tumor-reactive or antigen-specific T lymphocytes engineered to carry and deliver a unique cancer cell killing cytokine, i.e., MDA-7/IL-24, to achieve broader cancer recognition and destruction. This T cell-based platform for targeting MDA-7/IL-24 to the tumor sites confers advantages over current adenoviral delivery of MDA-7/IL-24, which can be potentially restricted by a host antiviral response (41). Since MDA-7/IL-24 can initiate production of an autocrine/paracrine production of MDA-7/IL-24 (38), the systemic administration of this purified cytokine protein to humans could be problematic. Genetically engineering tumor-reactive or antigen-specific T cells to carry MDA-7/IL-24 enhances expansion or prolongs persistence of TILs, and enables their efficient killing of antigen-loss tumor cell variants as well as antigen-positive targets, which can help overcome tumor resistance to the current adoptive cellular immunotherapies (e.g., CAR-T) to achieve long-lasting control of cancers and metastases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Statement of Significance

This research describes a novel strategy to overcome the antigenic heterogeneity of solid cancers and prevent tumor escape by engineering T lymphocytes to produce a broad-spectrum tumoricidal agent.

Fig. 1. MDA-7/IL-24-producing T cells destroy both antigen-positive and antigen-negative tumor targets.

RM1-OVA tumor-sensitized T cells were transduced with human MDA-7/IL-24-encoding lentivirus (LV-mda-7) or empty lentivirus (LV-vec). Culture media was collected 72 hours after infection to analyze MDA-7/IL-24 levels by immunoblotting (A). The frequency of CD62L^{low}CD44⁺ effector memory T cells (T_{EM}) or CD62L^{high}CD44⁺ central memory T cells (T_{CM}) was analyzed by flow cytometry after T cell expansion by IL-7/IL-15 (B). RM1 (left) or B16 (right) tumor-sensitized T cells were transduced with LV-mda-7 and co-cultured with RM1 or B16 tumor cells. Killing of tumor targets was assessed by LDH assay (C). LV-mda-7-engineered, OVA-specific OT-I cells (T-mda-7) were co-cultured with RM1-OVA or parental RM1 cells at a ratio of 20:1 or 10:1 for 24 hours. Non-transduced (control) or mock-transduced T cells (T-vec) were used as controls. Cytotoxicity was determined by LDH assay (D). Data shown are representative of at least three independent experiments. * $p < 0.05$, ** $p < 0.01$, using student t-test.

Fig. 2. MDA-7/IL-24 engineering confers T cells with improved therapeutic activity in eradication of prostate cancer.

C57BL/6 mice (n=5) were injected *s.c.* with 2×10^5 RM1-OVA tumor cells on day 0. Mice received three doses of LV-mda-7-transduced OT-I cells (T-mda-7) on days 2, 4 and 7. Mice receiving LV-vec transduced OT-I cells (T-vec) or left untreated served as controls. Tumor progression (**A**) and survival of mice (**B**) were recorded. The levels of human MDA-7/IL-24 in tumor tissues after T cell therapy was confirmed by immunoblotting (**A**, inner panel). Gene transcription of *ifng*, *tnfa*, and *tbet* was determined by qRT-PCR (**C**). The frequency of tumor-infiltrating OT-I cells was examined by flow cytometry (**D**). Recruitment of endogenous CD4⁺/CD8⁺ T cells as well as their activation were also examined (**E** and **F**). Data shown are representative of four independent experiments with similar results. * $p < 0.05$, ** $p < 0.01$, using two-way repeated measures ANOVA test (A), log-rank test (B) and student t-test (C, E and F).

Fig. 3. MDA-7/IL-24-producing T cells display enhanced therapeutic efficacy against melanoma. C57BL/6 mice with established B16 melanoma (n=5) received 1×10^7 LV-mda-7-engineered, tumor-sensitized T cells 5 (left) or 9 (right) days after tumor cell inoculation (A). Gene transcription of *ifng* and *tnfa* was determined by qRT-PCR (B). Tumor infiltration of adoptively transferred CD90.1⁺CD8⁺ T cells was assessed by flow cytometry (C). Number of CD90.1⁺CD8⁺IFN- γ ⁺ cells in the tumors (D) as well as the ratio of CD4⁺ Teff/Treg (CD4⁺CD25⁺FoxP3⁻/CD4⁺CD25⁺FoxP3⁺) or CD8⁺/Treg (CD8⁺/CD4⁺CD25⁺FoxP3⁺) (E) are shown. C57BL/6 mice with established B16 tumors received two doses of LV-vec or LV-mda-7-transduced, gp100₂₅₋₃₃-specific Pmel cells on days 4 and 9 post tumor injection. Tumor progression was monitored (F). Tumor-infiltrating CD90.1⁺CD8⁺ cells were analyzed by intracellular staining for IFN- γ (G). Number of CD90.1⁺CD8⁺IFN- γ ⁺

cells in the tumors is also shown (**H**). Data shown are representative of three independent experiments with similar results. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ using two-way repeated measures ANOVA test (A) and student t-test (B, C, D, E, G and H).

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Fig. 4. MDA-7/IL-24-producing T cells show improved ability to eliminate lung metastases. C57BL/6 mice ($n=5$) were injected *i.v.* with luciferase (Luc)-expressing RM1 prostate cancer cells (1×10^5) to establish experimental lung metastases (day 0). Mice were treated with RM1 tumor-sensitized T cells transduced with LV-vec or LV-mda-7 on days 2 and 8. Lung metastases were monitored by bioluminescent imaging. Representative images obtained three weeks after tumor injection are shown (A). Lungs were also collected and subjected to clonogenic assays to examine metastatic burden (B). Changes of lung-infiltrating CD90.1⁺ or CD8⁺CD90.1⁺ T cells (C) as well as endogenous (CD90.1⁻) or adoptively transferred (CD90.1⁺) CD8⁺ T cells expressing IFN- γ (D) were analyzed by flow cytometry. Gene transcription of *ifng* and *tnfa* in the lungs was assessed by qRT-PCR (E). Mice were established with lung metastases from RM1 tumor cells (day 0), followed

by treatment with CD8⁺ or CD4⁺ T cells sorted from LV-mda-7-engineered T cells, or a mixture of both on day 2. Lungs were collected for clonogenic assays two weeks after tumor injection (**F**). Data shown are representative of three independent experiments with similar results. * $p < 0.05$ using student t-test.

Fig. 5. MDA-7/IL-24 modification facilitates T cell proliferation through the STAT3 signaling pathway.

C57BL/6 mice bearing RM1-OVA lung metastases (n=5) were treated with tumor-sensitized T cells (A) or OVA-specific OT-I cells (B). Expansion of adoptively transferred T cells *in vivo* was examined by BrdU incorporation assays three days later. C. Mock- or LV-mda-7 transduced OT-I cells were stimulated with OVA₂₅₇₋₂₆₄ peptide and subjected to ³H-thymidine incorporation assays. D. Proliferation of OT-I cells in the presence or absence of recombinant MDA-7/IL-24 protein was also examined. E. Mock or LV-mda-7-engineered T cells were stimulated by anti-CD3 and anti-CD28 antibodies for 48 hours. Phosphorylation of STAT1 or STAT3 was assayed by immunoblotting. F. Cucurbitacin I (10 nM) were added to T cell culture to STAT3 during the last 16 hours of T cell stimulation. T cell proliferation was assessed by ³H-thymidine incorporation assays. Data shown are representative of three independent experiments. * $p < 0.05$, ** $p < 0.01$, using student t-test.

Fig. 6. MDA-7/IL-24 enhanced T cell activation induces endogenous mouse *mda-7/IL-24 (fisp)* in cancer cells.

A. C57BL/6 mice bearing RM1 lung metastases (n=5) (day 0) were treated with LV-vec or LV-mda-7-transduced, tumor-sensitized T cells on days 2 and 8. Transcription of human *mda-7* gene in tumors or draining lymph nodes (DLN) was assessed by qRT-PCR. **B.** mRNA levels of mouse *mda-7/IL-24 (fisp)* in the lung lesions or DLN were also examined. **C.** RM1 tumor cells were co-cultured with human MDA-7/IL-24-modified or mock-treated T cells (1:20) in the presence or absence of anti-CD3 and anti-CD28 antibodies. T cells were washed off and tumor cells were subjected to analysis of mouse *mda-7/IL-24 (fisp)* mRNA. **D.** RM1 tumor cells were cultured in the presence of conditioned media derived from T cells (T-CM) with or without activation, followed by examination of transcription of mouse

mda-7/IL-24 (fisp) in the tumor cells. **E.** Mouse B16 (*left*) or RM1 (*right*) tumor cells were transduced with a viral vector encoding mouse *mda-7/IL-24 (fisp)*. Cell proliferation was assessed using a CCK-8 kit. The levels of mouse MDA-7 in the culture media was determined by immunoblotting. Data shown are representative of at least three independent experiments. * $p < 0.05$, ** $p < 0.01$, NS, not significant using student t-test.

Fig. 7. Improved control of spontaneously developed prostate cancer by MDA-7/IL-24-producing T cells.

Schematic diagram of MDA-7/IL-24-engineered T cell therapy in Hi-Myc transgenic mice (A). Different cohorts of 4-month-old Hi-Myc transgenic mice received tumor-sensitized T cells that had been transduced with LV-vec (n=8) or LV-mda-7 (n=9) (once a week for a total of 4 doses). Animals were euthanized at 6 months and tumor burden was measured based on the weights of the prostates of individual mice (B). mRNA levels of human *mda-7/IL-24*, *tnfa*, or *ifng* in prostate tissues were assessed by qRT-PCR (C). Representative H/E sections of prostate from each experimental group are shown (D). Data shown are representative of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, NS, not significant using student t-test. E. A novel approach to improved T cell therapy. Antigen-specific or

tumor-reactive T cells can inherently destroy cancer cells *via* TCR or CAR-directed antigen recognition and cytotoxicity (IFN- γ , granzyme B). MDA-7/IL-24 engineering not only enhances T cell proliferation or persistence within the tumors, but also allows T cells to acquire additional tumoricidal capacity and to eliminate antigen-negative variants.