Sensitizing immune unresponsive colorectal cancers to immune checkpoint inhibitors through MAVS overexpression

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ABSTRACT

Background The majority of colorectal carcinomas (CRCs) are insensitive to programmed death protein-1/programmed death-ligand 1 (anti-PD-1/PD-L1) immune checkpoint inhibitor (ICI) antibodies. While there are many causes for ICI insensitivity, recent studies suggest that suppression of innate immune gene expression in tumor cells could be a root cause of this insensitivity and an important factor in the evolution of tumor immunosuppression.

Methods We first assessed the reduction of mitochondrial antiviral signaling gene (MAVS) and related RIG-I pathway gene expression in several patient RNA expression datasets. We then engineered MAVS expressing tumor cell lines and tested their ability to elicit innate and adaptive anti-tumor immunity using both in vitro and in vivo approaches, which we then confirmed using MAVS expressing viral vectors. Finally, we observed that MAVS stimulated PD-L1 expression in multiple cell types and then assessed the combination of PD-L1 ICI antibodies with MAVS tumor expression in vivo.

Results MAVS was significantly downregulated in CRCs, but its re-expression could stimulate broad cellular interferon-related responses, in both murine and patient-derived CRCs. In vivo, local MAVS expression elicited significant anti-tumor responses in both immune-sensitive and insensitive CRC models, through the stimulation of an interferon responsive axis that provoked tumor antigen-specific adaptive immunity. Critically, we found that tumor-intrinsic MAVS expression triggered systemic adaptive immune responses that enabled abscopal CD8+ T cell cytotoxicity against distant CRCs. As MAVS also induced PD-L1 expression, we further found synergistic anti-tumor responses in combination with anti-PD-L1 ICIs.

Conclusion These data demonstrate that intratumoral MAVS expression results in local and systemic tumor antigen-specific T cell responses, which could be combined with PD-L1 ICI to permit effective anti-tumor immunotherapy in ICI resistant cancers.

INTRODUCTION

Immune checkpoint inhibitor (ICI) antibodies targeting PD-1 and PD-L1 have revolutionized the treatment of cancer, triggering long-term regressions in a significant subset of patients with specific types of cancer;1-3 however, the majority of colorectal carcinomas (CRCs) are considered immunological cold and unresponsive to immunotherapy, including immune checkpoint inhibitors (ICIs).

What is already known on this topic

► Cancer immunotherapies have revolutionized the treatment of cancer; however, the majority of colorectal carcinomas (CRCs) are considered immunological cold and unresponsive to immunotherapy, including immune checkpoint inhibitors (ICIs).

What this study adds

► Mitochondrial antiviral signaling gene (MAVS) expression significantly upregulates type 1 interferon pathways in tumor cells, which leads to increased CD8+ T cell-dependent anti-tumor immunity. The combination of MAVS expression with ICIs showed the most improved tumor outcome in ICI irresponsible mouse CRC models.
low microsatellite instability (termed pMMR/MSI-L tumors) and have thus been largely unresponsive (~11% disease control rate) to anti-PD-1/PD-L1 ICIs.\textsuperscript{3,10–13,14} For these largely immune-non-responsive non-MSI CRCs, secondary analyses demonstrate a strongly diminished presence of infiltrating T cell and interferon (IFN) gamma signature in tumors,\textsuperscript{15} suggesting the importance of local T cell activity for responsiveness.\textsuperscript{16,17} Additional studies have also established that tumor mutations in the type I IFN pathway can confer immunotherapeutic resistance,\textsuperscript{18} further indicating that intact type I IFN signaling in the tumor microenvironment is critical for the generation of local and systemic T cell activity required for ICI responses.\textsuperscript{19} Collectively, these studies suggest that the majority of CRCs may have suppressed IFN responsiveness, potentially driven by selection against innate immune genes responsible for IFN signaling.

Given these observations, one potential mechanism for the loss of IFN signaling in CRC is reduced expression of the mitochondrial antiviral signaling gene (MAVS), which was recently reported to be reduced in CRCs, as well as lung and breast cancers.\textsuperscript{20} This study also demonstrated that MAVS knockout mice display an enhanced susceptibility to CRC development,\textsuperscript{20} which was linked to MAVS-regulated p53 stability; however, the potential role of MAVS in CRC immune surveillance was not interrogated, despite its critical role in initiating innate immune responses. In most cells, MAVS functions as the principal innate immune adaptor to transmit signals from the RIG-I and MDA5 RNA sensor genes.\textsuperscript{21–24} RNA sensing genes (RIG-I/MDA5) promote the polymerization of MAVS on the mitochondria to activate TBK1/IRF-3 induction of IFNβ that results in IFN signaling.\textsuperscript{25} Notably, the expression of different RNA/DNA sensing genes has also been found to be suppressed in more advanced cancers.\textsuperscript{26–27} Given this type of selection, significant efforts have been made to develop and exploit synthetic RNA/DNA agonists to enable direct anti-tumor immunotherapy.\textsuperscript{28–33} In a similar approach, de-methylating agents that activate dsRNAs have been shown to trigger MDA5/MAVS activation and colorectal anti-tumor immunity.\textsuperscript{34} Collectively, these findings suggest the primacy of innate immune pathways in initiating anti-tumor immune responses and support the use of alternative strategies to stimulate these responses in advanced tumors, where their function may be suppressed.

Supportive of this concept, it was recently demonstrated that melanoma intrinsic RIG-I plays a critical role in sensitizing to ICIs,\textsuperscript{29} indicating that suppression of the tumor intrinsic RIG-I/MAVS pathway may be a root cause for CRC immune suppression and ICI insensitivity. Structurally, MAVS functions as an intracellular scaffold bridging multiple RNA sensing genes to downstream innate immune signaling cascades, serving as the critical ‘knot’ for the bow-tie structure common in innate immune signaling pathways.\textsuperscript{30} From this vantage point, MAVS (and potentially other adaptors) is highly vulnerable to suppression during tumor development, but may be conversely exploited through its ability to polymerize to enable innate immune signaling.\textsuperscript{36–38} In our study, we validated that MAVS was significantly suppressed in colorectal cancer. We then developed a ligand-independent strategy to stimulate innate IFN signaling in colorectal cancers through the expression of MAVS, capitalizing on its capacity to constitutively polymerize and thereby avoiding ligand-sensor availability constraints encountered with ligand-based delivery approaches.\textsuperscript{39} Not reliant on a single agonist or receptor, we found that the expression of this pivotal adaptor served as an effective means to trigger a coordinated spectrum of IFN signaling genes, cytokines and chemokines in multiple type of murine cells, as well as in human CRC patient-derived xenografts (PDxs). We found that MAVS expression induced IFN responses that subsequently triggered systemic tumor-specific CD8+ T cell responses, resulting in abscopal adaptive immunity against distant tumors. This effect could also be achieved using intrasional MAVS expressing viral vectors and was effective in treating an ICI-resistant colorectal cancer mouse model. Moreover, we found that MAVS promoted the expression of PD-L1 and that anti-PD-L1 therapies were significantly enabled in combination with MAVS expression, thus suggesting their potential clinical utility in treating CRCs, as well as other ‘immunologically cold’ tumors currently resistant to anti-PD-L1/PD-1 ICIs.

**MATERIALS AND METHODS**

**Vector preparation**

Mouse MAVS, human MAVS and control GFP were cloned into E1 shuttle plasmids and used to generate [E1-E3-] Ad vectors using pAdEasy\textsuperscript{40} and generated vectors using previously described methods, except for Ad-MAVS. To generate MAVS expressing adenosial vectors, we engineered 293 cells using KD vectors for MAVS, TRAF6, IRAK4, as well as the overexpression of pp65 to mute innate immune responses that suppressed viral growth. Once Ad-MAVS vector stocks had been established, 293 cells could be infected at high titers to produce high titer stock purified as we described previously. Ad vector stocks were evaluated for replication-competent adenosial virus via real-time PCR (RT-PCR) and tittered using the AdEasy Titer kit (Stratagene). Lentiviral vectors were generated using a Gateway compatible inducible lentiviral system, as described.\textsuperscript{11}

**In vitro tumor cell lines, patient-derived human colon cancer culture, western blot and ELISA for condition media**

CT26 and CT26.CL25 (mouse colon carcinomas with CL25 expressing LacZ), MC38 (mouse colon carcinoma), B16-F10 (mouse melanoma), and 4T1 (mouse breast carcinoma) cells were obtained from ATCC and cultured accordingly. Generation of stable inducible vectors occurs through lentiviral transduction using modified inducible vectors as previously described.\textsuperscript{41} Patient-derived human colon cancer cells were established and described previously\textsuperscript{12} and following cells were used in the study: CRC167...
and CRC57. They are cultured in complete RPMI media and plated 5×10^4 cells/well in 12 well plate (Corning). C57/BL6 wild-type mouse embryonic fibroblasts (MEF) kind gifts from Joseph Nevins (Duke University, Durham, North Carolina, USA). Mouse dendritic cells (DC) were prepared by culturing bone marrow progenitors in Granulocyte-macrophage colony-stimulating factor (GM-CSF) (10 ng/mL) and interleukin (IL)−4 (10 ng/mL) for 5 days, whereas human DCs were prepared from PBMCs cultured in GM-CSF (10 ng/mL) and IL-4 (10 ng/mL) for 5−7 days as previously described.39 Ad-GFP or AdHu-Mavs were added on the day after plating. Because their sensitivity toward Ad-vector are widely different, each cell is treated with a serial dilution of Ad-viral from 1×10^12 to 1×10^9 vp per well. RNA and condition media (CM) were collected at 72 hours post Ad-infection. Samples are compared between cells which were treated with the same dilution of viral particles. Cell lysate were procured for MAVS (Cell Signal #4938) and β-actin (Cell Signal #8457) with western blot. CM was tested with the IFNβ ELISA kit with the procedure recommended by the manufacturer (R&D Systems, DY814 and DY8234). Where indicated, supernatants were assayed using Bio-Rad and Bio-Plex 23-Plex mouse cytokine kits, according to the manufacturer’s recommendations.

Animal procedures

BALB/C (Foxp3-EGFP), C57/Bl6 (Foxp3-IRES-DTR), RAG1 KO and Ifnar1 −/− were all purchased and bred from Jackson Laboratories, Bar Harbor, Maine, USA, while severe combined immunodeficiency (SCID)-beige mice (C.B-Igh-1b/GbmsTac-Pkdcsidc -Lystbg N7) were acquired and bred from Taconic Biosciences. All mice were bred, housed and experiments done at the Duke University Animal Facility. Tumor cell lines (CT26, CL25 or MC38) were injected at the indicated doses by s.c. administration in PBS into mouse flank. Mice (4−8 weeks of age and sex balanced for all experiments) were randomized based on sex and age matched. Tumor size was measured at the indicated time points by caliper measurement biweekly and tumor volume was calculated (small diameter)^2 × (large diameter/2). Mice were sacrificed if tumor volume exceeded 2000 mm^3 or by the end of mouse experiments. Data were collected using an LSR II flow cytometer (BD Bioscience) and analyzed with FlowJo software (TreeStar).

Microarray and quantitative RT-PCR (qRT-PCR) methods

RNA extraction and the qRT-PCR microarray method were described previously.43 44 In brief, RNA was extracted using an RNeasy kit (Qiagen) and assessed for quality and concentration with Nanodrop (ThermoFisher). For microarray, RNA was directly labeled and hybridized using the MO36k 70-mer probe sets from Operon (Mouse Genome Set version 4.0) by the Duke University microarray core. Analysis was performed using GeneSpring V.7.2 and DAVID V.6.0 (21) with data sets deposited at National Center for Biotechnology Information’s Gene Expression Omnibus in a MIAME-compliant form (along with complete details of all procedures and analyses) as accession numbers GSE18957 and GSE19006 using the platform GP6524. For qRT-PCR, RNA was first reverse transcribed using iScript (Bio-Rad). Q-RT-PCR was performed with and iTaq Universal SYBR Green Mix (Bio-Rad) with the QuantStudio 3 system using standard methods as recommended by the manufacturer.

Flow cytometry analysis

We performed two types of flow cytometry analysis (FACS): (1) surface staining of tumor-infiltrating immune cells (TICs). (2) cytokine production from splenocytes. Unless indicated, all mice were humanly sacrificed and flow cytometry was done on spleens and tumors from mice when tumors reached terminal endpoint volume (>2000 mm^3) or by the end of mouse experiments. Data were collected using an LSR II flow cytometer (BD Bioscience) and analyzed with FlowJo software (TreeStar).

FACS for IFNγ and TNFα production from splenocytes in response to antigen stimulation

Alloantigen-primed IFNγ and TNFα-producing T cells were analyzed using FACS. Briefly, splenocytes were prepared and incubated in stimulating media (25 000 000 cells/well). The stimulating media contains anti-CD28 (2 μg/mL), Brefeldin (ThermoFisher) and Monesin (ThermoFisher). The splenocytes were divided into three groups and treated with stimulating media containing firefly luciferase (negative control group, 1 mg/mL), LacZ (test group, 1 mg/mL), p15E (KSPWFTTL, test group, 1 mg/mL) or PMA/ionomycin mix (positive control group). The incubation started with for 5 hours in 37°C and then transfer to 4°C overnight. After the incubation, the splenocytes were first stained for surface marker (Biolegend) and the following panel was used: CD4 PE-TR (#1005666), CD8b APC-CY7 (#126620), CD45 PerCP-CV5.5 (#123128) and CD44 BV421 (#103039). The cells were fixed and permeabilized after surface staining, then followed by staining for IFNγ PE-Cy7 (#505826) and TNFα AF647 (#506314).

FACS analysis for TICs

Using flow cytometry (FACS) to analysis TICs was described before.33 44 In brief, whole tumors from mice were harvested and cut into small pieces (<1 mm) and incubated for 1 hour with 100 μg/mL collagenase, 0.2
U/mL DNase and 1 μg/mL hyaluronidase at 37°C. It was filtered with a 40 μm filter. The following panel of immune cell markers (Biologent) was used: CD44 PE-Cy7 (catalog 101216), LY6G APC (catalog 127614), CD62L BV410 (catalog 128032), CD45 PerCP-Cy5.5 (catalog 123128), CD8b APC-Cy7 (catalog 126620), CD4 PE-TR (catalog 1005666), and viability dye (Aqua; Thermo Fisher Scientific).

Enzyme-linked immunospot
Overall alloantigen-primed IFN-γ-producing splenocytes were quantified using an enzyme-linked immunospot (ELISpot) assay and was described previously. Briefly, splenocytes (500,000 cells/well) were incubated in RPMI1640 medium (Invitrogen) with 10% FBS for 24 hours. Cells were stimulated with LacZ peptides, p15E peptide, fire-fly luciferase peptides or PMA/ionomycin mix (same concentration as we used for FACS). Cells with no stimulation were also kept as a background control. The number of spots per well was determined using a KS ELISpot Automated Reader System with KS ELISpot V.4.2 Software (Carl Zeiss).

Statistics
The tumor volume data are expressed as mean±SEM and were analyzed with two-way analysis of variance. Statistics for ELISA, qRT-PCR, ELISpot and flow cytometry was analyzed with the Student’s t-test. All the statistical analyses are performed using the GraphPad software. The microarray data were analyzed using the limma package. Three replicates for the two conditions, Ad-GFP and Ad-MAVs, were available for MEFs and BMDCs. The expression data were first corrected for background noise. They were then normalized within the replicates and between the two conditions (Ad-GFP and Ad-MAVs) for each of the two cell types (MEFs and BMDCs). Differential gene expression analysis was done by fitting a linear model to the expression data for each probe followed by empirical Bayes moderation of the SEs towards a common value. Finally the p values were adjusted for multiple correction and the differentially expressed genes rank ordered based on the adjusted p value. Volcano plot and Heatmaps were generated for the differentially expressed genes using the R ggplot package.

RESULTS
MAVS expression is suppressed in human colon cancers but its expression can elicit IFN signaling
To determine if MAVS suppression could be associated with the ‘immunologically cold’ tumor microenvironment observed in most CRCs (as suggested from a recent study), we assessed its expression in multiple CRC clinical expression datasets (online supplemental figure S1A-H). These assessments revealed a significant suppression of MAVS transcripts in CRCs compared with normal colorectal tissue (online supplemental figure S1A-B), which did not vary by sex (online supplemental figure S1C), metastasis stage (online supplemental figure S1D), or specific colorectal site comparisons (online supplemental figure S1E). Moreover, subset analyses revealed the greatest MAVS reduction in MSI-hi, compared with MSI-lo CRCs (online supplemental figure S1F-G). This suggested further MAVS reduction due to elevated immune selective pressures in MSI-hi cancers, congruent with our findings that MAVS expression was positively associated with survival of patients with CRC (online supplemental figure S1H). Additionally, we also observed a reduction in MAVS expression in prostate cancer (online supplemental figure S1I), with significant survival associations in lung cancer, independent of smoking status (online supplemental figure S1J-K).

To determine if this transcriptional suppression was unique to MAVS, we performed additional bioinformatics analysis on other genes critical to both the MAVS and STING IFN signaling pathways in the TCGA Colorectal database (online supplemental figure S2A). This analysis revealed IRF-3 to be significantly suppressed in CRCs, while IRF-7, RIG-I and TBK1 had lower expression (although not significant) in CRCs compared with non-transformed colorectal tissue. Oddly, we found that STING and MDa5 expression were significantly elevated in CRCs (online supplemental figure S2A). To further interrogate the level of selection against this pathway, we performed single-sample GSVA enrichment scores of RIG-I, type 1 IFN and type 2 IFN gene sets in TCGA patients with CRC (online supplemental figure S2B). These analyses revealed a suppression of RIG-I/IFN-I gene sets in CRC samples, but not type II IF pathway genes, suggesting broad selective pressure across these pathways. While we did not observe striking genomic loss of MAVS in different datasets (online supplemental figure SSA-B), we also examined if somatic gene mutations in MAVS-related genes could account for suppressed RIG-I/IFN-I signatures (online supplemental figure S3C). These analyses revealed only ~12% of CRCs to have mutations (50 of 399) in any of these genes, with only a single missense mutation in MAVS detected. These analyses suggested that suppression of RIG-I/IFN-I signaling was mediated by the reduction of RNA expression rather than gene loss or mutation. Collectively, these significant expression associations support the premise that a reduction of MAVS expression may play a key role in the ability of CRCs to escape from immune surveillance. As such, we next directly tested if the restoration of MAVS expression would elicit innate immune signaling and subsequent development of anti-tumor immunity.

As MAVS polymerization had been described to elicit ligand-independent signaling in fish cells and autoimmune lupus disease, we sought to determine if this mechanism was conserved in mammalian colorectal cancer cells. In our initial studies, we expressed MAVS in both CT26 (a CRC line insensitive to PD-L1 ICI, online supplemental figure S4) and MC38 (a CRC line sensitive to PD-L1 ICI) murine colorectal cell lines. CT26 cells have a high ploidy number, but are without mutations in mismatch repair genes or hypermutation, and insensitive.
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to PD-L1/PD-1 ICI (online supplemental figure S4). In contrast, MC38 cells contain a hypermutational signature with mutations in BRAF, characteristic of MSI-hi tumors and are sensitive to PD-1/PD-L1 ICI. Using doxycycline-inducible vectors in CT26.CL25 cells (expressing LacZ), we found that doxycycline induction of MAVS (figure 1A) stimulated the expression of IFNβ (figure 1B), as well as multiple downstream IFN-stimulated genes (ISGs), such as ISG15, OAS1a, USP18 and CXCL10 (figure 1B). Using a constitutive MAVS expressing lentiviral vector in MC38 cells (figure 1C), we found comparable MAVS activation of IFNβ, although only induction of the ISG, OAS1a (figure 1D). While inducible IFN responses were insufficient to elicit apoptosis in CT26.CL25 cells (online supplemental figure S5A), constitutive MAVS expression was able to elicit a modest but significant induction of apoptosis in MC38 cells (online supplemental figure S5B). However, this level of MAVS expression and apoptosis was unable to affect the proliferation of either cell line (online supplemental figure S5C-D), suggesting a more muted apoptotic effect compared with studies using ligand stimulation of the RIG-I/MAVS pathway in other cell types. To determine if this effect was unique to MAVS polymerization, we also overexpressed STING in MC38 cells, finding it was unable to elicit IFN signaling (online supplemental figure S6A). We also found that overexpression of STING and multiple INF pathway genes were unable to stimulate IRF-1, Stat1/1, and Stat1/2 pathways to a similar degree in comparison to MAVS (online supplemental figure S6B-D). These results support the reduced ability of these genes to polymerize, dimerize or phosphorylate after overexpression to elicit downstream IFN signaling, as has been previously reported with wild-type STING expression in the absence of ligand.

Collectively, these studies suggested that MAVS expression in CRCs was capable of eliciting innate immune signaling, but had a more marginal effect in stimulating apoptosis and directly suppressing cellular proliferation.

**Tumor-intrinsic MAVS expression significantly suppresses tumor growth by triggering antigen specific anti-tumor immunity**

Having demonstrated the ability of MAVS expression to elicit innate immune signaling, we next determined its impact on anti-tumor responses through the implantation of inducible MAVS expressing CT26.CL25 (1×10^5 cells/site) cells, as well as parental controls, in immune competent BALB/c mice. In our initial experiment, doxycycline was administered at the time of implantation. We found that early MAVS induction suppressed tumor growth compared with non-doxycycline and parental controls, abrogating the development of ~29% of tumors (online supplemental figure S7A and B). To determine if this effect was enhanced in immune-sensitive lines, we conducted a similar experiment using our MC38 cell model counterparts. This experiment revealed that tumor-specific MAVS expression prevented the development of 80% of MC38 tumors and suppressed growth in the remaining tumors compared with controls (figure 2A). In contrast, overexpression of STING in MC38 cells had no effect on tumor growth (online supplemental figure S7C). Having demonstrated an impact on tumor development, we next assessed the effect of MAVS expression on established immune-insensitive tumors. To achieve this, we implanted parental or MAVS expressing CT26.CL25-MAVS cells and began doxycycline treatment when...
tumors had >2 mm diameter (7 days post injection). In this setting, we found that MAVS expression in established tumors caused a significant reduction in tumor growth with complete tumor regression occurring in 40% of mice (figure 2B). To determine if this regression could be due to the development of tumor-specific immunologic memory responses, we challenged mice that had complete tumor regression (1 month post regression in both MC38 and CT26.CL25 models) with parental CT26.CL25 or MC38 cells. In contrast to controls (figure 2C and D), we observed no tumor growth in any of the rechallenged mice (up to 3 weeks post challenge), which strongly suggested that innate immune induction mediated by MAVS expression triggered persistent adaptive immunological memory against tumors.

To directly determine the effect of MAVS expression on tumor-specific adaptive responses, we evaluated the induction of tumor-specific T cell responses from CT26.CL25-MAVS injected mice using LacZ peptides (specific for LacZ expressing CT26.CL25 cells; figure 2E). These assays revealed a significant systemic increase in LacZ-specific IFNγ responses in mice with MAVS expressing CT26.CL25 tumors (figure 2E). To further probe the nature of these systemic adaptive immune responses, we utilized intracellular flow cytometry to assess different immune response markers from tumor antigen-specific stimulated splenocytes. These studies demonstrated that MAVS expressing tumors had augmented populations of systemic IFNγ (figure 2F), TNFα+/IFNγ+ (online supplemental figure S8A) and CD4+TNFα+/IFNγ+ (online supplemental figure S8B) tumor antigen-specific CD8+ T cells. The induction of systemic antigen-specific activated poly-functional CD8+ T cells was also associated with a significant expansion of CD4+ T cells, especially CD4+ central memory T cells (CD4+CD62Lhigh) in the local tumor microenvironment (online supplemental figure S9), suggesting that MAVS expression enhances local CD4+ responses, which facilitate the systemic induction of CD8+ T cells. However, we did not observe a significant alteration in the overall level of CD8+ T cell infiltration in the tumor microenvironment (online supplemental figure S9). Overall, these results suggest that tumor-intrinsic expression of MAVS alters stimulation of systemic anti-tumor immunity, necessary for an abscopal effect.

To investigate if local MAVS intrinsic tumor expression could induce abscopal immunity against distant tumors, we employed a bilateral model using implantation of CT26.CL25-MAVS inducible cells into one flank and parental CT26.CL25 into the other flank (figure 3A). Using this model, MAVS expression was induced in established tumors at 7 days post implantation. As before, we found that MAVS significantly suppressed the growth of MAVS expressing tumors, but that it also equivalently suppressed the growth of non-MAVS expressing tumors.
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on contralateral flank (figure 3B). Similar to our previous results, we also observed a significant increase in tumor-specific IFNγ ELISPOT responses (figure 3C) in MAVS expressing tumor-bearing mice, as well as an enhanced expansion of antigen-specific IFNγ CD8+ T cells by intracellular flow cytometry (figure 3D). These results demonstrated that tumor intrinsic MAVS expression could induce systemic tumor antigen-specific T-cell immunity, in spite of an augmented overall tumor burden in the bilateral model, which permitted robust abscopal responses in vivo.

THE SUPPRESSED TUMOR GROWTH TRIGGERED BY TUMOR-INTRINSIC MAVS EXPRESSION IS DEPENDENT ON CD8+ T CELLS AND TYPE I IFN PATHWAY ACTIVITY

To confirm that MAVS suppresses tumor growth through an adaptive immune mechanism, we repeated our experiments in immunodeficient SCID mice, lacking T cells and B cells. These experiments revealed no significant difference in the tumor volume between control and MAVS expressing groups in both CT26.CL25 and MC38 models (figure 4A and B), consistent with a lack of proliferative impact in vitro (online supplemental figure S3). This suggested the importance of adaptive immunity in mediating tumor growth suppression, potentially through local IFN stimulation of T cell responses.18,19 Given MAVS induction of antigen-specific CD8+ T cell responses (figures 2 and 3), we next evaluated their importance in mediating anti-tumor responses by CD8-specific antibody depletion. These experiments revealed that CD8+ T cells were essential for MAVS-mediated anti-tumor responses against both MC38 cells (figure 4C) and CT26.CL25 cells (figure 4D). Assessment of antigen-specific responses after tumor-specific peptide stimulation by intracellular flow cytometry of splenocytes again confirmed systemic MAVS induction of antigen-specific immunity, characterized by polyfunctional IFNγ/TNFα CD8+ T cells (figure 4E–F) that were eliminated by CD8 T cell depletion (figure 4F). To determine the role of the type I IFN pathway in the induction of these responses, we repeated these experiments in the MC38 model using an IFNAR blocking antibody (figure 4G). We found that blockade of the type I IFN receptor also eliminated MAVS anti-tumor responses (figure 4G), demonstrating the importance of type I IFN signaling in the induction of adaptive anti-tumor immunity. These data support that IFN signaling is critical for MAVS induction tumor-specific, CD8+ T cell-mediated adaptive immunity.

Adenoviral vector-mediated MAVS overexpression alters gene expression and suppresses growth of established colorectal tumors

Having demonstrated the immunotherapeutic potential for MAVS expression in tumor cells, we next sought to test a more clinically translational approach using intratumoral delivery of MAVS expressed from adenoviral vectors. We found that MAVS expression significantly suppressed the generation of viral vectors; thus, we established 293 cells deficient in innate immune signaling to generate [E1-,E3-] adenoviral vectors expressing MAVS or GFP (see the Methods section). To determine if MAVS-mediated innate
responses were blunted, enhanced or unchanged due to the added variable of viral infection, we infected CT26.CL25 and MC38 colorectal lines with control and MAVS expressing adenoviral vectors and assessed IFNβ and ISGs expression (figure 5). We again found that MAVS induced the secretion of IFNβ, as well as the robust induction of ISG genes in both CT26.CL25 and MC38 cells compared with control Ad infected counterparts (figure 5A–D). We also found that while viral expression of MAVS enhanced apoptosis, this induction was not statistically significant in either MC38 or CT26.CL25 cell lines (online supplemental figure S10). To ascertain if this effect was restricted to CRC cells, we also

Figure 4  CD8 T cells and type 1 interferon (IFN) pathway plays a critical role in mitochondrial antiviral signaling gene (MAVS)- driven tumor growth suppression in vivo. (A) MC38 or MC38-MAVS cells (1×10^6 cells/site) were implanted into the flank of SCID-beige mice and growth monitored (N=5). (B) CT26.CL25 or CT26.CL25-MAVS cells (1×10^5 cells/site) were implanted as in (A) with doxycycline treated starting at day 0 (N=5). (C) MC38 or MC38-MAVS cells (1×10^6 cells/site) were implanted in mice with biweekly antibody injection to deplete CD8 T-cells (beginning on day 0; N=5). (D–F) CT26.CL25 or CT26.CL25-MAVS cells were engrafted into the flank of BALB/c mice (100k cells per mouse) with doxycycline administered at 1 week post implantation (all groups) and biweekly antibody injection to deplete CD8 T-cells (or isotype) starting on day 0. (E) At 20 days post tumor injection, mice were sacrificed and splenocytes from engrafted mice in both the control group and MAVS group were assessed for tumor-specific (anti-LacZ) T cell responses by IFN-γ/ TNFα+intracellular flow cytometry (E) and IFN-γ enzyme-linked immunospot (ELISpot) assay (F). (G) MC38 or MC38-MAVS cells (1×10^6 cells/site) were implanted in mice with IFN-α/β Receptor (IFNAR) antibody blockade. Biweekly antibody injection were started on the day of tumor cell injection and tumor volume were monitored biweekly as well (N=5). *, p<0.05; **, p<0.01, ***, p<0.001.

Figure 5  Ad-mitochondrial antiviral signaling gene (MAVS) infection elicits a significant induction of type I interferon (IFN) pathways and proinflammatory cytokines in vitro. (A–D) Ad-MAVS infection upregulates the expression of MAVS and the downstream type I IFN pathway genes via quantitative RT-PCR (QRT-PCR) (A) in CT26.CL25 (A) and MC38 cells (C) in vitro (n=3). The release of IFNβ was confirmed with ELISA (n=5) (B) and (D). (E–H) Ad-MAVS infection upregulates the expression of MAVS and the downstream type I IFN pathway genes via QRT-PCR in mouse melanoma B16-F10 cells (E) and breast cancer 4T1 cells (G) (n=3). The release of IFNβ was confirmed with ELISA (n=5) (F) and (H). (I–L) Ad-MAVS infection upregulates the expression of MAVS and the downstream type I IFN pathway genes via QRT-PCR in multiple patient-derived xenografts colorectal cancer cells in vitro, including CRC167(I), and CRC57(K) (n=3). The release of IFNβ was confirmed with ELISA (J) and (L). N=6–10, bars indicate SD, and ** represents p<0.01.
tested the impact of Ad-MAVS infection in B16-F10 melanoma cells, and a 4T1 breast carcinoma line. These studies also revealed robust induction of IFNβ and its downstream genes (figure 5E–H). Finally, to determine if these effects were constrained to mice and could apply to human CRCs, we also generated an [E1,E3]- adenoviral vector expressing human MAVS (Ad-hMAVS, see the Methods section). Using this vector, we infected several previously generated patient-derived tumor xenografts (PDX) colorectal cancer lines in vitro. Notably, we found that Ad-hMAVS infection significantly increased the secretion of IFNβ in multiple tested patient-derived colorectal cancer cells, suggesting that the effects of MAVS expression are highly conserved between different patients’ cancers (figure 5I–L). Collectively, these experiments revealed robust induction of IFNβ stimulation of innate immune responses is a basal function across different cancer cell types and has the potential to initiate anti-tumor immunity against human colorectal cancer.

These findings also prompted our exploration of other non-cancer cells, which constitute critical elements of the tumor microenvironment and could be transduced by different intratumoral delivery methods. As our previous studies had examined transcriptional data from bulk CRCs (online supplemental figure S1), we analyzed MAVS expression in a CRC single-cell RNA sequence (scRNAseq) dataset consisting of 23 individual CRC tumors (Fig S11). Notably, this broad scRNAseq analysis revealed that MAVS is mostly expressed in epithelial cells, with only modest expression in stromal cells and little expression in immune cells (such as T cells and B cells) in primary human CRC tumors (online supplemental figure S11). To further explore the possible role of MAVS in the CRC stromal cells, we used primary murine fibroblasts and bone-marrow derived DCs (representative of stromal and sentinel immune populations in tumors). We conducted RNA expression analysis experiments that revealed Ad-MAVS infection significantly increased the expression of MAVS, inflammatory cytokine/chemokines and immune-regulatory cytokines (online supplemental figures S12-14 and online supplemental table 1) in both cell types. These results indicated that MAVS expression not only triggered robust type I IFN signaling, but elicited a broader Th1 inflammatory profile (such as IL-12p70, GM-CSF, IL-2, and CCL2) that likely contributes to the T cell activity. Additionally, these experiments illustrate that infection of non-tumor cell types in the tumor microenvironment would lead to similar responses, due to the conservation of the MAVS innate immune pathway.

Having demonstrated the potential of Ad-MAVS to elicit robust IFN signaling in vitro, we then tested its therapeutic efficacy in established tumors in vivo. CT26.CL25 cells were bilaterally implanted into mice and Ad-MAVS or a control Ad-GFP were intratumorally injected at 7 days post implantation into the left flank tumor (figure 6A). In these experiments, we observed that Ad-MAVS injected tumors had

**Figure 6** Adenoviral delivery of mitochondrial antiviral signaling gene (MAVS) induces systemic, T-cell mediated anti-tumor immunity in vivo. (A–C) CT26.CL25 cells were engrafted bilaterally into the flanks of BALB/c mice. One week post implantation, left flank tumors were intralesionally challenged with control Ad-GFP or Ad-MAVS (5×10¹⁰ viral particles) and tumor growth monitored for 3 weeks (N=5). Splenocytes were then assessed for anti-LacZ and anti-GFP T cell responses by an interferon γ (IFN-γ) enzyme-linked immunospot (ELISpot) assay (N=5; C), (D–I) MC38 tumor which were engrafted bilaterally into the flanks of B6 mice (N=10 for Ctrl-Ad, N=14 for Ad-MAVS) and intralesionally vaccinated on one side at 1 week post implantation with a control Ad-LacZ or Ad-MAVS (5×10¹⁰ viral particles). Tumor growth were measured for 3 weeks (D). Splenocytes were assessed for MC38-specific anti-p15E T cell responses by IFN-γ ELISpots assay (E) and intracellular flow cytometry (F–I, N=5–10). In all panels, * represents p<0.05, **p<0.01, ****p<0.001.
significantly reduced growth compared with Ad-GFP injections. Moreover, we found that the contralateral flank from Ad-MAVS injected tumors displayed identical reductions of growth (figure 6B) and found a significant increase in systemic tumor antigen-specific IFNγ responses in Ad-MAVS treated mice compared with control Ad-GFP injected mice (figure 6C). We also confirmed that this anti-tumor effect was mediated by adaptive immune responses through identical intrallesional injection experiments using both SCID mice and Rag1−/− mice (online supplemental figure S15). As Rag1−/− mice contain functional populations of NK cells, this also revealed that NK cells did not have a significant impact on MAVS-mediated anti-tumor responses. To verify that this effect was not specific for CT26.CL25 cells, we also performed intrallesional injection of Ad-MAVS or a control Ad-GFP against a bilateral model of established MC38 tumors (figure 6D). Once again, we found that Ad-MAVS injection decreased contralateral tumor growth (figure 6D) and increased IFNγ responses against p15E, an endogenous antigen in MC38 colon cancer cells (34) (figure 6E), with specific expansion of tumor antigen-specific TNFα/IFNγ secreting CD44high CD8+ T cells (figure 6F–I and online supplemental figure S16). Collectively, these results confirmed our previous findings using modified tumor lines, suggesting the potential for the intratumoral delivery of viral vectors or other vectors encoding MAVS to stimulate innate and subsequent adaptive anti-tumor immune responses.

**Blockade of MAVS-stimulated PD-L1 enhances the anti-tumor responses in vivo**

While we observed that MAVS expression could elicit anti-tumor responses, we did observe heterogeneity in responsiveness between different cell lines (MC38 and CT26.CL25) and within groups, with some mice having complete tumor regressions while others experienced only diminished growth (figures 2–4). One possible explanation for this heterogeneity was the suppression of local responses by tumor immune checkpoint expression, such as PD-L1. PD-L1 expression has been well documented to be induced by type II IFN stimulation, with some studies demonstrating induction by type I IFN induction, as observed in B16 melanoma cells.55–58 To determine if MAVS expression could also elicit PD-L1 expression, we assessed PD-L1 expression in CT26.CL25 and MC38 cells after transduction with MAVS or GFP expressing vectors. These studies revealed a significant induction of PD-L1 expression by MAVS (online supplemental figure S17A–B), which we also verified in breast cancer (4T1) and melanoma (B16-F10) cells (online supplemental figure S17C–D). Additional experiments in CRC PDX lines revealed a similar effect for human MAVS in eliciting PD-L1 expression (online supplemental figure S17E–F). These studies demonstrated that MAVS induction of PD-L1 may permit resistance against CD8+ tumor-specific T cells, which might be overcome through combinations with anti-PD-L1 ICI.

To determine if MAVS anti-tumor immunity could be enhanced by PD-L1 ICI, we first explored its use in the PD-L1 insensitive MAVS inducible CT26.CL25 model (figures 1–2). As before, doxycycline treatment was started 7 days post implantation but with mice being randomized into anti-PD-L1 or control groups ten days post-doxycycline induction (to allow for the expression of MAVS and its downstream genes, including PD-L1). Consistent with our previous findings, these studies demonstrated that control CT26.CL25 growth was not significantly changed by anti-PD-L1 treatment (figure 7A, (online supplemental figure S4)) with MAVS expression eliciting significant growth suppression in control isotype treated groups (figure 7A). More importantly, these experiments revealed that the combination of MAVS expression and anti-PD-L1 treatment almost completely eradicated these cancers, with 91% (11 out of 12) completely regressing, compared with ~40% of tumors that regressed using MAVS expression alone (figure 7B). To determine if this effect could be achieved using viral vectors in larger tumors, we repeated these experiments with CT26.CL25 established tumors being intrallesionally injected with Ad-MAVS or Ad-LacZ (5×1010 vp) at 12 days post injection with PD-L1 or control mAbs administered on days 15, 18, 22, and 25 post-tumor injection. Using this more translational approach, we found the greatest anti-tumor response in the Ad-MAVS +PD-L1 ICI combination treated mice, with 50% of mice in this group experiencing complete tumor regression (figure 7C). Additionally, we observed no visible tumor growth when regressed mice were re-challenged with CT26.CL25 cell injection (on the contralateral flank), indicating that Ad-MAVS plus anti-PD-L1 ICI triggers systemic protective immunological memory against tumor cells (figure 7D). To further validate the potential of PD-L1 ICI in combination with Ad-MAVS, we also tested the impact of these therapies in advanced cancers from the more immunogenic MC38 model. In this model, we injected Ad-MAVS or control Ad-LacZ in established MC38 tumors at 7 days post injection, but administered anti-PD-L1 ICI (100 μg) just twice, on days 14 and 18 post implantation, which our studies demonstrate has no significant effect on tumor growth, even in this more PD-L1 sensitive model.49 We found that even with this suboptimal ICI administration, combination treatment had a profound effect on reducing tumor growth among the four groups (figure 7E). Assessment of intratumoral T cells in this group (figure 7F) revealed significantly increased CD4+ (figure 7G) and CD8+ T cell (figure 7H) infiltration in the tumors treated with the combination of Ad-MAVS and PD-L1 ICI, in contrast to either treatment alone. Collectively, these studies revealed that overexpression of MAVS using intrallesional viral vectors sensitizes the tumor for anti-PD-L1 ICI and significantly enhanced the infiltration/expansion of T-cells in the TME.

**DISCUSSION**

In our study, we identify that MAVS expression is reduced in colorectal cancers and that reintroduction of MAVS
expression stimulates robust IFN signaling in various types of tumor cells, fibroblasts, DCs, as well as human PDX colorectal tumor cells (figure 5). This likely occurs through constitutive, ligand-free MAVS polymerization after overexpression, which is the critical step in eliciting downstream signaling complexes through the formation of MAVS filaments. Our findings support the importance of MAVS suppression in cancer development, as demonstrated in studies using MAVS deficient mice. While this study did not attribute these reductions to immunity, it did note reduced expression of IFNβ and ISGs (such as CXCL10) in MAVS KO tumors. Similarly, other studies have demonstrated anti-tumor effects mediated by oncolytic viruses through their induction of the RIG-I/MAVS pathway. In our study, we found that MAVS expression failed to suppress CRC tumor growth in vitro and in immune deficient mouse models in vivo, as well as after CD8 depletion or IFNAR blockade, indicating that MAVS may primarily suppresses CRC growth through the type I IFN-dependent stimulation of adaptive immunity, specifically through stimulation of tumor antigen-specific CD8+ T cells (figure 4). Our findings thus may help explain why many colorectal cancers remain immunologically ‘cold,’ through their reduction of multiple innate immune regulators (online supplemental figure S1-2), such as MAVS, which are essential in the development of local immune responses. This idea is consistent with studies that have demonstrated enhanced CRC tumorigenesis in STING KO mice, in addition to MAVS KO mice, supporting the importance of dysregulating innate immune surveillance and IFN pathway stimulation in CRC development.

Innate immune responses are instrumental in enabling the induction of cytotoxic T cells to target intracellular pathogens. The importance of IFN induction to stimulate T cell responses and enable effective anti-tumor immunity has been known for decades, first demonstrated in the use of systemic interferon treatments to treat cancers (such as Kaposi's sarcoma, melanoma, etc), which have been unfortunately limited by toxicities accompanying systemic IFN delivery. In the past decade, there have been a plethora of approaches to stimulate these pathways to generate local anti-tumor immunity, notably in the development of STING and RIG-I agonists. Several of these agonists are now under investigation in clinical trials, with mixed results reported to date. It is thought that the effectiveness of these individual ligands may be highly dependent on their ability to transverse cell membranes, their rates of diffusion, as well as the expression of ‘sensor’ innate immune genes and cellular composition of the tumor microenvironment. Additionally, it is incompletely understood how long these ligands and their stimulation persist, or the schedule of administration that might yield maximum innate immune stimulation. To circumvent these challenges, we developed and demonstrate the utility of MAVS expressing vectors, which exploit its ability to elicit IFN stimulatory signaling in multiple cell types, regardless of innate immune gene ‘sensor’ activity. In our study, we found that MAVS expression alone is not enough to elicit robust IFN signaling in these cells, but that the addition of MAVS to the tumor can enhance the response.
expression. Surprisingly, we found that the therapeutic response was largely mediated through the induction of adaptive immunity, allowing for potent abscopal adaptive immune responses. This stands in contrast to many earlier studies that utilized delivery of systemic IFNβ to elicit anti-tumor immunity, due to apoptosis and NK cell mediated killing.  

We speculate that this is likely due to the complex induction of pathways downstream of MAVS, including TSLP, CXCL10 and CXCL11 (online supplemental figure S5, S12–14 and online supplemental table S1), as opposed to single expression of IFNβ, or a mix of different IFN-αs (utilized in systemic IFN therapies). The induction of these complex IFN responses in the tumor microenvironment and draining lymph nodes may be critical for the stimulation of tumor immunity, as studies have demonstrated that tumor antigen priming occurs in this setting and requires IFN signaling in DCs. Our studies support that intact host IFN signaling is an important aspect in eliciting adaptive anti-tumor responses, due to the reduction in efficacy by IFNAR blockade (figure 4). These responses allow for enhanced adaptive immune responses, specifically through the induction of CD4+ T cell in the TME and systemic induction of tumor-antigen specific CD8+ T cells (figure 4 online supplemental figure S8, S9 and S16). Thus, the local stimulation of multiple innate pathways appears to allow for stimulation of systemic tumor antigen-specific immunity to permit immune activity at distant tumor sites. While this mechanism also elicited PD-L1 expression, we found that the administration of anti-PD-L1 mAb mitigated this effect and allowed for synergistic anti-tumor responses, which may have important implications for expanded use of these anti-PD-L1/PD-L1 ICIs.

While anti-PD-L1 and anti-PD-L1 ICIs have demonstrated impressive clinical activity in certain cancers, this activity is restricted to a limited number of patients with specific types of cancer. Typical of this trend, the majority of CRC cases (non-MSI-H or microsatellite stable, MSS) have not been demonstrated to be highly responsive to these ICIs and have been described as immunologically ‘cold’ tumors with very low levels of immune cell infiltration. Our study suggests that part of this lack of immune involvement may be due to selection against innate immune sensing genes during tumor development, which would translate into reduced tumor-infiltrating lymphocytes and immune responsiveness. In MSI-H CRCs, our data suggest that this trend may actually be enhanced due to the instability of the genome, which allows for greater immune selection due to the more abundant expression of neoepitopes, as well as, potentially easier loss of innate immune gene expression due to altered regulatory control of DNA repair mechanisms. Thus, the use of MAVS therapy may have a profound impact on both MSI-H and MSS colorectal cancers. As the majority of CRC are MSS and predominantly treated with chemotherapy, it will be critical to identify these types of novel therapies that may enable long-term survival observed with anti-PD-L1 and anti-PD-1 ICIs. However, an important caveat is that our in vivo studies were performed with subcutaneous injection of murine CRC cells, which may not accurately reflect the MSS/MSI-H CRCs or the immune microenvironment of CRCs present in the intestinal tract. Thus, future studies will be needed to explore the role of MAVS in tissue-specific models. Additionally, this strategy may also be effective for other histological types of cancer, which have demonstrated reduced expression of MAVS such as lung and prostate cancers (Fig S1). While our preliminary studies in models of CRC require further validation and exploration of different gene delivery strategies, they suggest the potential of MAVS gene delivery as a local immunotherapeutic modality.

Finally, local MAVS gene delivery could have significant advantages over systemic delivery of IFNs or innate immune agonists in terms of reduced toxicity. While systemic administration of certain IFNαs has demonstrated efficacy for certain types of cancer (including metastatic melanoma, renal carcinoma and HIV associated-KS, the majority of cancers (including CRC) have been refractory to systemic IFNs treatment. While the reasons for this selectively are incompletely understood, they may be related to the type of IFN utilized, the short half-life and high toxicity of systemic IFN treatment, systemic regulatory feedback mechanisms, as well as genetic alterations that render tumors insensitive to IFN signaling. Vectors able to elicit MAVS expression in different cells within the tumor microenvironment may bypass genetic and epigenetic events that would short circuit these pathways in tumor cells, through their reliance on a highly conserved mechanism that is selectively suppressed during tumor evolution (Fig S1-2). Moreover, the induction of local innate responses may better stimulate appropriate tumor-specific adaptive immune responses, which will be critical in mediating systemic anti-tumor responses. This approach also carries the advantage of lowering systemic IFN/cytokine responses that would lead to undesirable side-effects. While we did not observe any toxicities associated with systemic IFN administration in mice for the durations of our experiments (up to 3 months), further studies will be needed to demonstrate adequate safety of this approach. This may also vary due to the type of vector utilized in delivery of MAVS, which may be further impacted by the type of tumors that can be intrasesionally injected and their vascularity. Recent advances in cancer gene therapy have demonstrated the feasibility of this approach through the advent of synthetic viral and nanoparticles, which may be particularly well suited for MAVS vector production and delivery.

In conclusion, our investigation highlights the importance of the MAVS innate pathway in colorectal cancers and demonstrates that the expression of MAVS can elicit profound innate IFN signaling responses across multiple cell types, including different CRC PDX models suggesting a highly conserved mechanism of action. Notably, modest MAVS overexpression did not alter cellular proliferation or strongly affect apoptosis. However, its stimulation of IFN signaling enabled the development of systemic tumor-specific adaptive immunity resulting in abscopal anti-tumor
responses. Additionally, we found MAVS stimulation of PD-L1 expression to be a critical immune feedback loop that could be exploited by combination with PD-L1 ICI in CRC models. Collectively, our studies suggest that targeted MAVS expression may offer a clinical means to enable effective anti-tumor immunity in combination with anti-PD-L1 and anti-PD-1 ICIs in colorectal tumors and other cancers that are currently unresponsive to these therapies.

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Contributors ZCH is the guarantor, initially conceived the project with HKL and wrote the manuscript with B-JH and input from all other coauthors. B-JH, L-CT and ZCH designed the experiments, analyzed and interpreted the data. B-JH performed the bulk of animal experiments, with assistance from TT, XY and TO. PA provided the microarray analysis and submission to GEO. CRA performed the bioinformatics analysis. TW provided technical support and suggestions for flow cytometry. JW, GL and ZCH generated various cell lines and early validation of their inducible activity. B-JH performed the in vitro cell culture assays. KL and MAM provided support for this work and also worked on the manuscript.

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