

An experimental model of anti-PD-1 resistance exhibits activation of TGF β and Notch pathways and is sensitive to local mRNA immunotherapy

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ABSTRACT

Immune checkpoint blockade elicits durable anti-cancer responses in the clinic, however a large proportion of patients do not benefit from treatment. Several mechanisms of innate and acquired resistance to checkpoint blockade have been defined and include mutations of MHC I and IFN γ signaling pathways. However, such mutations occur in a low frequency of patients and additional mechanisms have yet to be elucidated. In an effort to better understand acquired resistance to checkpoint blockade, we generated a mouse tumor model exhibiting *in vivo* resistance to anti-PD-1 antibody treatment. MC38 tumors acquired resistance to PD-1 blockade following serial *in vivo* passaging. Lack of sensitivity to PD-1 blockade was not attributed to dysregulation of PD-L1 or β 2M expression, as both were expressed at similar levels in parental and resistant cells. Similarly, IFN γ signaling and antigen processing and presentation pathways were functional in both parental and resistant cell lines. Unbiased gene expression analysis was used to further characterize potential resistance mechanisms. RNA-sequencing revealed substantial differences in global gene expression, with tumors resistant to anti-PD-1 displaying a marked reduction in expression of immune-related genes relative to parental MC38 tumors. Indeed, resistant tumors exhibited reduced immune infiltration across multiple cell types, including T and NK cells. Pathway analysis revealed activation of TGF β and Notch signaling in anti-PD-1 resistant tumors, and activation of these pathways was associated with poorer survival in human cancer patients. While pharmacological inhibition of TGF β and Notch in combination with PD-1 blockade decelerated tumor growth, a local mRNA-based immunotherapy potently induced regression of resistant tumors, resulting in complete tumor remission, and resensitized tumors to treatment with anti-PD-1. Overall, this study describes a novel anti-PD-1 resistant mouse tumor model and underscores the role of two well-defined signaling pathways in response to immune checkpoint blockade. Furthermore, our data highlights the potential of intratumoral mRNA therapy in overcoming acquired resistance to PD-1 blockade.

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Introduction

Immune checkpoint blockade targeting the PD-1/PD-L1 interaction elicits durable anti-cancer responses in patients with a broad range of cancer types.¹ However, many patients do not derive clinical benefit due to primary or acquired resistance to anti-PD-1/PD-L1 treatment. A current area of focus in the cancer immunotherapy field is to better understand the mechanisms that contribute to therapeutic resistance and identify combination therapies to improve the clinical response to anti-PD-1/PD-L1 agents. A number of different molecular and clinical biomarkers are associated with clinical response to anti-PD-1/PD-L1 treatments. The strongest association is linked to PD-L1 expression, while additional factors such as tumor mutational burden and preexisting T cell infiltrate also influence the response to checkpoint therapy.^{2–5} Furthermore, these characteristics are controlled in part by tumor cell-intrinsic activity of oncogenic and tumor suppressor signaling pathways. For example, oncogenic activation of Ras signaling and activation of PI3K through loss of the tumor suppressor PTEN both increase PD-L1 expression through distinct

mechanisms.^{6,7} PTEN loss, and other features such as activation of Wnt/ β -catenin, have been associated with exclusion of immune cells from the tumor microenvironment and may represent important drivers of immunologically cold tumors.^{8–10} Together these features influence primary resistance to immune checkpoint blockade, however the mechanisms that control acquired resistance are less well understood.

A proportion of patients that initially benefit from checkpoint blockade treatment later experience disease progression resulting from acquired resistance. Tumors with acquired resistance to immune checkpoint blockade may lack MHC I presentation, which allows tumor cells to evade T cell recognition and elimination. Inactivating genomic mutations in the beta-2-microglobulin (β 2M) subunit are detected in up to 30% of melanoma tumors from patients that relapse following checkpoint blockade therapy and are associated with poorer response to treatment.^{11,12} In tumors with functional MHC I, IFN γ is an important mediator of its expression levels. IFN γ produced by activated lymphocytes in the tumor microenvironment binds to the heterodimeric IFN γ receptor expressed on

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tumor cells to activate JAK/STAT signaling, resulting in transcription of IFN γ -responsive genes and upregulation of MHC I.¹³ Activation of IFN γ signaling in tumor cells can also inhibit proliferation and induce cell death. Loss of function mutations in Janus kinase 2 (JAK2) and other defects in the JAK/STAT signaling pathway have been observed in human melanoma tumors and render tumors insensitive to the direct cytotoxicity and immune control mediated by IFN γ .^{14–16} However, only a small proportion of patients exhibiting acquired resistance harbor these mutations, and additional mechanisms have yet to be defined.

In order to explore mechanisms of resistance to PD-1 blockade, we generated a mouse tumor model exhibiting *in vivo* resistance to anti-PD-1 treatment using an unbiased approach. MC38 colon adenocarcinoma tumors were serially passaged in anti-PD-1 treated mice. Tumors isolated after several rounds of passaging were refractory to treatment. Using transcriptomic and molecular analyses, we show that resistant tumors are less immune infiltrated and exhibit dysregulation of the two major signaling pathways TGF β and Notch. Pharmacological inhibition of TGF β and Notch combined with PD-1 blockade modestly inhibited growth of resistant tumors and extended survival of tumor-bearing mice. As systemic pathway inhibition induced a modest response, the potential efficacy of a local cancer immunotherapy was tested. Intratumoral administration of a mixture of mRNA-encoded cytokines induced a potent antitumor response, resulting in regression of resistant tumors and complete remission in a proportion of mice. Overall, this study describes a novel anti-PD-1 resistant tumor model and underscores the use of local mRNA-based immunotherapy as a mechanism to promote antitumor immunity.

Methods

Cell culture

MC38 cells were a gift from Dr. S. A. Rosenberg (National Institute of Health, Bethesda, MD, USA). Short Tandem Repeat (STR) DNA profiling was performed to verify cell identity. MC38 and MC38-derived cell lines were cultured in DMEM with sodium pyruvate and L-glutamine supplemented with 10% FBS, 1% NEAA and 1% HEPES (ThermoFisher Scientific, Waltham, MA, USA) at 37°C in a humidified environment with 5% CO₂. For IFN γ treatment, cells were incubated overnight with 10 U/ml recombinant mouse IFN γ (Peprotech, Rocky Hill, NJ, USA) in complete media.

In vivo studies

Female C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME, USA) aged 6 to 8 weeks were housed in a temperature-controlled environment on 12-h light cycle with free access to food and sterile water. All mice were acclimated for at least 3 days prior to experimentation. To implant tumors, 0.5 or 1 million cells were suspended in 200 μ l DPBS and injected subcutaneously into the right flank of mice. Mouse body weight and tumor volume were measured twice weekly until the experimental endpoints. Tumor volume is expressed as the

product of perpendicular diameters using the following formula: $(\text{length} \times \text{width}^2)/2$. All procedures were approved by the Sanofi Institutional Animal Care and Use Committee and were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals. For survival analysis, Z-scores were calculated using trimmed mean and standard deviation with top and bottom 10% trimmed. Individuals with scores greater than or equal to 3 were removed and survival compared among treatment groups. Animal survival was plotted on a Kaplan-Meier curve and the log-rank test performed to determine whether distribution was statistically different. Survival analysis was performed with “survival” package in R or GraphPad Prism version 8.0.2.¹⁷

Serial in vivo passaging

C57BL/6J mice bearing MC38 tumors were treated with 5 mg/kg anti-PD-1 antibody (clone RMP1-14, BioXCell, West Lebanon, NH, USA) or control antibody (clone 2A3, BioXCell) twice weekly by intraperitoneal (i.p.) injection. Tumors selected for passaging were excised, dissociated using a mouse tumor dissociation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) and cultured *in vitro* until implantation into naïve mice. This process was repeated for a total of three rounds of passaging, with anti-PD-1 antibody dose increased to 10 mg/kg for the second and third rounds.

In vivo drug treatments

Antibodies were obtained from BioXCell unless stated otherwise and administered twice weekly by i.p. injection. Anti-PD-1 and IgG2 isotype (clone 2A3) antibodies were administered at a dose of 10 mg/kg. Anti-TGF β (clone 1D11, produced internally) and IgG1 isotype (clone MOPC-21) antibodies were administered at a dose of 25 mg/kg. LY3039478 (Selleck Chemicals, Houston, TX, USA) was formulated in 1% sodium carboxymethyl cellulose, 0.25% Tween 80 and administered by oral gavage (p.o.) three times per week at a dose of 8 mg/kg. For triple combination experiments, treatments were administered for three weeks. mRNA was co-developed with and manufactured by BioNTech as previously described.¹⁸ For mRNA treatments, mice were anesthetized with isoflurane and 40 μ g mRNA in saline solution was injected intratumorally every four days for four doses. When tested in combination with mRNA, anti-PD-1 antibodies were administered for two weeks for a total of six doses. For antitumor efficacy studies, drug treatment was initiated when tumors reached an average volume of 50–60 mm³, unless otherwise noted in the figure legends.

RNA-sequencing and gene expression analysis

Total RNA was isolated from tumor tissue using RNeasy 96 kit according to the manufacturer’s instructions (Qiagen, Germantown, MD, USA). Libraries were prepared using KAPA RNA HyperPrep Kit (KAPA Biosystems, Wilmington, MA, USA) and analyzed on an Illumina HiSeq 1500 sequencer. Gene-level estimation of expression in transcripts per million was performed using STAR aligner¹⁹ and Cufflinks.²⁰ The data

were then quantile normalized and \log_2 -transformed. Differentially expressed genes were identified using one-way ANOVA with post-hoc contrast analysis comparing groups. Gene expression analysis was performed using Array Studio (Qiagen). Genes with absolute fold change greater than or equal to 1.5 and p -value less than or equal to 0.05 were considered differentially expressed. Differentially expressed genes were then analyzed by Ingenuity Pathway Analysis (IPA, Qiagen) and Causal Network Analysis was performed to identify upstream regulators. For upstream regulator analysis, extrinsic factors such as chemical drugs were excluded. Relative abundance of various immune cell types in tumor was estimated using Microenvironment Cell Populations-counter (MCP-counter)²¹ using the same statistical analysis described above. IFN γ regulated genes, based on IPA annotation, were subjected to hierarchical clustering in which the expression value of each gene is robust Z-normalized across all tumor samples and complete-linkage clustering was performed based on Pearson correlation coefficients.

Flow cytometry

Cultured cells were trypsinized and washed once prior to staining. Tumor tissue was dissociated using a mouse tumor dissociation kit (Miltenyi Biotec). Samples were then subjected to red blood cell lysis (Miltenyi Biotec), washed once in buffer (1X PBS, 0.5% FBS, 0.1% sodium azide) and then stained with Zombie NIR viability dye (Biolegend, San Diego, CA, USA). Following another wash, samples were incubated with anti-mouse CD16/32 (Biolegend) and fluorescent antibodies (Supplemental Table 1) for 20 minutes on ice in the dark, washed, incubated in 1.6% paraformaldehyde, washed and resuspended in buffer. Samples were analyzed on a FACSCanto II or LSR Fortessa machine (BD Biosciences) and data were analyzed using FlowJo software version 10.

Results

Generation of a syngeneic tumor model resistant to PD-1 blockade

We sought to develop an *in vivo* mouse tumor model insensitive to PD-1 blockade to better understand mechanisms of

resistance to immune checkpoint therapies. We utilized the MC38 colon adenocarcinoma model, which is sensitive to a range of cancer immunotherapies including anti-PD-1,^{22,23} and performed serial *in vivo* and *in vitro* passaging in the presence of anti-PD-1 treatment (Figure 1a). Following three cycles of passaging, an MC38 cell line exhibiting resistance to anti-PD-1 antibody treatment (MC38-resistant) was derived. While parental MC38 tumors exhibited growth delay when treated with anti-PD-1 antibody, MC38-resistant tumors were unresponsive to anti-PD-1 treatment *in vivo* (Figure 1b).

PD-L1, β 2M and IFN γ pathways remain intact in MC38-resistant cells

Resistance to PD-1 blockade in the clinic has been attributed to several molecular mechanisms, including dysregulation of PD-L1 expression, lack of IFN γ sensitivity and lack of MHC I presentation through loss of β 2M.²⁴ We first investigated whether any of these known mechanisms were observed in MC38-resistant cells. Notably, no differences in surface expression of PD-L1, β 2M or the IFN γ receptor subunits IFNGR1 and IFNGR2 were observed between the MC38 parental and resistant lines (Figure 2a). The IFN γ signaling pathway is an important determinant of sensitivity to anti-PD-1 treatment as mutations in Janus kinase 1 (JAK1) or JAK2 have been linked to acquired resistance.²⁵ To examine functionality of the pathway, we monitored surface expression of PD-L1, which is upregulated by IFN γ .²⁶ IFN γ treatment induced a 3-fold increase in PD-L1 expression based on mean fluorescence intensity in both parental and MC38-resistant cells (Figure 2b), consistent with a functional signaling pathway. Expression of PD-L2, an alternative ligand for PD-1, was not detected in untreated or IFN γ treated cells (Supplemental Figure 1). To test the functionality of the antigen processing and presentation pathway, we expressed full-length ovalbumin protein in both the MC38 parental and MC38-resistant cell lines and monitored surface expression of the immunodominant SIINFEKL peptide, reasoning that any defect in antigen processing would lead to a reduction of surface expressed SIINFEKL peptide H2-K^b. Surface expression of the SIINFEKL peptide by H2-K^b MHC class I was similar for MC38 and MC38-resistant cells, consistent with functional antigen processing (Figure 2c). Together, these results show

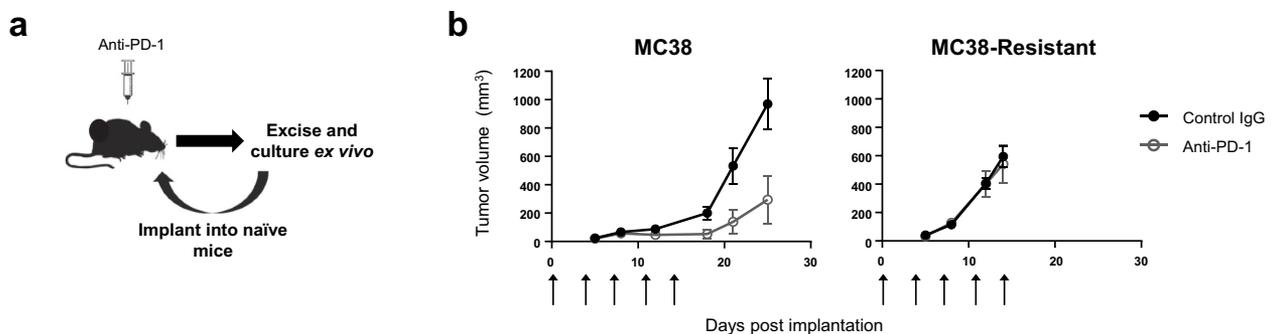


Figure 1. Generation of a tumor line resistant to PD-1 blockade. (a) Diagram of *in vivo* passaging method. Briefly, C57BL/6J mice bearing MC38 tumors were treated with anti-PD-1 antibody (clone RMP1-14), growing tumors were excised, and cells were cultured *ex vivo* prior to implantation into naïve mice. (b) Tumor growth curves for MC38 and MC38-resistant tumor cell lines implanted in C57BL/6J mice treated with 10 mg/kg anti-PD-1 antibody ($n = 5$ /group). Treatment was initiated on the day of tumor implantation. Arrows below the x-axis indicate antibody treatments.

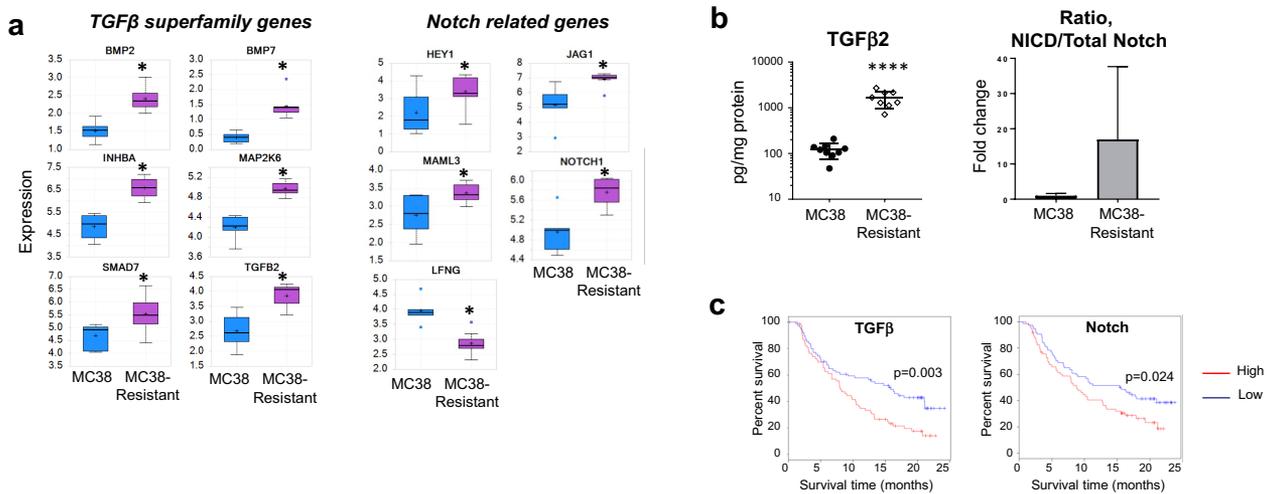


Figure 4. TGFβ and Notch pathways are activated in anti-PD-1 resistant tumors. (a) TGFβ superfamily and Notch pathway genes for which expression was significantly dysregulated in MC38-resistant tumors compared to parental MC38 tumors. Data is from RNA-sequencing experiment shown in Figure 3. (b) Total protein was isolated from subcutaneous tumor tissue and TGFβ and Notch proteins analyzed. Left, TGFβ2 protein levels were quantified by MSD. Right, cleaved and total Notch1 protein was quantified by western blot. Ratio of cleaved to total protein is shown (n = 4/group). (c) TGFβ and Notch pathway activation was evaluated in a published dataset derived from human metastatic urothelial cancer patients.²⁷ Overall survival of patients with high and low pathway activation scores is shown.

upstream regulators in the resistant model (Figure 3b). The most significantly inhibited pathways included regulators of immune response, including IFNG and SPI1, a transcription factor involved in immune cell generation and differentiation. We further examined the IFNγ pathway and observed broad dysregulation of IFNγ target genes in resistant tumors (Supplemental Figure 2b) suggestive of reduced IFNγ in the tumor microenvironment. As IFNγ in the tumor microenvironment is primarily produced by activated T cells and NKs,¹³ we used Microenvironment Cell Populations-counter (MCPCounter) to investigate changes in tumor immune infiltration based on gene expression in subcutaneous tumors.²¹ The frequency of CD3⁺ T cells, NK cells, B cells, monocytes, and CD8⁺ T cells was decreased in MC38-resistant compared to parental tumors, consistent with overall reduced immune infiltration in resistant tumors (Figure 3c). Overall, unbiased analysis revealed global differences in gene expression between MC38 and MC38-resistant tumors and revealed modulation of several pathways with important immune regulatory roles in the tumor microenvironment in anti-PD-1 resistant tumors.

Immune infiltration is reduced in anti-PD-1 resistant tumors

Gene expression data indicated reduced immune infiltration within tumors resistant to PD-1 blockade. We next sought to confirm these observations by performing flow cytometry on dissociated tumors (Supplemental Figure 3a). The frequency of total CD45⁺ immune cells was markedly reduced in MC38-resistant compared to parental tumors. Likewise, the percentage of several immune cell types, including CD4⁺ and CD8⁺ T cells, macrophages, and NKs was also decreased (Figure 3d). Immunohistochemistry of parental and resistant MC38 tumors showed a significant reduction in CD45 staining in MC38-resistant compared to parental tumors (Supplemental Figure 3b,c), confirming the reduced immune cell abundance

observed by other methods. While exclusion of immune cells from the tumor represents a potential mechanism of resistance, tumor cells may also lose sensitivity to T cell-mediated killing. To address this possibility, cytotoxic T lymphocytes (CTLs) were generated from mice that exhibited complete regression of MC38 parental tumors in response to anti-PD-1 treatment and utilized in an *in vitro* killing assay. Parental MC38 cells were more effectively lysed by CTLs compared with MC38-resistant (Supplemental Figure 4a). Further, CTLs co-cultured with MC38 cells produced more IFNγ compared to CTLs cultured with MC38-resistant (Supplemental Figure 4b), consistent with stronger activation. Taken together, these results suggest that anti-PD-1 resistant MC38 tumors exhibit an immunologically cold tumor microenvironment in which fewer immune cells infiltrate the tumor and malignant cells are less sensitive to immune-mediated killing.

Dysregulation of signaling pathways in resistant tumors

Tumor intrinsic activation of oncogenic signaling pathways has been associated with lack of tumor immune infiltration.^{8,28} We hypothesized that activation of TGFβ and Notch signaling observed by pathway analysis could be associated with reduced immune infiltration and sought to further characterize TGFβ and Notch activity in MC38-resistant tumors. Dysregulated genes in the TGFβ and Notch pathways included ligands such as TGFβ2 and JAG1 and the receptor NOTCH1 (Figure 4a). We next examined whether TGFβ proteins were also upregulated as suggested by mRNA expression. Strikingly, total TGFβ2 was upregulated more than 10-fold in protein lysates from MC38-resistant compared to parental MC38 tumors (Figure 4b). TGFβ3 was upregulated 3-fold, while TGFβ1 was unchanged (Supplemental Figure 5a). As gene expression analysis also revealed upregulation of JAG1 and NOTCH1, we investigated whether these were upregulated at the protein level. Flow cytometry of cultured cells showed

increased Jagged1 surface expression in MC38-resistant cells (Supplemental Figure 5b). Activation of the membrane-associated Notch1 receptor triggers a series of cleavage events that results in the release of the intracellular domain, which translocates to the nucleus and activates gene transcription.²⁹ Resistant tumors exhibited increased abundance of cleaved Notch1, consistent with elevated signaling activity (Figure 4b, Supplemental Figure 5c). Altogether, these results confirm activation of TGF β and Notch pathways in MC38-resistant tumors.

TGF β and Notch activation correlate with poorer response to checkpoint blockade

To examine whether these observations are relevant to human cancers, we investigated whether pathway activation was correlated with response to checkpoint blockade immunotherapy in patients. TGF β gene expression has been correlated with poorer survival in metastatic urothelial cancer patients treated with atezolizumab.²⁷ By further probing this dataset, we found that a higher activation score for TGF β and Notch pathways significantly correlated with poorer patient survival (Figure 4c). This analysis suggests that TGF β and Notch pathway activation is associated with worse therapeutic outcomes in cancer patients.

Combined inhibition of TGF β and Notch plus PD-1 blockade delays growth of resistant tumors

Our results suggest that activation of TGF β and Notch signaling pathways is associated with lack of response to checkpoint

blockade through reduced immune infiltration, and furthermore suggest that activation of these pathways may be relevant to anti-PD-1 responsiveness in human cancers. We next investigated whether pharmacological inhibition of these pathways could overcome resistance to PD-1 blockade. Mice bearing established MC38-resistant tumors were treated with a combination of anti-PD-1, anti-TGF β neutralizing antibody, and the small molecule gamma-secretase inhibitor LY3039478. Triple combination treatment significantly inhibited tumor growth and prolonged survival in mice bearing MC38-resistant tumors, while no significant response was observed in response to combinations of two agents (Figure 5a,b, Supplemental Figure 6). Interestingly, when treatment was initiated at an earlier timepoint before formation of established tumors, the growth delay observed in response to combination treatment was less pronounced, demonstrating that the antitumor efficacy could not be improved by earlier intervention (Supplemental Figure 7). The lack of efficacy of early triple combination treatment could be attributed to the dual functions of TGF β and Notch as both oncogenes and tumor suppressors, and there is evidence that the role of TGF β shifts from tumor suppressive during early tumor development to tumor-promoting in late tumorigenesis.^{29,30} Next, the pharmacodynamic effects of the anti-PD-1 + anti-TGF β + LY3039478 triple combination was investigated in MC38-resistant tumors after one week of treatment. Activation of the TGF β receptor results in phosphorylation of SMAD2, which associates with SMAD4 and translocates to the nucleus to activate gene expression.³⁰ Following triple combination treatment, SMAD2 phosphorylation was reduced relative to untreated tumors (Supplemental Figure 8a). Treated tumors also showed decreased abundance of cleaved Notch1 relative to total Notch1 levels (Supplemental Figure 8b). RNA-sequencing

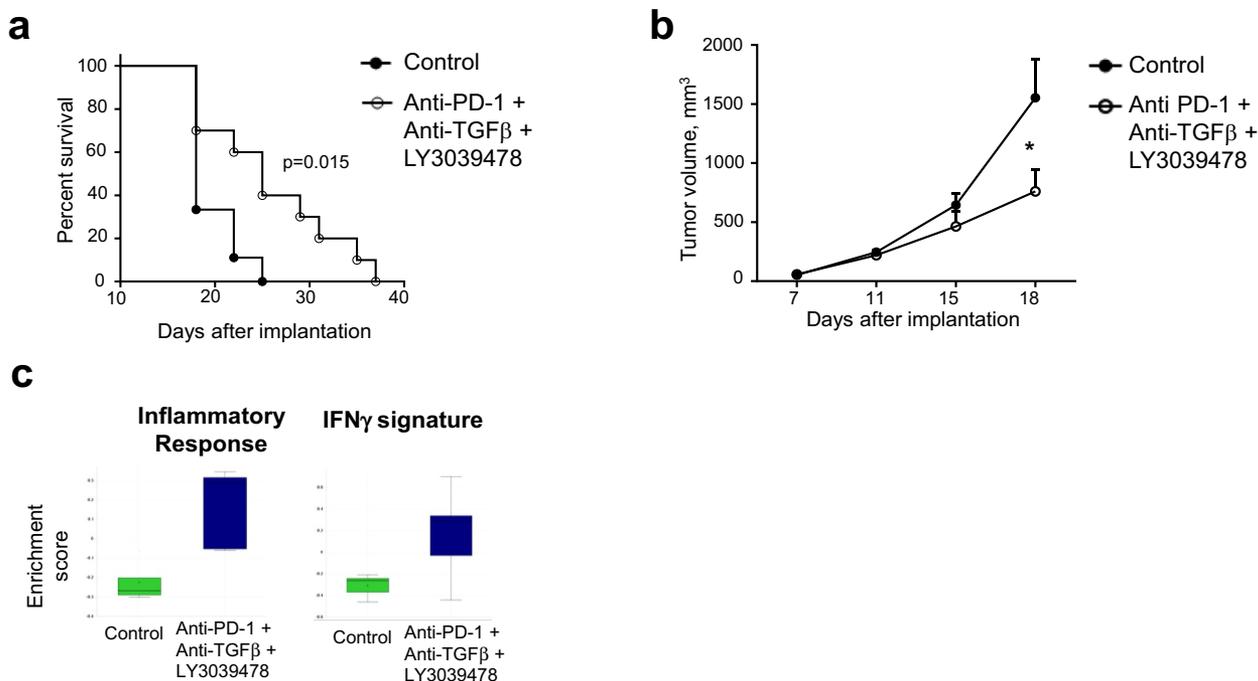


Figure 5. Combination of TGF β and Notch inhibition with PD-1 blockade delays growth of resistant tumors. (a) Survival of MC38-resistant tumor-bearing mice treated with vehicle and anti-PD-1, anti-TGF β and LY3039478. (b) Median tumor volume for experiment shown in (a). (c) RNA was isolated from tumors (n = 5/group) treated as in (a) and gene expression was analyzed by RNA-sequencing and pathway analysis. Shown are enrichment scores for inflammatory response hallmark pathway (left) and IFN γ signature in treated tumors. * indicates $p < 0.05$.

and pathway analysis revealed inhibition of Notch signaling and upregulation of TGF β inhibited genes in treated tumors, consistent with effective TGF β neutralization and Notch inhibition (Supplemental Figure 8c,d). Finally, treatment-induced changes in immune contexture were analyzed based on gene expression data. Combination treatment induced both an inflammatory signature and IFN γ signature, consistent with reduced immunosuppression (Figure 5c). Overall, these results confirm that systemic treatment with anti-PD-1 + anti-TGF β + LY3039478 inhibited tumor progression by suppressing TGF β and Notch activity and increasing inflammation in the tumor microenvironment. However, combination treatment was insufficient to induce complete tumor regression, potentially resulting from the reduced sensitivity to T cell-mediated killing observed *in vitro*. We, therefore, explored other therapeutic approaches that activate antitumor immunity.

Intratumoral therapy with a mixture of cytokine-encoding mRNAs induces profound therapeutic response in anti-PD-1 resistant tumors

A mixture of mRNAs encoding single-chain IL12 (fusion of IL12p40 and IL12p35 subunits), IFN α , GM-CSF, and IL15-sushi (fusion of IL15 to sushi domain of IL15 receptor) was previously shown to induce potent tumor regression and development of antitumor immunity and enhance the efficacy of

checkpoint blockade in multiple mouse cancer models.¹⁸ Intratumoral cytokine mRNA treatment was tested in parental and MC38-resistant tumors. Mice bearing parental or MC38-resistant tumors were treated with intratumoral injection of cytokine mRNA or a control mRNA encoding firefly luciferase. Cytokine mRNA treatment induced regression of parental MC38 tumors and prolonged survival of tumor-bearing mice, with complete regression observed in 5/8 mice (Figure 6a,b,i). No antitumor effect was observed in response to control mRNA. Cytokine mRNA treatment also induced regression of MC38-resistant tumors and prolonged survival, with 3/8 mice exhibiting complete tumor regression (Figure 6e,f,i). Finally, we tested whether cytokine mRNA treatment would render MC38-resistant tumors sensitive to anti-PD-1. Mice bearing MC38-resistant tumors were treated with intratumoral injection of cytokine mRNA as a single agent or in combination with systemic anti-PD-1 antibody. Treatment with cytokine mRNA alone induced tumor regression in 2/10 mice and significantly prolonged survival relative to control treatment, while no tumor regression or survival improvement was observed in response to anti-PD-1 (Figure 6c,d,j). Combination of cytokine mRNA and anti-PD-1 induced more potent antitumor efficacy, with 8/10 mice exhibiting tumor regression, and significant improvement in overall survival relative to control (Figure 6h,j). Overall, a multitargeted intratumoral immunotherapy capable of activating both innate

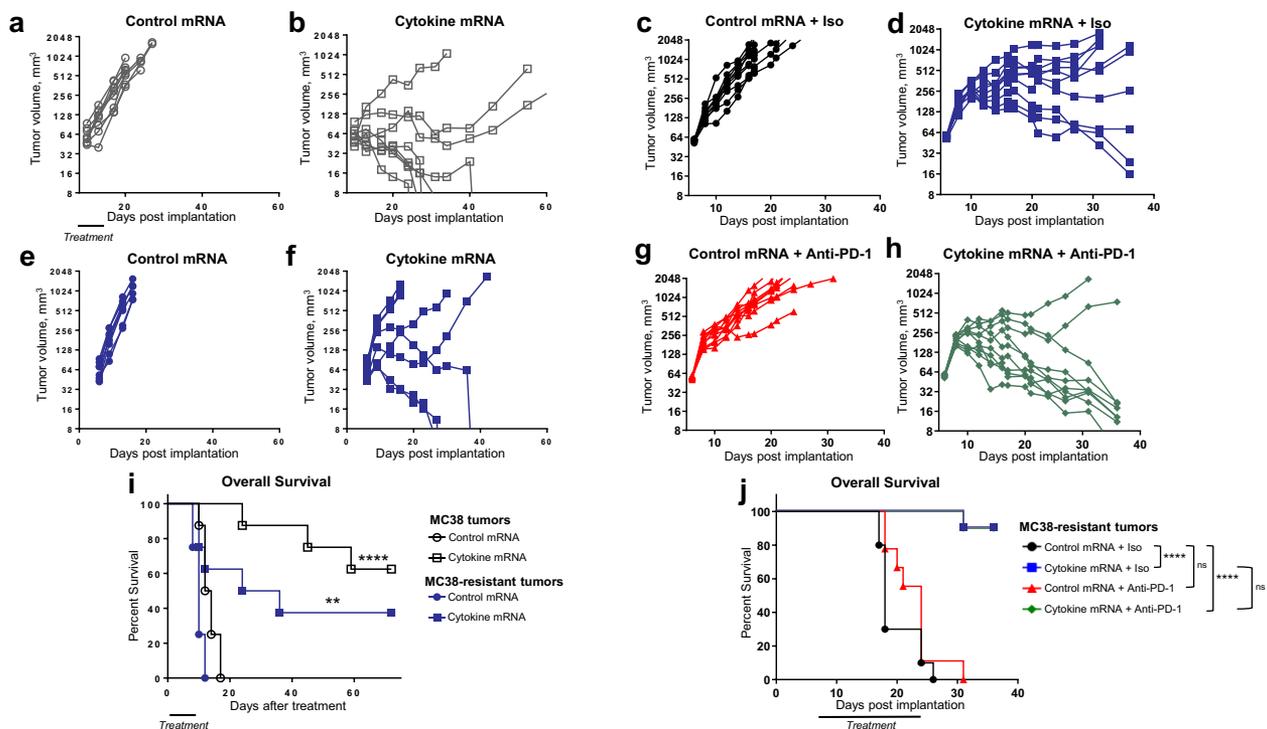


Figure 6. Intratumoral injection of a cytokine-encoding mRNA mixture induces regression of tumors resistant to anti-PD-1 and sensitizes tumors to anti-PD-1 treatment. (a, b, e, f) Individual tumor growth trends for mice bearing parental MC38 (a, b) or MC38-resistant (e, f) tumors treated with control mRNA or cytokine mRNA. (c, d, g, h) Individual tumor growth trends for mice bearing MC38-resistant tumors treated with intratumoral mRNA as a single agent or in combination with anti-PD-1. (i) Overall survival for experiment shown in (a, b, e, f). (j) Overall survival for experiment shown in (c, d, g, h). Overall survival was analyzed by log-rank test with Bonferroni correction comparing the survival of each treatment group to control. *** indicates $p < 0.01$, **** indicates $p < 0.0001$. Iso, isotype antibody.

and adaptive immunity exhibited antitumor efficacy in tumors with acquired resistance and resensitized tumor to PD-1 blockade.

Discussion

Immune checkpoint blockade exhibits strong antitumor activity in preclinical models and in the clinic, however many patients do not benefit from treatment due to primary and acquired resistance. While several factors that correlate with sensitivity to immune checkpoint blockade treatment have been identified, including PD-L1 expression, neoantigen burden, and immune infiltration, none are predictive in all tumor contexts. In an effort to better understand mechanisms underlying resistance to PD-1 blockade, we generated a mouse tumor model refractory to anti-PD-1 treatment using serial *in vivo* passaging. Other groups have utilized this experimental approach to study resistance to checkpoint blockade. A murine lung tumor model generated by serial *in vivo* passaging exhibited acquired resistance to anti-PD-1 treatment through decreased tumor cell expression of MHC I. In this model, combination with radiotherapy induced secretion of IFN β , which upregulated MHC I expression on cancer cells and improved antitumor efficacy of anti-PD-1 treatment.³¹ In contrast, a B16 melanoma tumor model with acquired resistance to a combination of triple checkpoint blockade and vaccination harbored a dysregulated metabolic phenotype, which impeded effective antitumor immune responses.³² The anti-PD-1 refractory MC38-resistant tumor model described here did not exhibit MHC I downregulation, PD-L1 dysregulation or loss of IFN γ sensitivity, nor were tumor metabolic pathways dysregulated, suggesting that other mechanisms are involved.

Although PD-1 resistant MC38 tumor cells retained MHC I expression and sensitivity to IFN γ , they were less sensitive to T cell-mediated killing relative to parental MC38 cells. Reduced sensitivity to T cell-mediated killing could be a consequence of reduced expression of tumor antigens following *in vivo* passaging. Tumors that develop in immunocompetent hosts are subject to immunoediting, a process which shapes the antigenic repertoire of the developing lesion.³³ Tumors that escape elimination often exhibit reduced immunogenicity resulting from loss of tumor antigens, which could contribute to the immune resistance observed in our model. The mutational landscape has not yet been explored in mouse models of acquired resistance, and future studies should further investigate its role as well as potential interaction with other mechanisms of resistance.

Other factors that could contribute to the resistance phenotype were explored using gene expression analysis. RNA-sequencing comparing sensitive and resistant tumors revealed considerable changes in immune-related genes, consistent with reduced immune infiltration, as well as activation of TGF β and Notch signaling pathways. These results suggest that TGF β and Notch activation may contribute to resistance to anti-PD-1 therapy. While both TGF β and Notch pathways have been associated with tumor progression^{34,35} and poorer response to chemotherapy³⁶ and targeted therapy,^{37,38} emerging evidence suggests a similar role in response to cancer immunotherapy. TGF β is a pleiotropic cytokine with well-documented immunosuppressive functions, including the expansion of T regulatory

cells and suppression of proliferation and activity of effector T cells and NK cells.³⁹ Further, TGF β protein is abundant in the tumor microenvironment, where it contributes to the dysfunction of antitumor immune responses. Mariathasan et al. demonstrated that high TGF β levels were associated with poorer lymphocyte infiltration into the tumor bed and functioned to restrict immune cells to the surrounding stromal tissue,²⁷ suggesting that a major mechanism of TGF β -mediated immunosuppression is the exclusion of immune cells from the tumor microenvironment.

In our anti-PD-1 insensitive model, pharmacological inhibition of TGF β and Notch pathways combined with PD-1 blockade extended survival of tumor-bearing mice and increased intratumoral inflammation in response to anti-PD-1 but was insufficient to induce complete tumor remission. Our results, while implicating activated TGF β and Notch signaling in anti-PD-1 resistance, do not exclude the possibility that other factors, such as compensatory upregulation of suppressive immune checkpoint molecules and T cell exhaustion, limit the antitumor efficacy of PD-1 blockade. Indeed, T cells infiltrating tumors that progress during PD-1 blockade are functionally exhausted and exhibit increased expression of inhibitory immune checkpoints such as TIM3.⁴⁰ Combination treatments that both increase immune infiltration and reprogram dysfunctional immune cells may be required to overcome resistance to checkpoint blockade.

Local administration of a mixture of mRNAs encoding the immune stimulatory cytokines IL12, IFN α , GM-CSF and IL15-sushi was previously shown to elicit potent antitumor immunity in a range of mouse cancer models.¹⁸ Cytokine mRNA treatment increased immune infiltration, enhanced priming and increased T cell polyfunctionality in mouse melanoma tumors, and showed improved antitumor efficacy in combination with anti-PD-1.¹⁸ Our work further demonstrates the potent antitumor efficacy of cytokine mRNA in anti-PD-1 resistant tumors and underscores its potential to resensitize resistant tumors to PD-1 blockade. Based on these observations, clinical trials of intratumoral cytokine mRNA, as monotherapy and in combination with anti-PD-1, are under way in patients with solid tumors (NCT03871348). The ability to both counteract lack of immune infiltration and enhance immune cell function may underlie the antitumor efficacy observed with cytokine mRNA mixture in our anti-PD-1 resistant MC38 tumors.

Current knowledge suggests that immune checkpoint blockade resistance is dictated by many tumor cell-intrinsic features, including genomic alterations, mutational burden, and oncogenic pathway activation, as well as tumor cell-extrinsic features such as nonmalignant stromal cells, organization of the extracellular matrix, and abundance and functional state of tumor-infiltrating immune cells. The mechanisms that control sensitivity to immune checkpoint blockade are likely multifactorial and may vary among different cancer types and patient populations. Significant efforts are underway to overcome immunotherapy resistance through combination therapies, with hundreds of combinations currently being tested in

clinical trials.⁴¹ Our data suggest that local intratumoral therapy that delivers potent immuno-stimulation directly into the tumor microenvironment may have utility in overcoming acquired resistance to anti-PD-1. The development of new biomarkers to predict which patients will benefit from specific combination treatments will further maximize the impact of cancer immunotherapies in the clinic.

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