Downregulation of Interferon- γ Receptor Expression Endows Resistance to Anti-Programmed Death Protein 1 Therapy in Colorectal Cancer[§]

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ABSTRACT

Immune checkpoint inhibitors have emerged as a frontline treatment of a variety of malignancies. However, only a subset of patients respond to these therapies, and many initial responders eventually develop resistance, leading to tumor relapse. Programmed death protein 1 is one of the checkpoint inhibitors that is expressed on activated T cells and suppresses the antitumor immune response when binding to its ligand, programmed death ligand 1, on tumor cells. Previous studies indicated that loss-of-function mutations in the IFN- γ pathway could result in acquired resistance to immune checkpoint inhibitors in human patients with cancer. Here, we investigated the effects of the IFN- γ receptor downexpression on the response to an anti-PD-1 antibody (α PD1) in a murine colorectal cancer model and the underlying mechanisms of resistance. IFN- γ receptor (IFNGR) 1 was knocked down in MC38 cells, a murine colon adenocarcinoma cell line using *IFNGR1* short hairpin RNA (shRNA) lentiviral particles. Then, MC38 IFNGR1 knockdown (KD) cells and negative control (SC) cells were used in this study. In the C57BL/6 xenograft model, the KD tumor

demonstrated resistance to α PD1 in comparison with SC cells. The observed treatment resistance might be associated with reduced tumor-infiltrating immune cells (TILs). When mixed, the resistant (KD) and control cells (SC) grew in spatially separated tumor areas, and α PD1 did not impact this pattern of spatial distribution. Our findings have proved that downregulation of the IFNGR1 endowed resistance to α PD1 and provided the potential mechanisms involving the TILs.

SIGNIFICANCE STATEMENT

Immunological checkpoint blockades have achieved substantial efficacy in a variety of tumors. However, only a subset of patients respond to these therapies, and innate and acquired resistance is widely present. Our study found that the downregulation of the IFN- γ receptor caused resistance to an anti-PD-1 antibody in a murine colorectal cancer model associated with the reduced tumor-infiltrating lymphocytes. Our findings have substantial implications for improving the efficacy of checkpoint blockades.

Introduction

The blockades of immune checkpoints such as programmed death protein 1 (PD-1) and cytotoxic-lymphocyte antigen 4 have ushered in a new era in cancer therapy (Philips and Atkins, 2015; Gao et al., 2016; Chen et al., 2018). The PD-1/PD-L1 pathway has become an attractive therapeutic target in treating melanoma, squamous non-small-cell lung cancer, colorectal cancer, renal cell cancer, and many others (Taube et al., 2014). PD-1 is expressed on activated T cells and

suppresses the antitumor immune response when binding to its ligand, PD-L1, on tumor cells (Topalian et al., 2012; Champiat et al., 2017; Chen et al., 2018). Blocking the PD-1/PD-L1 axis can unleash the immune function, resulting in durable tumor control (Chen et al., 2015). Approximately 30% of patients with renal cell cancer or melanoma treated with nivolumab, one of the prominent anti-PD-1 (α PD1) agents, experienced durable tumor regression (Juneja et al., 2017). However, not all patients demonstrated durable tumor regression, even in the initially well responsive patients (Callahan, et al., 2016). The rate of innate and acquired resistance is nearly 60%–70% to the α PD1 drugs (Juneja et al., 2017), which encumbers the effective clinical use of α PD1 (Peng et al., 2012; Hugo et al., 2016). Studies of the innate and acquired resistance mechanisms to α PD1 agents are underway.

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ABBREVIATIONS: IFN, interferon; IFNGR, IFN- γ receptor; JAK, Janus kinase; KD, knockdown IFN- γ receptor in MC38 cells; KD-RFP, KD cells labeled with RFP; PD-1, programmed death protein 1; α PD1, anti-PD-1 antibody; PD-L1, programmed death ligand 1; SC, scramble/control MC38 cells; SC-GFP, SC cells labeled with GFP; TIL, tumor-infiltrating lymphocyte; WT, wild-type shRNA short hairpin RNARFP red fluorescent protein FoxP3 forkhead box P3.

IFN- γ signaling pathway is an essential component in the responsive immune microenvironment in tumor (Ayers et al., 2017). Acquired resistance to α PD1 therapy in patients with melanoma was associated with loss-of-function mutation in the IFN- γ downstream signaling pathway, such as Janus kinase (JAK) 1 and 2 (Sucker et al., 2017). The IFN- γ responses are receptor-mediated signaling through IFN- γ receptors, including two isoforms: IFNGR1 and IFNGR2. When IFN- γ binds to IFNGR1 or IFNGR2, the complex could drive the receptors to be oligomerized and activate the receptor-associated JAK1 and JAK2, which in turn activates signal transducer and activator of transcription 1 and initiates the transcription of IFN- γ -associated genes (Miller et al., 2015; Gao et al., 2016; Kak et al., 2018). With defective IFN- γ receptors, tumor cells lose IFN- γ signaling and fail to present neoantigens to activate antitumor immunity, thereby escaping the immune surveillance (Tau and Rothman, 1999). However, IFN- γ is also one of the most potent inducers of PD-L1 expression, which impedes immune checkpoint therapy (Grabie et al., 2007; Shi, 2018). The two faces of IFN- γ in immune checkpoint therapy complicate the revealing of the mechanism of resistance.

In the present study (Fig. 1D), which uses a murine model, we investigated whether the downregulation of IFN- γ R1 could lead to resistance to α PD1 therapy, and if so, the potential immunologic mechanisms. One recent study has found a strong clonal cooperation between sensitive and resistance clones in tumor microenvironments, which further influence the immune selection of tumor clones, resulting in immune escape of the resistance clones (Williams et al., 2020). When the malfunction of the IFN- γ receptor occurs in a subset of tumor cells, we evaluated whether there is any clonal interaction between the clones with dysfunctions and the ones with normal functions of IFN- γ receptor.

Materials and Methods

Reagents and Antibodies. Dulbecco's modified Eagle's medium (with 4500 mg/l glucose, L-glutamine, sodium pyruvate), nonessential amino acid, penicillin-streptomycin, 0.05% trypsin-EDTA, gentamicin, and protease inhibitor were all purchased from Gibco-Thermo Fisher Scientific. Puromycin (10 mg/ml) and ammonium-chloride-potassium (ACK) lysis buffer were from Thermo Fisher Scientific. HEPES was from Cellgro. Fetal bovine serum and radioimmunoprecipitation assay buffer were purchased from Sigma-Aldrich. *IFNGR1* shRNA lentiviral particles (sc-35636-V), negative control shRNA lentiviral particles (sc-108080), and copepod green fluorescent protein (copGFP) control lentiviral particles (sc-108084) were purchased from Santa Cruz Biotechnology. LentiBrite GFP control lentiviral biosensor (17-10387) and LentiBrite RFP control lentiviral biosensor (17-10409) were obtained from Millipore. Collagenase/Dispase and DNase were obtained from Roche. Hoechst 33258 was purchased from Invitrogen. Cyanine dyes Cy3 and Cy5 were bought from PerkinElmer. All the antibodies with their catalog number are shown in Supplemental Table 1.

Cell Culture, shRNA Lentiviral Transfection, and Fluorescence Labeling. The MC38 cell line derived from C57BL/6 murine colon adenocarcinoma cells was purchased from Kerafast and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 μ M nonessential amino acid, 2 mM glutamine, 100 mM HEPES, 1% penicillin-streptomycin, and 50 mg/l gentamicin.

MC38 IFNGR1-knockdown (KD) cells and negative control (SC) cells were prepared as shown in Fig. 1A. KD cells were prepared by transducing MC38 cells with *IFNGR1* shRNA lentiviral particles at multiplicity of infection 20. Stable clone expressing the shRNA was then selected via puromycin dihydrochloride selection at 40 μ g/ml. The multiplicity of infection and puromycin dihydrochloride concentration were optimized using MC38 cells transduced with copepod green fluorescent protein (copGFP) control lentiviral particles. SC cells were prepared and selected similarly using shRNA lentiviral particles encoding a scrambled shRNA sequence that will not lead to degradation of any specific cellular message. Western blot was used to confirm the knockdown of IFNGR1 in KD cells and not in SC cells.

Fig. 1. Knockdown of IFN- γ receptor in MC38 colorectal cells. (A) The workflow for the development of the SC-GFP and KD-RFP cell lines. (B) The flow cytometry results for sorting SC-GFP and KD-RFP cell lines after 1 month of culture. SSC-A is for side-scatter area. (C) Western blot confirmed knockdown of IFN- γ R1 protein expression in KD cells and KD-RFP cells and not in SC cells and SC-GFP cells in comparison with MC38 WT cells. (D) The schematic and research questions that will be addressed in this study.

Next, KD cells and SC cells were labeled with GFP and RFP, respectively, using LentiBrite GFP control lentiviral biosensor and LentiBrite RFP control lentiviral biosensor. The fluorescently labeled cells (SC-GFP and KD-RFP) were selected using flow cytometry. Western blot was repeated to confirm the knockdown of IFNGR1 in KD-RFP cells and not in SC-GFP cells.

Western Blot Analysis. Cells were collected and lysed with radioimmunoprecipitation assay buffer supplemented with protease inhibitors. In total, 50 μ g lysate proteins were analyzed by SDS-PAGE and Western blotting under reducing condition. IFNGR1 was detected using mouse IFN- γ R α Antibody F-6 (sc-74450; Santa Cruz). β -Actin was used as a loading control.

Animal Study. The animal study, including tumor inoculation, drug administration, and measurement of tumor size, was performed by University of North Carolina at Chapel Hill Animal Studies Core (blinded to the treatment). In total, 108 female C57BL/6 mice (6–8 weeks; Jackson Laboratories, Bar Harbor, ME) were randomized into three tumor groups ($n = 36$). Each mouse group was randomized into two treatments ($n = 18$): BioXCell InVivoMab anti-mouse PD1 (CD279) antibodies (clone RMP1-14, catalog number BE0146, lot 71791801, anti-PD-1 group) or BioXCell InVivoMab rat IgG2a isotype control antibody (clone 2A3, catalog number BE0089, lot 686318A3, control group). As shown in Fig. 2A, the mice were inoculated with 5×10^5 SC-GFP cells, 5×10^5 KD-RFP cells, or 5×10^5 SC-GFP cells: KD-RFP cells 1:1 in the flank at day 0. At days 9, 12, and 15, the mice were treated with α PD1 antibody or control antibody at 0.2 mg per mouse (i.p., 10 mg/kg). The tumor size was measured every other day with a caliper. The tumor volumes were calculated by the following formula: tumor volume = (width² $\times$ length)/2. Mice were euthanized when the tumor reached a size of 3000 mm³ or by day 53. Upon euthanasia, the tumor was removed and cut into halves. One of the halves was analyzed by flow cytometry, and the other was analyzed by immunofluorescence staining for tumor-infiltrating lymphocytes (TILs).

All animal care was performed according to institutional guidelines at the School of Pharmacy, the University of North Carolina at Chapel Hill. All experimental procedures were approved by the Institutional Animal Care and Use Committee.

Tumor-Infiltrating Lymphocytes. TILs were analyzed according to the published method (Lau et al., 2017). Briefly, one-half of the freshly excised tumors were digested in PBS solution containing 1 mg/ml collagenase D and 0.2 mg/ml DNase I at 37°C for 1 hour. After filtering the digested suspension through a 40- μ m cell strainer, single cell suspension was prepared. Red blood cells were removed by incubating the cells in ammonium-chloride-potassium (ACK) lysis buffer at 25°C for 5 minutes. TILs were enriched using a Ficoll gradient (Histopaque 1083; Sigma). The obtained cell suspension was then stained for flow cytometry analysis of TILs. All the dead cells were removed by LIVE/DEAD Fixable Blue Dead Cell Stain Kit (Thermo Fisher Scientific). Cell surface markers including CD45, CD8, CD4, PD-1, and PD-L1 were stained using fluorescently labeled antibodies listed in Supplemental Table 1. Stained samples were analyzed using an LSRII cytometer (BD Biosciences).

Immunofluorescence Staining. The other half of the tumor was cut into two pieces. One piece was flash-frozen on dry ice and then fixed in optimal cutting temperature compound (OCT). The frozen tumors were sectioned to assess the spatial location of KD-RFP cells and SC-GFP cells using a fluorescence microscope (Nikon Ti2). The other piece was fixed in formalin for further analysis of the distribution of CD4, CD8, Ki67, and FoxP3-positive cells in tumors. The formalin-fixed tissues were sectioned into 5- μ m-thin slices. These slices were processed with H&E staining, thoroughly washed, and then dual-stained in the Bond fully automated slide staining system (Leica Microsystems). Slides were dewaxed and hydrated, and then heat-induced antigen retrieval was performed at 100°C for 20 minutes, followed with 10 minutes of peroxide blocking. After pretreatment, one slide was incubated with a CD4 rat anti-mouse antibody (1:500) and a CD8 rat anti-mouse antibody (1:200). One slide nearby the former

one was incubated with Ki67 rabbit anti-mouse antibody (1:200) and FoxP3 rabbit anti-mouse antibody (1:100). Goat anti-rat secondary antibody for CD4 and CD8 and goat anti-rabbit secondary antibody for Ki67 and FoxP3 were used. Stained slides were counterstained with Hoechst 33258 and mounted with prolong gold antifade reagent. Tyramide Signal Amplification systems with Cy3 or Cy5 were used for visualization.

Modeling of Tumor Growth. The individual tumor growth and mean tumor growth over time were analyzed using the exponential growth model (eq. 1) and the Gompertz function (eq. 2), respectively.

$$Y = Y_0 \times e^{kt} \quad (1)$$

$$Y = Y_0 \times (Y_0/Y_M)^A e^{kt} \quad (2)$$

Y_0 is the initial size (cubic millimeters), k is the growth rate constant (1 per day), t represents time (days), and Y_M indicates the tumor growth capacity limit. All model fittings were conducted using ADAPT-5 (Biomedical Simulations Resource; University of Southern California).

Statistics Analysis. All results were expressed as means $\pm$ S.D. and evaluated using a two-tailed t test. Statistical analysis was performed in GraphPad 8.0; P values < 0.05 were considered as statistically significant.

Results

The Knockdown of IFNGR1 in the MC38 Cell Line.

As shown in Fig. 1A, the wild-type (WT) MC38 colorectal cancer cells were transduced with *IFNGR1* shRNA lentiviral particles to produce *IFNGR1* knockdown cells or transduced with scrambled shRNA lentiviral particles to produce the negative control (SC) cells. These cells were then labeled with RFP or GFP for tracking purposes (KD-RFP and SC-GFP). As shown in Fig. 1B, high percentages of labeled cells remained after 1 month of culture, indicating the stability of fluorescent transfection. In comparison with the WT cells, Western blotting in Fig. 1C confirmed the knockdown of *IFNGR1* in KD cells and KD-RFP cells and not in the negative control cells, SC cells, and SC-GFP cells.

Varied Individual Tumor Response to α PD1 Therapy. As shown in Fig. 2A, 36 C57BL/6 mice were inoculated with 5×10^5 SC-GFP cells, 5×10^5 KD-RFP cells, or 5×10^5 1:1 mixture of SC-GFP cells and KD-SC cells on day 0, respectively. In the following text, the tumor groups are termed as SC, KD, and KD-SC tumors, respectively, for simplicity. Each tumor group was randomized at 1:1 to receive either isotype control IgG or α PD1 at 0.2 mg per mouse (10 mg/kg) on days 9, 12, and 15. In total, 21 in 108 mice had tumor sizes that never exceeded 100 mm³ throughout the study. These mice were excluded from analysis because of concern of tumor inoculation failure.

The individual tumor growth trajectory is shown in Fig. 2B. High interindividual variability in tumor growth trajectory within each tumor type and each treatment group was observed. In the SC group, 2 of 11 mice treated with α PD1 first experienced exponential tumor growth until day 25 (10 days after last α PD1 dosing); the tumor remained stable for about 10 days (until day 35) and then started to shrink. Mice bearing SC tumor had smaller maximum tumor volume during the study when treated with α PD1 in comparison with the control IgG group. In the KD tumor group, most mice showed exponential growth even when treated with α PD1. In total, 1 of 18 mice bearing the KD tumor had a rapid tumor

shrinkage on day 28, on the 13th day after the last α PD1 dose. In the mice inoculated with a 1:1 mixture of SC and KD cells, 2 of 14 mice experienced delayed exponential growth. The percentage of mice with tumor volume above 1000 mm³ on day 25 was lower for the α PD1 group than the control IgG group for all tumor types. It appears that the α PD1 therapy was most effective in the SC tumor type.

The diverse tumor growth trajectories in response to α PD1 treatment and even within the control IgG group may reflect the high variability of the immune function of the mice and their interaction with α PD1 treatment. The interindividual

variability of tumor response was assessed by fitting the individual tumor growth trajectory to an exponential growth model. The exponential function captured most of the curves, except for the tumors showing delayed or multiphasic response patterns, for which only the exponential growth phases (the last few tumor sizes before the end of experiments) were included in the modeling. Despite the diverse growth patterns, the last few tumor sizes were more closely associated with animal survival than earlier tumor responses. The individual fitting results were summarized in Supplemental Fig. 1. The growth rate K and the time when tumor size reached

Fig. 2. Tumor growth trajectories for SC-GFP, KD-RFP, and SC-GFP/KD-RFP 1:1 xenografts in C57BL/6 mice treated with control IgG or α PD1. (A) Animal study plan. (B) Individual tumor growth trajectory group by tumor type and treatment. The number of mice with tumor volume above 1000 mm³ at day 25 was labeled. SC, KD, and KD-SC are mice inoculated with SC-GFP cells, KD-RFP cells, or a 1:1 mixture of SC-GFP and KD-RFP cells, respectively.

1000 mm³ (*T1000*) were compared in Supplemental Fig. 2A. The SC group treated with α PD1 showed the lowest growth rate and longest time to reach 1000 mm³, even though no significant level was not reached due to high variability.

The Knockdown of IFNGR1 Conferred Resistance to α PD1 Therapy. The effectiveness of α PD1 treatment in the three tumor types was further evaluated by plotting the population average tumor growth trajectory. Figure 3 presented the population average tumor growth curves and Kaplan-Meier survival curves. α PD1 significantly suppressed the tumor growth and improved survival in the SC tumor group. In contrast, α PD1 therapy was not significantly superior to control IgG in suppressing KD tumor growth and improving survival, suggesting the knockdown of IFN- γ R1 conferred resistance to α PD1. Interestingly, the KD-SC tumor was partially responsive to the α PD1 therapy. The effect size was about the average suppressive effect on the SC and the KD tumors. The average effect size of α PD1 in the KD-SC group may reflect the suppression on the sensitive SC cells, which were inoculated at 50%.

As shown in Supplemental Fig. 2B, the Gompertz function captured the average growth data well ($R^2 > 0.99$). In three control IgG groups, the tumor growth potential (Y_M) is similar ($P = 0.466$). The tumor growth potential in the α PD1 group is KD > KD-SC > SC ($P = 0.021$). The tumor growth potential in the α PD1-treated SC group was the lowest (1294 mm³)—about 60% of the levels in the other groups (>2000 mm³). These results indicated the significant suppressive effects of α PD1 in the SC tumor, but the effects dissipated in KD tumor, suggesting knockdown of *IFNGR1* conferred resistance to α PD1 therapy in MC38 colorectal cancer.

IFNGR1 Knockdown Tumor Had Less Infiltration of Immune Cells. IFN- γ could induce the upregulation of class I major histocompatibility complex and antigen processing machinery, and it could also promote productions of

chemokines that can result in the recruitment of effector T cells (Yang et al., 1995; Minn and Wherry, 2016). As shown in Fig. 4, the infiltrating immune cells (CD45+) in the KD control IgG group was significantly lower than in the SC control IgG group ($P = 0.001$), suggesting the reduced infiltration of immune cells in the *IFNGR1* knockdown xenograft. The KD-SC control group (IgG) had a significant increase in the infiltrating immune cells (CD45+) than in the KD group ($P = 0.006$). Interestingly, α PD1 significantly decreased the infiltrating immune cells in the SC group compared with the control IgG ($P = 0.026$), in contrast to the KD group, in which α PD1 modestly increased infiltrating immune cells ($P = 0.103$). A similar suppressive effect of α PD1 on infiltrating immune cells was observed in the KD-SC group compared with the SC group. Because of the high variabilities, the difference between the control IgG and α PD1 in the percentage of cytotoxic (CD8+) and regulatory T cells (CD4+) within the CD45+ population did not reach significance in all tumor groups. However, the percentage of cytotoxic T cells appeared to be higher after the treatment of α PD1 in the SC group but not in the KD group and the KD-SC group. On the other hand, IFN- γ could induce the expression of PD-L1 and alter the immune response of tumor cells (Grabie et al., 2007; Shi, 2018). In the SC group, α PD1 increased PD-1+ T cells compared with the control IgG ($P = 0.031$). On the other side, PD-L1+ tumor cells were significantly lower in the KD control IgG group compared with the SC control IgG group ($P = 0.026$). α PD1 increased the fraction of PD-L1+ tumor cells, suggesting the responsive regulation of checkpoint by tumor cells.

The Spatial Distribution of Immune Cells Was Not Altered by the Knockdown of IFNGR1. The spatial distribution of CD8+, CD4+, Ki67+, and FoxP3+ cells in the SC-KD tumors was assessed through immunofluorescence staining. As shown in Fig. 5, the cytotoxic T cells (CD8+) were much higher than the regulatory T cells (CD4+) in all groups.

Fig. 3. The knockdown of IFN- γ R1 conferred resistance to α PD1 therapy. (A) Mean tumor growth curves of C57BL/6 mice bearing different MC38 tumors and treated with control IgG or α PD1. Student's *t* test was used to compare the tumor volume at the end of the study between the two treatments. (B) Kaplan-Meier survival analysis of the same mice. SC, KD, and KD-SC are mice inoculated with SC-GFP cells, KD-RFP cells, or a 1:1 mixture of SC-GFP and KD-RFP cells, respectively.

Fig. 4. Analysis of TILs and tumor cells by flow cytometry. Percentage of CD45+ cells in TILs, percentage of CD8+ T cells in CD45+ cells, percentage of CD4+ T cells in CD45+ cells, percentage of CD8+ and CD4+ T cells in CD45+ cells, percentage of PD-1+ cells in CD45+ cells, and PD-L1+ cells in tumor cells. Statistical significance was determined by *t* test. SC, KD, and KD-SC are mice inoculated with SC-GFP cells, KD-RFP cells, or a 1:1 mixture of SC-GFP and KD-RFP cells, respectively.

The proliferating tumor cells (Ki67+) resided at the edge of the tumor. The natural regulatory T cells (FoxP3+) evenly distributed throughout the tumor. Importantly, no apparent difference (Fig. 5B vs. Fig. 5C) in cell spatial distribution was observed between the α PD1 and control IgG groups. We also evaluated the distribution of the SC-GFP and KD-RFP tumor cells in the mixed group. Interestingly, two types of tumor cells grew in spatially exclusive tumor areas, with moderate mixing (Fig. 5A). Although SC-GFP cells are more responsive to α PD1, this growing pattern was not noticeably shifted by α PD1. This spatially aggregated growing pattern warrants further investigation.

Discussion

The blockades of PD1 or PD-L1 immune checkpoints are efficacious in treating multiple cancer types by reactivating T cells (Chen and Han, 2015; Koyama et al., 2016). However, only a small fraction of patients have a long-term survival benefit, with the majority either not responding or quickly developing resistance shortly after an initial response (Bertrand et al., 2017; Kakavand et al., 2017). Some studies had confirmed the association between mutation in the IFN- γ signaling pathway and acquired resistance to α PD1 in patients with melanoma (Sucker et al., 2017). The disruption or downregulation of IFN- γ receptors in tumor cells could be caused by the immune selection, which leads to the deficiency of antigen presentation machinery and results in treatment resistance (Tau and Rothman, 1999). Recent investigations revealed dysfunctions of IFN- γ signaling in tumor cells derived from patients who acquired resistance after initial response to anti-PD-1 therapy. The functional roles played by IFN- γ in antitumor immunity are dynamic and involve both positive and negative regulatory activities (Shin et al., 2017).

In this study, we investigated the effect of the knockdown of the IFNGR1 on the response to α PD1 therapy in a murine colorectal cancer model. We prepared IFNGR1 knockdown MC38 murine colorectal cancer cells as well as control cells (SC) using shRNA lentiviral particles as reported previously (Gao et al., 2016). The response to α PD1 dissipated in the KD group, confirming that the disruption of IFN- γ signaling led to resistance.

We further explored the resistance mechanism by analyzing the compositions and the spatial distributions of TILs. The tumor is the home to many types of immune cells, and the abundance and spatial distribution of TILs are closely associated with the response to immunotherapy (Pancione et al., 2014). Multiple reports demonstrated that elevated numbers of TILs have been noted in responsive cancers and that the increase in TILs might represent the higher response to the therapy (Fares, et al., 2019). Although our studies were challenged by high variability, we found that the disruption of IFNGR1 expression is associated with decreased TILs (CD45+). Interestingly, α PD1 increased TILs in the KD group but decreased TILs in the SC group. The opposing effect was probably associated with the distinct tumor immune micro-environments between the SC and the KD xenografts, suggesting the crucial role of immune baseline status to the response of checkpoint blockades (Zemek et al., 2020). In addition to the abundance of TILs, the spatial locations of TILs related to tumor cells, within distinct tumor regions, have also been evaluated as a prognostic factor for routine clinical practice (Chen and Mellman, 2013). Unfortunately, our study did not observe the spatial shifts of TILs in the tumors. The therapeutic pressure can favor the selection of resistant clones, which has been demonstrated to be one of the primary drivers of resistance. We showed that knockdown of IFNGR1 endows MC38 colorectal cancer cells resistance to α PD1 therapy. We mimic the clinical heterogeneity in tumor

Fig. 5. The spatial distribution of the tumor cells and immune cells (CD8+, CD4+, FoxP3+) in KD-SC xenografts by immunofluorescence staining. (A) Distribution of the SC-GFP and KD-RFP tumor cells. (B) Staining of CD4+ regulatory cells (Treg) and CD8+ cytotoxic T cells. DAPI (4',6-diamidino-2-phenylindole) is a fluorescent stain that binds strongly to DNA. (C) Staining of Foxp3 regulatory T cells and Ki67+ proliferating cells.

clonality by inoculating the KD-SC tumor, a mixture of sensitive cells (SC-GFP) and resistant cells (KD-RFP), and tested the tumor growth in the absence or presence of α PD1 therapy. In the absence of α PD1 therapy, the SC tumor, KD tumor, and the mixture xenograft exhibited similar growth, suggesting similar fitness between the sensitive cells and resistant cells in the absence of treatment. Although not statistically significant, there was a partial suppression of α PD1 on the growth of KD-SC xenograft, which was approximately the average effect size between the high effect in the SC group and minimal effect in the KD group. The average effect size in the KD-SC xenograft may come from the suppressive effect on the SC cells, which were inoculated at 50%. It seems that the presence of resistant cells in the xenograft did not strongly interfere with the growth and response of the sensitive cells in our experimental settings. Interestingly, in the KD-SC group, two types of cells grew spatially separate with minimal mixing, which was not altered by α PD1. The reason for this spatially aggregating phenomenon of each kind of cell warrants further investigation.

Limitations

Our study has one significant limitation: the flow cytometry analysis of TILs using tumor samples resected at the end of

the study. Because of the diverse growth trajectories, the tumor was resected at varying sizes and timings postinoculation. Several slowly growing tumors were very small by the time of resection so that the obtained sample was limited for repeated flow cytometry analysis of TILs. In addition, our study only provided a snapshot characterization of the TILs, which should be interpreted with caution when the tumor exhibited multiphasic growth patterns.

Conclusion

In conclusion, our study confirmed that the downregulation of the IFN- γ receptor caused treatment resistance to α PD1, which appeared to be associated with the reduced infiltration of lymphocytes in the tumor. The presence of resistance cells did not appear to interfere with the growth and response of sensitive cells in our experimental settings. These findings have important clinical implications for disclosing the resistance mechanisms to checkpoint blockades.

Authorship Contributions

Participated in research design: Yuan, Cao.

Conducted experiments: Lv, Yuan.

Performed data analysis: Lv, Yuan, Cao.

Wrote or contributed to the writing of the manuscript: Lv, Yuan, Cao.

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