Down-regulation of the IFN-γ receptor expression endows resistance to anti-PD-1 therapy in colorectal cancer

Chunxiao Lv 1, 2#, Dongfen Yuan 2#, Yanguang Cao 2, 3*

1Department of Clinical Pharmacology, Second Affiliated Hospital of Tianjin University of Traditional Chinese Medicine, Tianjin, 300250, China.

2Division of Pharmacotherapy and Experimental Therapeutics, UNC Eshelman School of Pharmacy, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina. 27599, United States.

3Lineberger Comprehensive Cancer Center, School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, United States

# Authors contribute equally to the work.
Running Title: Down-regulation of the IFN-γR endows resistance to αPD1

* To whom correspondence should be addressed:

Yanguang Cao, UNC Eshelman School of Pharmacy, UNC at Chapel Hill. Tel: (919) 966-4040, Email: yanguang@unc.edu

Pages: 34
Tables: 0
Figures: 5
References: 33
Abstract Word Count: 231
Introduction Word Count: 517
Discussion Word Count: 763
**Abbreviation:**

αPD1: anti-PD1 antibody

BMSR: Biomedical simulations resource

CTLA-4: cytotoxic-lymphocyte antigen 4

DMEM: dulbecco's modified eagle medium

FBS: Fetal bovine serum

H&E: haemotoxylin and hosin

HEPES: hydroxyethyl piperazineethanesulfonic acid

IFNGR1: IFN-γ receptor 1

IFNGR2: IFN-γ receptor 2

JAK1: Janus kinases 1

JAK2: Janus kinases 2

KD cells: knocked down INF-γ receptor in MC38 cells

KD-RFP: KD cells labeled with RFP

MHC: major histocompatibility complex

MOI: multiplicity of infection

NEAA: non-essential amino acid

PBS: phosphate buffer saline

PD-1: programmed death protein 1

PD-L1: programmed death ligand 1

Pen/Strep: penicillin-streptomycin

RIPA: radioimmunoprecipitation assay

SC cells: scramble/control MC38 cells

SC-GFP: SC cells labeled with GFP

SDS-PAGE: standard sodium dodecyl sulfate polyacrylamide gel electrophoresis
STAT1: signal transducer and activator of transcription 1
TILs: tumor-infiltrating lymphocytes
TSA: tyramide signal amplification
UNC: University of North Carolina
WT: wild-type

**Recommended section:** Special Issue on “Drug Discovery and Translational Medicine”
Abstract

Immune checkpoint inhibitors have emerged as a frontline treatment for 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 (PD-1) is one of the checkpoint inhibitors which expressed on activated T cells and suppresses the antitumor immune response when binds to its ligand PD-L1 on tumor cells. Previous studies indicated that loss-of-function mutations in INF-γ pathway could result in acquired resistance to immune checkpoint inhibitors in human cancer patients. Here, we investigated the effects of the INF-γ receptor down-expression on the response to an anti-PD1 antibody (αPD1) in a murine colorectal cancer model and the underlying mechanisms of resistance. INF-γ receptor1 (IFN-γR1) was knocked down in MC38 cells (KD), a murine colon adenocarcinoma cell line using IFNGR1 shRNA lentiviral particles. Then, MC38 IFN-γR1 knockdown (KD) cells and negative control (SC) cells were used in this study. In C57BL/6 xenograft model, the KD tumor demonstrated resistance to αPD1 in comparison to 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 αPD1 did not impact this pattern of spatial distribution. Our findings have proved that down-regulation of the IFN-γR1 endowed resistance to αPD1 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 down-regulation of the INF-γ receptor caused resistance to an anti-PD1 antibody (αPD1) 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.
1. Introduction

The blockades of immune checkpoints such as programmed death protein 1 (PD-1) and cytotoxic-lymphocyte antigen 4 (CTLA-4) have ushered in a new era in cancer therapy (Philips and Atkins, 2014, Chen et al., 2018, Gao et al., 2016). 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 binds to its ligand PD-L1 on tumor cells (Chen et al., 2018, Topalian et al., 2012, Champiat et al., 2017). 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 (αPD1) 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 αPD1 drugs (Juneja et al., 2017), which encumbers the effective clinical use of αPD1 (Hugo et al., 2016, Peng et al., 2012). Studies of the innate and acquired resistance mechanisms to αPD1 agents are undergoing.

IFN-γ signaling pathway is an essential component in the responsive immune microenvironment in tumor (Ayers et al., 2017). Acquired resistance to αPD1 therapy in melanoma patients was associated with lose-of-function mutation in the IFN-γ downstream signaling pathway such as Janus kinases 1 and 2 (JAK1 and JAK2) (Jesse et al., 2016, Sucker et al., 2017). The IFN-γ responses are receptor-mediated signaling through IFN-γ receptors, including two isotypes receptor 1 (IFNGR1) and 2 (IFNGR2). When the 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 activate signal transducer and activator of transcription 1 (STAT1) and initiates the transcription of IFN-γ associated genes (Gao et al., 2016, Miller et al., 2015, Kak et al., 2018). With defective IFN-γ receptors, tumor cells lose IFN-γ signaling and fail to present neo-antigens 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 (Figure 1d), using a murine model, we investigated whether the down-regulation of IFN-γR1 could lead to the resistance to αPD1 therapy, and if so, what are the potential immunological 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 INF-γ receptor occurs in a subset of tumor cells, we evaluated if there is any clonal interaction between the clones with dysfunctions and the ones with normal functions of INF-γ receptor.
2. Materials and Methods

2.1 Reagents and antibodies

Dulbecco's modified eagle medium (DMEM, with 4500 mg/L glucose, L-glutamine, sodium pyruvate), non-essential amino acid (NEAA), penicillin-streptomycin (Pen/Strep), 0.05% trypsin-EDTA, gentamicin and protease inhibitor were all purchased from Gibco-Thermo Fisher Scientific (USA). Puromycin (10 mg/mL) and ACK lysis buffer were from Thermo Fisher Scientific (USA). Hydroxyethyl piperazineethanesulfonic acid (HEPES) were from Cellgro (USA). Fetal bovine serum (FBS) and radioimmunoprecipitation assay (RIPA) buffer were purchased from Sigma-Aldrich (USA). IFNGR1 shRNA lentiviral particles (sc-35636-V), negative control shRNA lentiviral particles (sc-108080), copGFP control lentiviral particles (sc-108084) were purchased from Santa Cruz Biotechnology (USA). LentiBrite GFP control lentiviral biosensor (17-10387) and LentiBrite RFP control lentiviral biosensor (17-10409) were obtained from Millipore (USA). Collagenase/Dispase and DNase were obtained from Roche (USA). Hoechst 33258 was purchased from Invitrogen (USA). Cyanine dyes, Cy3, and Cy5 were bought from PerkinElmer (USA). All the antibodies with the catalog number were shown in Table S1 (Supplemental Data).

2.2 Cell culture, shRNA lentiviral transfection, and fluorescence labeling

MC38 cell line derived from C57BL6 murine colon adenocarcinoma cells were purchased from Kerafast and maintained in DMEM medium supplemented with 10% FBS, 100 µM NEAA, 2 mM glutamine, 100 mM HEPES, 1% Pen/Strep, 50 mg/L gentamicin.

MC38 IFN-γR1-knockdown (KD) cells and negative control (SC) cells were prepared as shown in Figure 1a. KD cells were prepared by transducing MC38 cells with IFNGR1 shRNA lentiviral particles at multiplicity of infection (MOI) 20. Stable clone
expressing the shRNA was then selected via puromycin dihydrochloride selection at 40 µg/mL. The MOI and puromycin dihydrochloride concentration were optimized using MC38 cells transduced with 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 IFN-γR1 in KD cells and not in SC cells. 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 IFN-γR1 in KD-RFP cells and not in SC-GFP cells.

2.3 Western blot analysis
Cells were collected and lysed with RIPA buffer supplemented with protease inhibitors. 50 µg lysate proteins were analyzed by standard sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting under reducing condition. IFN-γR1 was detected using mouse IFN-γRα Antibody F-6 (Santa Cruz sc-74450). β-actin was used as a loading control.

2.4 Animal study
The animal study including tumor inoculation, drug administration, and measurement of tumor size was performed by UNC 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 mice group were randomized into two treatments \((n = 18)\): BioXCell InVivoMAb anti-mouse PD1 (CD279) antibodies (clone RMP1-14, cat# BE0146, lot# 71791801, Anti-PD-1 group) or BioXCell InVivoMAb rat IgG2a isotype control antibody (clone 2A3, cat# BE0089,
lot#686318A3, control group). As shown in Figure 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 day 9, 12, 15, the mice were treated with αPD1 antibody or control antibody at 0.2 mg/mouse (i.p., 10 mg/kg). The tumor size was measured every other day with a caliper. The tumor volumes were calculated by the formula: 
\[ \text{tumor volume} = \frac{\text{Width}^2 \times \text{Length}}{2} \]
Mice were euthanized when the tumor reached a size of 3000 mm^3 or by the day 53. Upon euthanization, the tumor was removed and cut into halves. One was analyzed by flow cytometry and the other 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 (UNC). All experimental procedures were approved by the Institutional Animal Care and Use Committee.

2.5 Tumor-infiltrating lymphocytes (TILs)
TILs were analyzed according 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 h. After filtering the digested suspension through 40 µm cell strainer, single cell suspension was prepared. Red blood cells were removed by incubating the cells in ACK lysis buffer at 25 °C for 5 min. 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, PD-L1 were stained using fluorescently labeled antibodies listed in Table S1 of Supplemental Data. Stained samples were analyzed using an LSRII cytometer (BD Biosciences).

2.6 Immunofluorescence staining
The other half of tumor was cut into two pieces. One piece was flash frozen on dry ice and then fixed in 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 μm thin slices. These slices were processed with haemotoxylin and eosin (H&E) staining, thoroughly washed, and then dual-stained in the Bond fully-automated slide staining system (Leica Microsystems). Slides were dewaxed and hydrated, then heat-induced antigen retrieval was performed at 100°C for 20 min, followed with 10 min 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, 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 (TSA) systems with Cy3 or Cy5 were used for visualization.

2.7 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.

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

$$
Y = Y_0 \times \left(\frac{Y_0}{Y_M}\right)^{e^{kt}} \quad (2)
$$

$Y_0$ is the initial size (mm$^3$), $k$ is the growth rate constant (1/day), $t$ represents time (days), $Y_M$ indicates the tumor growth capacity limit. All model fittings were conducted using ADAPT-5 (Biomedical simulations resource (BMSR), University of Southern
California).

2.8 Statistics analysis

All results were expressed as mean ± SD 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.
3. Results

3.1 The knockdown of IFN-γR1 in the MC38 cell line.

As shown in Figure 1a, the wild-type (WT) MC38 colorectal cancer cells were transduced with \textit{IFNGR1} shRNA lentiviral particles to produce IFN-γR1 knockdown (KD) 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 purpose (KD-RFP and SC-GFP). As shown in Figure 1b, high percentages of labeled cells remained after the one-month culture, indicating the stability of fluorescent transfection. In comparison to the WT cells, western blotting in Figure 1c confirmed the knockdown of IFN-γR1 in KD cells and KD-RFP cells and not in the negative control cells, SC cells and SC-GFP cells.

3.2. Varied individual tumor response to αPD1 therapy

As shown in Figure 2a, 36 C57BL/6 mice were inoculated with $5 \times 10^5$ SC-GFP cells, $5 \times 10^5$ KD-RFP cells, or $5 \times 10^5$ 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 were randomized at 1:1 to receive either isotype control IgG or αPD1 at 0.2 mg/mouse (10 mg/kg) on days 9, 12, and 15. There were 21/108 mice with tumor size never exceeded 100 mm$^3$ throughout the study. These mice were excluded from analysis due to concern of tumor inoculation failure.

The individual tumor growth trajectory is shown in Figure 2b. High inter-individual variability in tumor growth trajectory within each tumor type and each treatment group
was observed. In the SC group, 2/11 mice treated with αPD1 firstly experienced exponential tumor growth till day 25 (10 days after last αPD1 dosing), the tumor remained stable for about ten days (till day 35), and then started to shrink. Mice bearing SC tumor had smaller maximum tumor volume during the study when treated with αPD1 in comparison to the control IgG group. In the KD tumor group, most mice showed exponential growth even when treated with αPD1. 1/18 mouse bearing the KD tumor had a rapid tumor shrinkage on day 28, on the 13th day after the last αPD1 dose. In the mice inoculated with a 1:1 mixture of SC and KD cells, 2/14 mice experienced delayed exponential growth. The percentage of mice with tumor volume above 1000 mm$^3$ on day 25 was lower for the αPD1 group than the control IgG group for all tumor types. It appears that the αPD1 therapy was most effective in the SC tumor type.

The diverse tumor growth trajectories in response to αPD1 treatment and even within the control IgG group may reflect the high variability of the mice immune function and their interaction with αPD1 treatment. The inter-individual 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 Figure S1. The growth rate $K$ and the time when tumor size reached 1000 mm$^3$ ($T_{1000}$) were compared in Figure S2a. The SC group treated with αPD1 showed the lowest growth
rate and longest time to reach 1000 mm$^3$, even though no significant level was not
reached due to high variability.

3.3. The knockdown of IFN-γR1 conferred resistance to αPD1 therapy

The effectiveness of αPD1 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. αPD1
significantly suppressed the tumor growth and improved survival in the SC tumor
group. In contrast, αPD1 therapy was not significantly superior to control IgG in
suppressing KD tumor growth and improving survival, suggesting the knockdown of
IFN-γR1 conferred resistance to αPD1. Interestingly, the KD-SC tumor was partially
responsive to the αPD1 therapy. The effect size was about the average suppressive
effect on the SC and the KD tumors. The average effect size of αPD1 in the KD-SC
group may reflect the suppression on the sensitive SC cells, which was inoculated at
50%.

As shown in Figure S2b, the Gompertz function captured the average growth
data well ($R^2 > 0.99$). In three control IgG groups, the tumor growth potential ($Y_m$) are
similar ($p = 0.466$). The tumor growth potential in the αPD1 group is KD > KD-SC > SC
($p = 0.021$). The tumor growth potential in the αPD1 treated SC group was the lowest
(1,294 mm$^3$), about 60% of the levels in the other groups (> 2,000 mm$^3$). These
results indicated the significant suppressive effects of αPD1 in the SC tumor, but the
effects dissipated in KD tumor, suggesting knockdown of IFN-γR1 conferred
resistance to αPD1 therapy in MC38 colorectal cancer.
3.4. IFN-γR1 knockdown tumor had less infiltrations of immune cells

IFN-γ could induce the upregulations of class I major histocompatibility complex (MHC) and antigen processing machinery; and it also could 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 Figure 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 IFN-γR1 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, αPD1 significantly decreased the infiltrating immune cells in the SC group compared to the control IgG ($p = 0.026$), in contrast to the KD group, where αPD1 modestly increased infiltrating immune cells ($p = 0.10 3$). A similar suppressive effect of αPD1 on infiltrating immune cells was observed in the KD-SC group compared to the SC group. Due to the high variabilities, the difference between the control IgG and αPD1 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 αPD1 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, αPD1 increased PD-1+ T cells compared to the control IgG ($p = 0.031$). On the other side, PD-L1+ tumor cells were significantly lower in the KD control IgG group compared to the SC control IgG group ($p = 0.026$). αPD1 increased the fraction of PD-L1+ tumor cells, suggesting the responsive regulation of checkpoint by tumor cells.

3.5 The spatial distribution of immune cells was not altered by the knockdown
of IFN-γR1.

The spatial distribution of CD8+, CD4+, Ki67+, and FoxP3+ cells in the SC-KD tumors was assessed through immunofluorescence staining. As shown in Figure 5, the cytotoxic T cells (CD8+) were much higher than the regulatory T cells (CD4+) in all groups. The proliferating tumor cells (Ki67+) cells resided at the edge of the tumor. The natural regulatory T cells (FoxP3+) evenly distributed throughout the tumor. Importantly, no apparent difference (Figure 5b vs. 5c) in cell spatial distribution was observed between the αPD1 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 (Figure 5a). Although SC-GFP cells are more responsive to αPD1, this growing pattern was not noticeably shifted by αPD1. This spatially aggregated growing pattern warrants further investigation.
4. Discussion

The blockades of PD1 or PD-L1 immune checkpoints are efficacious in treating multiple cancer types by reactivating T cells (Koyama et al., 2016, Chen and Han, 2015). 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 (Kakavand et al., 2017, Bertrand et al., 2017). Some studies had confirmed the association between mutation in the IFN-γ singling pathway and acquired resistance to αPD1 in melanoma patients (Jesse et al., 2016, Sucker et al., 2017). The disruption or downregulations 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 IFN-γR1 on the response to αPD1 therapy in a murine colorectal cancer model. We prepared IFN-γR1 knocked down MC38 murine colorectal cancer cells (KD) as well as control cells (SC) using shRNA lentiviral particles as reported previously (Gao et al., 2016). The response to αPD1 dissipated in the KD group, confirming 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). Most reports demonstrated
the elevated numbers of TILs have been noted in responsive cancers, the more 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 IFN-γR1 expression are associated with decreased TILs (CD45+). Interestingly, αPD1 increased TILs in the KD group while decreased TILs in the SC group. The opposing effect was probably associated with the distinct tumor immune microenvironments 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 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 INF-γR1 endows MC38 colorectal cancer cells resistance to αPD1 therapy. We mimic the clinical heterogeneity in tumor 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 αPD1 therapy. In the absence of αPD1 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 αPD1 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 was inoculated at 50%. It seems that the presence of resistant
cells in the xenograft did not strongly interfere 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 αPD1. The reason for this spatially aggregating phenomenon of each kind of cells warrant 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. Due to the diverse growth trajectories, the tumor was resected at varying sizes and timings post-inoculation. Several slowly growing tumors were very small by the time of resection so that the obtained sample were 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 the down-regulation of the IFN-γ receptor caused treatment resistance to αPD1, which appeared to be associated with the reduced infiltration of lymphocytes in the tumor. The presence of resistance cells did not appear to interfere 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.

Acknowledgements: This study was supported by National Institute of Health GM119661

Authorship Contributions:

Participated in research design: Yuan D, Cao Y
Conducted Experiments: Lv C, Yuan D

Performed data analysis: Lv C, Yuan D, Cao Y

Wrote or contributed to the writing of the manuscript: Lv C, Yuan D, Cao Y
Reference


Shin DS, Zaretzky JM, Escuin-Ordinas H, Garcia-Diaz A, Hu-Lieskovan S, Kalbasi A


Footnotes

This work was supported by the National Institutes of Health [GM119661].

No author has an actual or perceived conflict of interest with the contents of this article.
Figure Legends

Figure 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 one-month culture. c. Western blot confirmed knockdown of IFN-γR1 protein expression in KD cells and KD-RFP cells and not in SC cells and SC-GFP cells in comparison to MC38 wild-type (WT) cells. d. the schematic and research questions that will be addressed in this study.

Figure 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 αPD1. 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 1:1 mixture of SC-GFP and KD-RFP cells, respectively.

Figure 3. The knockdown of IFN-γR1 conferred resistance to αPD1 therapy. a. Mean tumor growth curves of C57BL/6 mice bearing different MC38 tumors and treated with control IgG or αPD1. The 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 1:1 mixture of SC-GFP and KD-RFP cells, respectively.

Figure 4. Analysis of tumor infiltrating lymphocytes (TILs) and tumor cells by flow cytometry. CD45+ cells percentage in tumor-infiltrating lymphocytes (TILs), CD8+ T cells, CD4+ T cells, CD8+CD4+ T cells, PD-1+ cells percentage in CD45+ cells, 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 1:1 mixture of SC-GFP and KD-RFP cells, respectively.

Figure 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+ Treg and CD8+ cytotoxic T cells; c. Staining of Foxp3 regulatory T cells and Ki67+ proliferating cells.
Female C57BL6 mice

Tumor inoculation

0 9 12 15 53

Day

Total 108 mice

Fig. 2

IP injection of control or anti-PD-1 drug (0.2 mg/mouse)

Control

Anti-PD-1

Days post tumor inoculation
Fig. 4

Box plots showing the % of lymphocytes and CD45+ cells in different groups: Control, Anti-PD-1, SC, KD, KD-SC.

CD45+ % of Lymphocytes:
- Control: 0.001, 0.006
- Anti-PD-1: 0.026, 0.103, 0.253

CD8+ % of CD45:
- SC: 0.330, 0.971, 0.844
- KD: 0.012
- KD-SC: 0.065

PD-1+ % of CD45:
- SC: 0.031
- KD: 0.452
- KD-SC: 0.587