Tumor heterogeneity and clonal cooperation influence the immune selection of IFN-γ-signaling mutant cancer cells

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PD-1/PD-L1 blockade can promote robust tumor regression yet secondary resistance often occurs as immune selective pressure drives outgrowth of resistant tumor clones. Here using a genome-wide CRISPR screen in B16.SIY melanoma cells, we confirm Ifngr2 and Jak1 as important genes conferring sensitivity to T cell-mediated killing in vitro. However, when implanted into mice, these Ifngr2- and Jak1-deficient tumors paradoxically are better controlled immunologically. This phenotype maps to defective PD-L1 upregulation on mutant tumor cells, which improves anti-tumor efficacy of CD8⁺ T cells. To reconcile these observations with clinical reports of anti-PD-1 resistance linked to emergence of IFN-γ signaling mutants, we show that when mixed with wild-type tumor cells, IFN-γ-insensitive tumor cells indeed grow out, which depends upon PD-L1 expression by wild-type cells. Our results illustrate the complexity of functions for IFN-γ in anti-tumor immunity and demonstrate that intratumor heterogeneity and clonal cooperation can contribute to immunotherapy resistance.
immune checkpoint blockade therapy targeting the negative regulatory receptors CTLA-4 and/or PD-1 has transformed cancer care, being FDA approved in at least 14 distinct cancer entities. However, despite these successes, many patients do not respond clinically, and some patients initially show clinical tumor regression yet subsequently progress with therapeutic resistance. The mechanisms of primary resistance are only beginning to be understood, and include tumor-cell-intrinsic oncogenic alterations, parallel immune suppressive pathways, adaptive resistance mechanisms, and the composition of the commensal microbiota that set the overall immune system tone. Secondary resistance arises under strong immune selective pressure and is also beginning to become characterized, with evidence for the emergence of tumor cells that lose antigen expression, or are deficient for expression of class I MHC or antigen processing machinery. Recent investigations uncovered deficiencies in IFNγR signaling in resistant tumor cell variants derived from patients who progressed after initially responding to anti-PD-1 therapy. This latter phenomenon is of particular interest, as the functional roles of IFN-γ in anti-tumor immunity are complex and include both positive and negative regulatory activities. IFN-γ induces upregulation of class I MHC and of antigen processing molecules, exerts anti-proliferative effects, and also promotes production of chemokines that have antiangiogenic properties in addition to promoting effector T cell recruitment. However, IFN-γ also induces upregulation of key negative regulatory molecules, including PD-L1 and indolamine-2,3-dioxygenase (IDO), and additionally can support T cell apoptosis during immune responses in vivo. It is thus conceivable that the functional consequences of loss of IFNγR signaling on tumor cells will depend on the net sum of complex activities of this cytokine in individual contexts, and whether positive versus negative immune regulatory effects are dominant in a given scenario.

Generation of IFN-γ-insensitive B16.SIY tumor cells. To be able to examine the role of tumor-intrinsic IFN-γ signaling in the antitumor immune response in vivo, we generated IFNγR2- and Jak1-mutant B16.SIY tumor cell lines by single-cell cloning using the sgRNAs identified from the CRISPR/Cas9 screen. To minimize founder effects that can arise from single-cell cloning, we began by generating monogenetic founder B16.SIY tumor cell clones and selected one that behaved similar to polyclonal B16. SIY tumors in vivo. Several criteria were imposed to select an optimal founder population; these included similar SIY expression based on DsRed intensity, comparable tumor growth kinetics to polyclonal B16.SIY tumors in vivo, and normal efficacy response to anti-PD-L1 blockade in vivo. As a control, the founder cell population was transduced with empty vector (EV-BFP). After transduction of sgRNAs we transfected cells with a Cas9-encoding vector and selected for Cas9-expressing cells with blasticidin. Single cells were sorted based on BFP expression and screened for loss of responsiveness to IFN-γ by failure to upregulate H-2Kb after IFN-γ stimulation in vitro.

We used two different sgRNAs for both IFNγR2 and Jak1 to control for off-target effects. One clone was chosen for each sgRNA and IFN-γ-insensitivity was tested (Fig. 1a). To confirm loss-of-function mutations in both alleles the genotype was determined by sequencing (Fig. 1b). We found a premature stop codon in all alleles, with the exception of Jak1 Mutant 1 (Jak1 Mut1) allele 2, which obtained an in-frame six base pair deletion (Fig. 1b). To validate our initial in vitro CRISPR/Cas9 screen we exposed IFNγR2- and Jak1-mutant tumor cells to activated 2C/ RAG2−/− T cells to evaluate lysis. Indeed, IFN-γ-insensitive tumor cells were relatively resistant to T cell-mediated killing in vitro, confirming our genome-wide screen results (Fig. 1c).
IFN-γ-insensitive tumors are spontaneously controlled in vivo.

In order to assess the behavior of IFNγR2- and Jak1-mutant tumor cells in vivo, we subcutaneously implanted mutant or WT tumor cells into C57BL/6 mice and tracked tumor growth. Unexpectedly, both IFNγR2- and Jak1-mutant B16.SIY tumors were spontaneously rejected by day 14. This was not due to increased immunogenicity from BFP expression as WT-BFP B16.SIY tumors grew progressively (Fig. 2a). To rule out the possibility that spontaneous tumor control was due to off-target effects from CRISPR/Cas9 mutagenesis, we retrovirally reintroduced IFNγR2 or Jak1 into IFNγR2- and Jak1-mutant cell lines, respectively, and confirmed successful IFN-γ signaling by upregulation of H-2Kβ after IFN-γ stimulation in vitro (Fig. 2b, c). When implanted into mice, tumors with restored IFN-γ signaling exhibited progressive growth kinetics comparable to WT tumors (Fig. 2d, e). The observed restoration of tumor growth kinetics indicates that no increase in immunogenicity due to on- or off-target mutations from CRISPR/Cas9 mutagenesis was responsible for the spontaneous control of IFN-γ-insensitive tumors.

To determine if increased tumor control was specific to the B16.SIY model system or if this phenomenon might be observed in other models, we assessed whether MC38 tumors similarly mutated exhibited delayed tumor growth. As with the generation of IFNγR2- and Jak1-mutant B16.SIY tumor cells, we generated MC38 IFNγR2- and Jak1-mutant tumors by establishing a founder cell line and a similar single-cell CRISPR/Cas9 mutagenesis method. We confirmed the loss of IFN-γ signaling in IFNγR2- and Jak1-mutant MC38 tumor cell clones (Supplementary Fig. 3a). Similar to the B16.SIY model, IFN-γ-insensitive MC38 tumors exhibited slower tumor growth in vivo (Supplementary Fig. 3b), which was reversed after the reintroduction of IFNγR2 (Supplementary Fig. 3c and d). B16.SIY and MC38 were selected because they are known to be immunogenic; therefore, we tested whether this phenotype is observed in a less antigenic model. To this end, we mutated IFNγR2 and Jak1 in B16F10 tumor cells using the same single-cell method and confirmed successful disruption of IFN-γ signaling (Supplementary Fig. 3e).

Because B16F10 tumors are highly aggressive, we utilized the standard number of 100,000 tumor cells to allow for more time for the immune system to mount a response. Consistent with our previous observations, IFNγR2- and Jak1-mutant B16F10 tumors also exhibited slower tumor growth, although the difference in growth rate was less pronounced presumably due to decreased antigenicity (Supplementary Fig. 3f). These data show that, in vivo assessment for loss of IFN-γ signaling. Tumor cell clones were stimulated with 10 ng/mL IFN-γ for 16 h and measured for H-2Kβ upregulation by flow cytometry.

Fig. 1 A genome-wide CRISPR/Cas9 screen identifies Ifngr2 and Jak1 as essential for T cell-mediated killing of B16.SIY cells in vitro. a In vitro analysis of resistance of IFNγR2- and Jak1-mutant B16.SIY tumor cells to T cell-mediated killing in vitro. Tumor cells were incubated with pre-primed 2C T cells for 24 h and remaining cells were measured by live/dead staining. n = 7 assays; data are pooled from three independent experiments. Results are expressed as mean ± s.e.m. Statistical significance was determined by a two-way ANOVA Bonferroni post-hoc test (c). p < 0.05.
adaptive immunity, we inoculated Rag2−/− mice with WT, IFNγR2-, or Jak1-mutant tumor cells and tracked tumor growth. No difference in tumor growth between IFNγR2-mutant and WT tumors was observed, although Jak1-mutant tumor growth was slightly delayed (Fig. 3a). Knowing that tumor control was dependent on the adaptive immune system we investigated which immune cell compartment was necessary for tumor control. B16 melanoma is known to express low levels of class I MHC at baseline, which is upregulated by IFN-γ. Therefore, it was conceivable that failure to upregulate MHC molecules by IFN-γ-insensitive tumors could lead...
to tumor control by NK cells. Alternatively, a low level of MHC-I may be sufficient to escape destruction by NK cells, and the spontaneous tumor control was dependent on other immune cell subsets. Therefore, we depleted CD8^+ T cells, NK cells or both with depleting antibodies in C57BL/6 mice 1 day before and 5 and 10 days after engrafting WT or IFNγR2-mutant tumor cells. Successful depletion was confirmed 3 days after antibody administration (Supplementary Fig. 4). While tumor control was maintained in NK-depleted mice (Supplementary Fig. 5a), CD8-depleted mice failed to control IFNγR2-mutant tumors (Fig. 3b) and no synergy was observed when both NK and CD8^+ T cells were depleted (Supplementary Fig. 5b). These data indicate that CD8^+ T cells were required to control IFNγ-insensitive tumors.

The antitumor CD8^+ T cell response is augmented. Because CD8^+ T cells were necessary for control of IFNγ-insensitive tumors in vivo, we characterized the endogenous antitumor CD8^+ T cell response. The frequency of SIY-reactive CD8^+ T cells was evaluated in the spleen by an IFNγ-ELISPOT assay. On day 7 after tumor inoculation, we observed a modest increase in the frequency of IFNγ-producing effector cells specific for the SIY antigen (Fig. 4a). We also measured the frequency of tumor-antigen-specific CD8^+ T cells within the tumor microenvironment by H-2Kb/SIY-pentamer staining. On day 7 after tumor challenge, a 3-fold increase in the frequency of H-2Kb/SIY^+ CD8^+ tumor infiltrating lymphocytes (TILs) was observed in IFNγR2- and Jak1-mutant tumors compared to WT tumors (Fig. 4b). To rule out the possibility that off-target mutations were responsible for the increase in H-2Kb/SIY^+ CD8^+ TIL we measured the frequency of antigen-specific CD8^+ TILs in IFNγR2- and Jak1-mutant tumors with reintroduced IFNγR2 or Jak1. Restoration of IFNγ signaling in tumor cells reverted the frequency of antigen-specific CD8^+ TILs in IFNγR2- and Jak1-mutant tumors with reintroduced IFNγR2 or Jak1. Restoration of IFNγ signaling in tumor cells reverted the frequency of antigen-specific CD8^+ TILs in IFNγR2- and Jak1-mutant tumors with reintroduced IFNγR2 or Jak1. Together, these data indicate that the absence of IFNγ signaling in tumor cells resulted in increased accumulation of tumor-antigen-specific CD8^+ T cells in the tumor microenvironment.

IFNγ drives a tumor-intrinsic immune modulatory program. Knowing that CD8^+ T cells were the mediator of tumor control we next sought to determine whether CD8^+ TILs exhibited improved effector functions in the context of IFNγR2- or Jak1-mutant tumors. However, CD8^+ TILs isolated on day 7 after tumor engraftment showed no difference in expression of Ifng, GzmB, Tnfa, Prf1, and Il2 by qRT-PCR in WT, IFNγR2-, and Jak1-mutant tumor contexts (Supplementary Fig. 6). These data suggest that the CD8^+ TIL compartment contains the necessary cytotoxic functions to eradicate IFNγR2- and Jak1-mutant tumors, indicating that an alteration on the tumor cell side may be responsible for the improved spontaneous tumor control observed. To investigate whether IFNγ-insensitive tumor cells showed decreased expression of a negative immune regulatory factor, RNASeq was performed on purified tumor cells from WT, IFNγR2-, and Jak1-mutant tumors on day 7 after tumor engraftment. Many of the differentially expressed genes found were shared between IFNγR2- and Jak1-mutant tumor cells (Fig. 5a and Supplementary Data 1). Overlapping downregulated genes included those involved in antigen presentation (H2-K1, H2-Aa, H2-T23, Tap1, Tap2, B2m), immune functions (CD274, Serpinb9, Icam2, Cxcl9, Cd5, Sema7a), extracellular matrix (Lgals3bp, Gip2, Igfb3), ubiquitination (Trim21, Dtx3l, Her6), and GTPase activity (Lig1p, Tgtp1, Gpb2, Ghp7) (Fig. 5b, c). However, a key negative regulatory molecule was also expressed at lower levels, CD274 (PD-L1). Indolamine-2,3-deoxygenase (IDO), another known IFNγ-induced negative immune regulatory gene27, was minimally expressed by tumor cells and not different between conditions. Since total tumor digests have been shown to upregulate IDO in previous work27, we isolated tumor cells and host APCs from tumors on day 7 and analyzed IDO expression by qRT-PCR. We found that tumor cells themselves expressed very little transcript for IDO whereas significant levels of IDO transcript were observed among the host APCs (Supplementary Fig. 7). These data point to a broad IFNγ-induced genetic program induced in WT but not IFNγR2- or Jak1-mutant tumor cells early in the antitumor immune response, with most of these genes being positive factors for antitumor immunity, but the key negative regulator PD-L1 is also induced in WT tumor cells yet lost in IFNγR expressing mutants.

Restored PD-L1 expression re-establishes tumor growth. We hypothesized that one possibility to explain the spontaneous tumor control of IFNγ-insensitive tumors was their failure to upregulate PD-L1, an important adaptive resistance mechanism. We first measured PD-L1 expression on the host and tumor compartments in WT, IFNγR2-, and Jak1-mutant tumors following implantation in vivo. We found a high level of PD-L1 expression on host APCs and on WT tumor cells; in contrast, IFNγ-insensitive tumor cells showed minimal PD-L1 expression (Fig. 6a–c). This was confirmed by at the transcript level by qRT-PCR analysis on sorted tumor cells (Fig. 6d). These results suggested that reduced PD-L1 expression might be responsible for improved tumor control. To test this hypothesis, we retrovirally restored expression of PD-L1 in IFNγR2- and Jak1-mutant tumor cells (Fig. 6e). Restored expression of PD-L1 in IFNγR2- and Jak1-mutant tumor cells re-established the progressive growth kinetics comparable to WT tumors (Fig. 6f). These results suggested that PD-L1 may be acting directly at the T cell/tumor cell interface to inhibit CD8^+ T cell cytotoxicity. If these were true, by removing antigen-dependent tumor recognition by CD8^+ T cells, IFNγ-insensitive tumors should no longer be spontaneously rejected. To test this, we deleted the H-2Kb gene from the IFNγR2-mutant tumor cells by CRISPR/Cas9 mutagenesis and tracked tumor growth. Indeed, IFNγR2-mutant tumors lacking H-2Kb grew progressively (Fig. 6g). These results indicate that IFNγR2- and Jak1-mutant tumors are better controlled immunologically through defective expression of PD-L1, in a manner dependent on direct tumor recognition by CD8^+ TILs.

IFNγR2-mutants are selected when mixed with WT tumor cells. Our data thus far implicate tumor-intrinsic IFNγ signaling as a critical factor to blunt the initial T cell insult and to establish the immunosuppressive microenvironment through the upregulation of immune negative regulatory molecules, particularly PD-L1. In addition, PD-L1 expressed on host cells may partly compensate for dampening the antitumor immune response when PD-L1 is not expressed on IFNγ-insensitive tumor cells. In recent reported cases of acquired immune resistance, tumors or patient-derived cell lines were found to contained loss-of-function mutations in components of the IFNγ-signaling pathway and tumor cells harboring these mutations increased in proportion after anti-PD-1 therapy15–17. Therefore, to investigate why IFNγ-insensitive tumors were spontaneously controlled instead of being more aggressive, we reasoned that our mouse system differed in two major ways. First, the human tumor scenario almost certainly involves intratumoral heterogeneity, in which a minor subset of IFNγ-signaling-mutant tumor cell clones was selected out among a population of IFNγ-signaling competent tumor cell clones. Second, the clinical scenario also occurred in the context of anti-PD-1 antibody therapy, which
would neutralize the negative effect of PD-L1 on the WT tumor cell clones. In theory, this would leave the antiproliferative and pro-immunogenic effects of IFN-γ to dominate, which would spare the IFN-γ signaling-mutants and allow their outgrowth. To test this hypothesis, we inoculated mice with a mixture of WT and IFNγR2-mutant tumor cells at a 1:1 ratio, and then treated the mice with anti-PD-L1 antibody. When tumors were measured over time, we observed slow but progressive tumor growth of the mixed tumor population, suggesting that the presence of WT tumor cells provided missing negative regulatory signals (Fig. 7a). In approximately half (15/28) of mice with WT:IFNγR2-mutant mixed tumors that received anti-PD-L1 therapy, the tumor escaped. When these tumors were harvested and re-analyzed by flow cytometry, selection for the IFNγR2-mutant tumor cells was observed in 12/15 of these cases (Fig. 7b). To rule out the possibility that selection of IFNγR2-mutant tumor cells was due to factors independent of the immune system, Rag2−/− mice were inoculated with either mixed WT:WT or WT:IFNγR2-mutant
tumor cells, and no differences between the tumor clones was observed (Fig. 7c). We reasoned that this immune selection for IFN-γR2 mutants would occur most robustly in the presence of a strong tumor-antigen-specific CD8+ T cell response, as the selection mechanism would involve direct tumor cell recognition of antigen. We therefore analyzed the SIY-specific T cell response using H-2Kb/SIY-pentamer staining among the TILs. In fact, we found that in the 12 mice where selection of IFNγR2-mutant tumor cells occurred, the frequency of H-2Kb/SIY+CD8+ TILs was greater compared to the three mice that grew out WT tumor cells (Fig. 7d, e). In fact, a greater frequency of H-2Kb/SIY+CD8+ TILs correlated with a greater fraction of IFNγR2-mutant tumor cells emerging (Fig. 7f). Together, these data suggest that in the context of a strong tumor antigen-specific CD8+ T cell response and PD-L1 blockade, IFN-γ-insensitive tumor cells have a selective growth advantage out of a mixture with WT tumor cells, allowing escape which equates to therapeutic resistance.

IFN-γ from CD8+ TILs drives selection for mutant tumor cells.

To address the mechanism behind the selection process of IFNγR2-mutant tumor cells, we hypothesized that IFN-γ-insensitive tumor cells could escape the direct antitumor effects of

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**Fig. 5** A complex genetic program is induced by IFN-γ signaling in tumor cells that includes PD-L1. 

a Volcano plot of differentially expressed genes (DEGs) from IFNγR2- and Jak1-mutant tumor cells compared to WT tumor cells. Tumor cells were sorted on day 7 after tumor engraftment. 

b The number of unique and shared DEGs between IFNγR2- and Jak1-mutant tumor cells. 

c Selected downregulated genes in IFNγR2- or Jak1-mutant tumor cells compared to WT tumor cells grouped by biological pathway. Numerical values in heat map are expressed as Z-scores.
IFN-γ, which would be preserved in tumor cells that have intact IFN-γ-signaling. Under this premise, we reasoned that the relative intratumoral quantity of IFN-γ would correlate with the selection of IFNγR2-mutant tumor cells. To test this, we inoculated mice with mixed WT:IFNγR2-mutant tumors and once tumors emerged they were excised. The tumor was split into two fractions, one fraction was mashed and assessed for IFN-γ content by ELISA within the interstitial fluid, and the other was used to measure the frequency IFNγR2-mutant and WT tumor cells.

We found that the concentration of intratumoral IFN-γ positively

**Fig. 6 Re-establishment of PD-L1 restores progress tumor growth of IFN-γ-insensitive tumors.** a–c Representative histograms of PD-L1 expression on tumor cells (a) or CD45+ MHCII+ APCs (b) from IFNγR2 Mut.1, Jak1 Mut.1, or WT tumors and summary of percent PD-L1+ tumor cells (c). Analysis was performed on day 7 after tumor engraftment. n = 15 mice; data are pooled from three independent experiments. d mRNA expression of PD-L1 from sorted tumor cells on day 7 after tumor engraftment. n = 8 mice; data are pooled from two independent experiments. e Representative histogram of PD-L1 expression after retroviral introduction of PD-L1 in IFNγR2- and Jak1-mutant tumor cells in vitro. Expression of PD-L1 was compared to WT tumor cells with or without IFN-γ stimulation. f Tumor outgrowth curves when PD-L1 was re-expressed in IFNγR2- and Jak1-mutant tumor cells. n = 10 mice (WT, IFNγR2 Mut1, Jak1 Mut1, and IFNγR2 Mut1 + PD-L1) and n = 5 mice (Jak1 Mut1 + PD-L1); data are pooled from two independent experiments (IFNγR2) or from one experiment (Jak1). g Tumor outgrowth curves when H-2Kb was deleted in IFNγR2-mutant tumor cells. n = 10 mice (WT and IFNγR2 Mut1), n = 15 mice (IFNγR2 Mut1 H-2Kb Mut), and n = 5 mice (H-2Kb Mut); data are pooled from two independent experiments. Results are expressed as mean ± s.e.m. Statistical significance was determined by a Kruskal-Wallis (non-parametric) test (b, c) and a two-way ANOVA Bonferroni post-hoc test (f, g). *p < 0.05, **p < 0.001.
**Fig. 7** When mixed with WT tumor cells, IFN-γ-insensitive tumor cells selectively grow out following to anti-PD-L1 therapy in vivo. **a** Tumor outgrowth of mixed WT and IFN•γ-R2-mutant tumors with and without anti-PD-L1 therapy. n = 24 mice (WT:WT), n = 14 mice (WT:WT + anti-PD-L1), n = 25 mice (IFN•γ-R2 Mut.1:WT), and n = 28 (WT:IFN•γ-R2 Mut.1 + anti-PD-L1); data are pooled from four independent experiments. **b** Log2(BFP/ZsGreen) fold-change after anti-PD-L1 therapy. **c** Fold-change of mixed tumors in Rag2−/− mice. n = 9 mice (WT:WT) and n = 10 mice (WT:IFN•γ-R2 Mut.1); data are pooled from two independent experiments. **d** Representative flow plots of tumor composition of mixed WT:IFN•γ-R2-mutant tumors in the context of a strong and weak immune response. **e** Tumor composition of mixed tumors from individual mice treated with anti-PD-L1. **f** Correlation between H-2Kb/SIY+ CD8+ TILs and selection for WT-BFP or IFN•γ-R2 Mut.1 BFP. For (b, e, f) n = 13 mice (WT:WT + anti-PD-L1) and n = 15 mice (WT:IFN•γ-R2 Mut.1 + anti-PD-L1); data are pooled from five independent experiments. Results are expressed as mean ± s.e.m. Statistical significance was determined by a two-way ANOVA Bonferroni post-hoc test (a) and a Kruskal-Wallis (non-parametric) test (b, c). Least squares regression was performed in (f), **p < 0.01, ***p < 0.001.
correlated with both selection of IFNγR2-mutant tumor cells and also the frequency of SIY/H-2Kβ CD8+ TILs (Fig. 8a, b). Consistent with our previous observation, the total number of H-2Kβ/SIY+ CD8+ TILs correlated with selection for IFNγR2-mutant tumor cells (Fig. 8c). Selection was mainly driven by IFNγ produced by CD8+ T cells as no selection was observed when CD8+ T cells were depleted (Fig. 8d, e). To further test the role of IFNγ in tumor cell selection, we reasoned that administration of additional IFNγ could push the system further towards IFNγR2-mutant tumor cells. To this end, we inoculated mice with WT:WT or WT:IFNγR2-mutant tumor cells and administered 50 μg of recombinant mouse IFNγ i.p. on days 7, 10, 13, and 16 and analyzed tumor cell composition on day 18 after tumor cell inoculation. We observed a 3.9-fold increase in the frequency of IFNγR2-mutant tumor cells after IFNγ administration compared to mice that did not receive IFNγ (Fig. 8f).

Taken together, these results suggest a scenario in which IFNγ-insensitive tumor cells gain a selective advantage by avoiding the direct antitumor effects of IFNγ. This process likely occurs after the initial T cell insult diminishes to a point where PD-L1 expression on tumor cells is no longer required and can be compensated by PD-L1 on the mixed WT tumor cells. To test whether PD-L1 on the admixed WT tumor cells was necessary for the selective outgrowth of IFNγR2-mutant tumor cells, the PD-L1 gene was disrupted using CRISPR. Indeed, under these conditions, progressive tumor growth was no longer observed, with no detectable outgrowth of IFNγR2-mutant tumor cells (Fig. 8g). These results indicate the involvement of clonal cooperation for immune selection of these mutants to occur, as WT tumor cells provide exogenous PD-L1 into the system. This impedes the immune response sufficiently to prevent total rejection of the mutant clones, allowing the differential effects of secreted IFNγ to select for IFNγR2-mutants.

Discussion
Our results utilizing an in vitro CRISPR screen revealed that tumor cell-intrinsic IFNγ-signaling is necessary for optimal T cell-mediated tumor cell killing in vitro. These results are consistent with other recently published CRISPR screens that similarly identified IFNγ-signaling-mutants using in vitro T cell selection systems23,24. However, resistance to T cell-mediated killing in vitro did not translate to aggressive tumor growth in vivo, as these IFNγ-signaling-mutant tumors showed...
improved immune-mediated tumor control (Supplementary Fig. 11). These discrepant phenomena may be explained by differing requirements for the levels of class I MHC upregulation required for tumor control in vitro versus in vivo, and also potentially distinct mechanisms of tumor cell destruction in the two settings. Short-term in vitro lysis assays are thought to be highly sensitive to the level of class I MHC expression by target cells, and cultured B16 melanoma cells show very low expression of H-2Kb by flow cytometry in vitro. It is clear that IFN-γ produced by the CD8+ effector T cells in vitro induces class I MHC upregulation that improves tumor cell lysis in a short-term assay. Following subcutaneous implantation into mice, tumor cells rapidly upregulate class I MHC, which is likely modulated by additional factors beyond IFN-γ. Mechanistically, in vitro lysis by CD8+ T cells is dependent on perforin/granzyme release, yet we have previously published that T-cell-mediated control of B16 melanoma in vivo is intact in perforin-deficient mice28. Immunologic control of tumors in vivo can occur through additional killing mechanisms, such as engagement of death receptors28–30, the antiproliferative effects of cytokines, both direct and through inhibition of angiogenesis31,32, and even indirectly through the effects of macrophages or other innate immune cells33,34. Our use of CRISPR/Cas9 to delete H-2Kb from tumor cells proved that, despite class I MHC expression being low on IFN-γ-signaling-mutant tumor cells, H-2Kb was nonetheless required for anti-PD-L1 efficacy and CD8+ T cell-mediated tumor control in vivo. Together, our results suggest that in vitro screens and in vivo confirmatory experiments offer complementary information that together can reveal the complexity of immune resistance within the tumor microenvironment.

Deeper analysis of the antitumor immune response to IFN-γ-insensitive tumors revealed an increased frequency of antigen-specific CD8+ T cells within the tumor microenvironment of IFN-γ-insensitive tumors, while T cell priming systemically was only minimally affected. Three possibilities could explain the increase in H-2Kb/SIY+ CD8+ TILs: first, it could be that T cell recruitment to the tumor site is increased; second, intratumoral proliferative expansion of SIY-reactive CD8+ TILs might be augmented; and third, CD8+ TIL cell death might be diminished. Because the augmented antitumor immune effect required H-2Kb expressed by tumor cells, it is likely that TIL proliferation and/or death are affected through this cognate interaction. IFN-γ has been shown to upregulate expression of FasL, which has been reported to contribute to TIL apoptosis in vivo21. In addition, IFN-γ upregulates expression of class II MHC, which in addition to presenting peptides to CD4+ T cells also is a ligand for the inhibitory receptor LAG-335,36 expressed by tumor antigen-specific CD8+ TILs37. Some of these considerations also may help to explain previous reports in which tumor cells expressing a dominant negative IFNγR1 exhibited increased tumor growth, as this dominate negative receptor can still bind IFN-γ and perhaps sequester it from other cells within the tumor. Future work will be required to continue to dissect the additional complexities of IFN-γ functions among the array of cells present within the tumor microenvironment.

The functional contribution of PD-L1 on tumor cells versus host APCs in engaging PD-1 on TILs and inhibiting T cell function has been controversial but likely varies with the tumor model or cancer histology being studied, and also the degree of antigenicity. In human cancer patients, T cell-infiltrated tumors can show PD-L1 upregulation on either tumor cells or myeloid cells, and each can be associated with anti-PD-1 therapeutic efficacy in defined contexts38. In mouse models, PD-L1+/− hosts have been reported to show improved antitumor immunity in some model systems39,40 but in other models CRISPR/Cas9-mediated disruption of the PD-L1 gene in tumor cells can be sufficient for improved tumor control41. In our current work, we utilize the B16.SIY and MC38 models, which have high antigenicity and prime strong CD8+ T cell responses, even though the tumors eventually grow progressively. It now seems clear that a major mechanism for ultimate tumor growth in these models is through PD-L1 upregulation on tumor cells via persistent IFN-γ production in the tumor microenvironment. Our data highlight that tumor cells can afford to lose PD-L1 expression through disruption of the IFN-γ signaling pathway when PD-L1 expression on WT tumor cells can compensate for this loss.

The spontaneous in vivo control of IFN-γ-signaling-mutants in our current work seems initially to be at odds with other published reports showing a loss of IFN-γ signaling on tumor cells that grew out in association with acquired resistance to anti-PD-1 therapy15,23–25. While our final experiments studying selection following immunotherapy were performed with anti-PD-L1 and the available clinical data were obtained in the context of anti-PD-1 Ab therapy, both Abs are thought to function by a similar mechanism. We used single-cell cloning to establish our IFN-γ-insensitive tumor cell populations, therefore when engrafted into mice the starting tumor population lacked IFN-γ signaling on all tumor cells. In other experimental models, due to incomplete CRISPR/Cas9 mutagenesis, it is possible that a fraction of the starting tumor population retained IFN-γ signaling and was in fact a cellular mixture. Therefore, our in vivo results from the in vitro CRISPR screen might have been masked had we not taken the reductionist approach of deriving genetically homogenous tumor cell populations by single-cell cloning. One caveat of this method is that single-cell clones can remain subject to genetic drift as they expand in culture. However, reintroducing IFNγR2 in two tumor models restored progressive tumor growth indicating that the lack of IFN-γ signaling was the causative factor resulting in spontaneous tumor control.

The difference in growth rate between the B16F10 and B16.SIY models is likely a result of the differing immunogenicity between these tumors. Strong antigens that arise as a result of mutations can lead to T cell killing of immunogenic tumor cell populations42. Furthermore, the variable immunogenicity among clonal tumor cell populations is likely a determining factor in immunoselection, while the net immunogenicity of all tumor cells may dictate the relative contribution that immune-inhibitory pathways play in this process. Our collective data are consistent with the notion that strong immune selection for resistant mutants is dependent upon tumor cell antigenicity.

Clinically, tumors that grow out under immune selective pressure likely start out as a heterogeneous mixture, in which sensitive tumor cells are eliminated and resistant tumor cells grow progressively. To better align our mouse model with this clinical scenario, we found that when WT tumor cells were mixed with IFNγR2-mutant tumor cells and implanted in vivo, the IFNγR2-mutant cells were indeed selected out under strong immune pressure upon anti-PD-L1 therapy. Since PD-L1 is the major negative immune regulatory pathway upregulated by IFN-γ on tumor cells, once the PD-L1/-PD-1 interaction is masked, then it appears that the antitumor effects of IFN-γ dominate, selecting for IFN-γ-signaling-mutant tumor cells. Intratumor heterogeneity has several implications for the immunobiology of the tumor microenvironment. Perhaps most importantly, heterogeneity in the expression of mutant antigens (trunk versus branch mutations) can influence the degree to which a dominant T cell population may eliminate cancer cells following immunotherapy. Treatment with anti-PD-1 has been shown to prune the tumor cell population in clinical responders, as specific mutational epitopes become eliminated as assessed by on-treatment biopsies. Increased intratumor heterogeneity has been associated with inferior clinical activity of anti-PD-1 therapy in patients43,44. Our current work...
suggests that intratumor heterogeneity in other molecular pathways also contributes to secondary resistance, beyond tumor antigen expression. In addition, our results suggest that two different tumor cell clones can cooperate to allow selective outgrowth of one clone under immune selective pressure.

**Methods**

**Mouse cancer cell lines.** All cell lines were routinely tested for mycoplasma contamination using the HEKBlue (InvivoGen) reporter cell line, following the manufacturer’s protocol. B16F10 (ATCC) cells were engineered to express DsRed fused in-frame with the model antigen SIYRYG124,25. The MC38 cell line was a gift from Dr. Yang-Xin Fu (UT Southwestern). DsRed expression was routinely monitored as it can shift over time, and flow cytometric sorting was performed periodically to ensure uniform expression.

To establish a founder B16.SIY.DsRed tumor cell line, single-cell B16.SIY.DsRed clones were tested for their relative intensity of DsRed expression compared to the B16.SIY.DsRed polyclonal population. Two B16.SIY.DsRed founder lines with comparable DsRed expression to the polyclonal B16.SIY.DsRed tumor cell population were tested for normal tumor growth and responsiveness to anti-CD3 + anti-CTLA-4 blockade. B16.SIY.DsRed clone #9 was chosen for subsequent experiments.

**Lentiviral packaging and transduction.** Individual sgRNA expression vectors were constructed as follows. Forward and reverse 26 nt oligonucleotides (Supplementary Table 2) were mixed at 10 mM each in 10 mM Tris-HCl (pH8.0) and 5 mM MgCl2 in a total volume of 100 μL. The mixture was incubated at 95 °C for 5 min and cooled to room temperature slowly to form duplex oligonucleotides. The duplexes were then cloned into the BamHI and KpnI sites of the packaging plasmid pCAGGS-PGKpuro2ABFP (Addgene, #50946). sgRNA sequences targeting IFNγR2 and Jak1 were chosen from a genome-wide CRISPR library described in Koike-Yusa et al.26. To generate the gRNA sequences for H-2Kb, the online ATUM-guide RNA design tool was used (www.atum.bio/eCommerce/cas9/input).

For Cas9 expression either a lentiviral vector encoding Cas9 and GFP (Sigma: pLV-U6-EPCG) or a vector encoding Cas9, a piggyBac transposon element, and blasticidin resistance gene (Bsr) was used (Cas9-Bsr). Vector transfection was performed using Lipofectamine 3000 reagent (Thermo Fisher Scientific) according to the manufacturer’s protocol. For lentivirus packaging, Two hundred and ninety-three 0.5 mL vectors were transduced with the genome-wide pooled gRNA library in 24-well plates. The transduction mixture was added to tumor cells with polybrene at 10 μg/mL Ampicillin) were sequenced using the primer 5' gagggcctatttcccatgatt-3' (GFP) and 5' taccgggcc-3' (Bsr) in a 1% agarose gel. The vectors were then cloned into the BamHI and KpnI sites of the packaging plasmid pCAGGS-PGKpuro2ABFP (Addgene, #50946). gRNA sequences targeting IFNγR2 and Jak1 were chosen from a genome-wide CRISPR library described in Koike-Yusa et al.26. To generate the gRNA sequences for H-2Kb, the online ATUM-guide RNA design tool was used (www.atum.bio/eCommerce/cas9/input).

**Genome-wide gRNA lentiviral library (Addgene, #50947) was packaged as described above in 293 T cells. Viruses containing media was collected 48 h post-transfection. A 1 x 10^5 B16.SIY cells were seeded in each well of a 24-well plate one day before transduction. Five hundred microliter virus mix was added to B16.SIY cells. Transduction efficiency of B16.SIY cells was analyzed by flow cytometry based on GFP expression.**

**Generation of targeted deletion mutants.** To generate loss-of-function mutations in the IFNγR2 and Jak1 genes, the B16.SIY.DsRed founder line was transduced with lentivirus encoding the targeting gRNA and a BFP reporter. Cells were allowed to recover for 3 days and then transduced with the Cas9-Bsr plasmid. Cells were selected with blasticidin for 2 days and then single-cell sorted based on BFP expression. To confirm successful disruption of Ifngr2 and Jak1 the targeted region was amplified by PCR, TA-cloned (Invitrogen, TOPO-TA), and transformed into E. coli. Competent E. coli cells were transformed using M13R primer on 10–15 bacterial colonies to obtain sequences for both alleles. The primers used for amplification of the targeted region in Ifngr2 and Jak1 are shown in Supplementary Table 3.

To generate IFNγR2/H-2Kb double mutant cells, #9 B16.SIY.DsRed cell line was transduced with lentivirus encoding the targeting gRNA and a BFP reporter. Cells were allowed to recover for 3 days and then transduced with the Cas9-Bsr plasmid. Cells were selected with blasticidin for 2 days and then single-cell sorted based on BFP expression. Cells were then cloned for the inability to upregulate H-2Kb in response to IFN-γ (5 ng/mL) stimulation. One clone was chosen, expanded and transduced with Cas9-GFP and pKLV-U6-sgKGNfi(gr2)-PGKpuro2ABFP vectors. Cells were sorted and tested for the inability to upregulate PD-L1 in response to IFN-γ stimulation. The #9 B16.SIY IFNγR2 H-2Kb double mutant cell line was then transduced with the empty vector pKLV-U6-PGKpuro2ABFP lentivirus to equalize BFP expression to the IFNγR2 Mut.1 tumor cells.

For experiments using MC38 and B16F10 tumor cells, Cas9-GFP (Sigma: pLV-U6-EPCG) was retrovirally introduced and cell sorted 2-3 times for GFP expression. To generate loss-of-function mutations in the IFNγR2 and Jak1 genes, cells were transduced with the corresponding pKLV-U6-sgRNA-PGKpuro2ABFP vectors and single-cell sorted based on GFP and BFP expression and tested for disruption of the targeted gene by IFN-γ (5 ng/mL) stimulation.

**Reintroduction of IFNγR2 mutant cell line.** The codon-optimized IFNγR2 gBlock (IDT) with mutated gRNA sites and flanking BamHI and NotI cut sites was digested and inserted into the pRETO-IRE-S-ZsgGreen1 (Clontech) expression vector. For Jak1, due to the large size of the Jak1 gene, three codon-optimized gBlock fragments were synthesized and assembled into the pRETO-IRE-S-ZsgGreen vector by BioGen assembly. Sequencing was performed in Supplementary Fig. 10. Reintroduction of Jak1 into the Jak1 Mut.1 tumor cell line did not restore IFN-γ signaling, possibly due to a dominant negative effect from the endogenous mutated Jak1 protein. However, reintroduction of Jak1 into the Jak1 Mut.2 cell line did not restore IFN-γ signaling and was used for subsequent experiments.

**Mice and tumor inoculation.** C57BL/6Ntac mice were purchased from Taconic farms. Rag2-/- (NCl) mice were bred in house under specific pathogen-free conditions. 2 C Rag2-/- mice were bred in our facility. For tumor inoculation, unless otherwise stated, 2 × 10^6 tumor cells were subcutaneously injected into the right flank of female mice. Tumor volume was measured around twice per week using calipers. Tumor volume was calculated: \( V = \frac{1}{2} \times L \times W^2 \), where \( T \) is the tumor length, \( L \) is the tumor width, and \( W \) is the tumor width. All studies were approved according to the National Institute of Health Animal Care guidelines and utilized under IACUC-approved protocols. All experiments were approved by the Institutional Animal Care and Use Committee at the University of Chicago and followed all international guidelines.

For IFNγR2 Mut.1 and WT mixing experiments, 1 × 10^6 of each tumor cell line was used for a total of 2 × 10^6 tumor cells. The ratio of tumor cells was analyzed by flow cytometry before tumor inoculation to ensure a near 50:50 percent ratio. Mixed tumor cells were inoculated into Rag2-/- mice to control for differences in tumor growth rate and viability. The BFP/ZsGreen ratio from Rag2-/- mice was used to normalize the ratio from experimental groups.

**Antibody administration.** For antibody treatments, mice received 100 μg of anti-CD11 (10F.G2: BioXcell) and/or 100 μg anti-CTLA-4 (BE1031: BioXcell) intraperitoneally on days 7, 10, and 13 after tumor engraftment. For depletion
studies, mice received 200 μg of either anti-CD8 (TYS 169.4: BioXCell) and/or anti-NK1.1 (PK136: BioXCell) or a combination of anti-CD8+ anti-NK1.1 2 days before tumor engraftment and once every 7 days after. Depletion of the relevant cell populations was confirmed by flow cytometry analysis of blood 3 days after antibody administration and in the tumor at endpoint.

TIL and tumor cell isolation. Tumors were harvested from mice at the indicated time points. Tumors were digested using an enzyme mixture containing Collagenase (1 mg/mL Sigma: C5138), DNase (200 μg/mL Sigma: D5025), and Hyaluronidase (100 μg/mL Sigma: H6254) for 20–30 min at 37°C while rotating. The tumor suspension was filtered through a 50 μm filter and washed with PBS and purified by Ficoll-Hypaque density gradient centrifugation. For TIL sorting, cells were washed twice with PBS before staining and maintained in complete DMEM (cDMEM) (DMEM: 10% PBS, 100 U/mL Penicillin-Streptomycin, 1% MEM Non-Essential Amino Acids, 50 μM β-mercaptoethanol, 0.01 M MOPS) and sorted into RLT buffer using an Aria II flow cytometric cell sorter (BD). Sorted cells were rapidly frozen on dry ice. Gating strategy for cell sorting experiments are represented in Supplementary Fig. 8.

Flow cytometry and antibodies. For samples not sorted, cells were washed with PBS and stained in FACS buffer (2% PBS, 2 mL EDTA, and 0.001% NaN₃). All gating strategies are represented in Supplementary Fig. 9. Cells were first stained for H-2Kd/SIY-pentamer (PE; ProImmune) for 10 min at room temperature at a 1:20 dilution, followed by staining with remaining antibodies for 20 min on ice. Antibodies against the following molecules were used: CD3 (clone 17A2, BioLegend, 100216), Thy1.2 (clone 30-H12, BioLegend, 105320), CD45.2 (clone 104, BioLegend, 109806), CD8α (clone 53-6.7, BioLegend, 107747), CD4 (clone RM-4, BioLegend, 100547), PD-L1 (clone 10 F.9.G2, BioLegend, 124312), CD19 (clone 6D5, BioLegend, 115545), A-I-E-1 (clone MS114.15.2, BioLegend, 107630), and H-2Kd (clone A6F6-88.5, BioLegend, 742862). All antibodies were used at a 1:100 dilution. Fixable Viability Dye eFluor 506 or 780 (ebioscience) was used for live/dead discrimination and was used at a 1:200 dilution. All flow cytometric analysis was conducted on either an LSRFortessa or X20 (BD) and analyzed using FlowJo software (Tree Star).

Quantitative real-time PCR and RNA sequencing. Total RNA was extracted from sorted cell populations using the RNeasy Micro Kit (QIAGEN) following manufacturers protocol. For qRT-PCR, cDNA was synthesized using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) according to manufacturer’s protocol. Transcript levels were measured using primer-probe sets (Supplementary Table 2) developed through the online ProbeFinder Software (Roche). RNA sequencing was performed by the University of Chicago Genomics Core facility using the Illumina HiSeq platform. Alignment was performed using the Kallisto software. All submitted samples passed quality control using the R package FastQC. Raw read counts were processed by TMM normalization followed by log transformation. Genes expressed in fewer than three samples were removed from further analysis. Limma voom was used to identify differentially expressed genes between each mutant cohort and the WT cohort. Genes expressed in fewer than three samples were removed from further analysis. Limma voom was used to identify differentially expressed genes between each mutant cohort and the WT cohort.

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**Author contributions**

S.L., H.H. and T.F.G. conceived of the project with assistance from J.B.W.; J.B.W., S.L., H.H. and T.F.G. designed the project and wrote the manuscript with assistance from A.C. and E.F.H.; J.B.W., S.L. and T.F.G designed experiments; J.B.W. and S.L. performed experiments and acquired data with assistance from A.C. and E.F.H.; E.F.H. performed the RNASeq analysis and visualization. X.W. provided the Cas9 plasmid and helped design the initial CRISPR screen. T.F.G. and H.H. contributed equally.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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