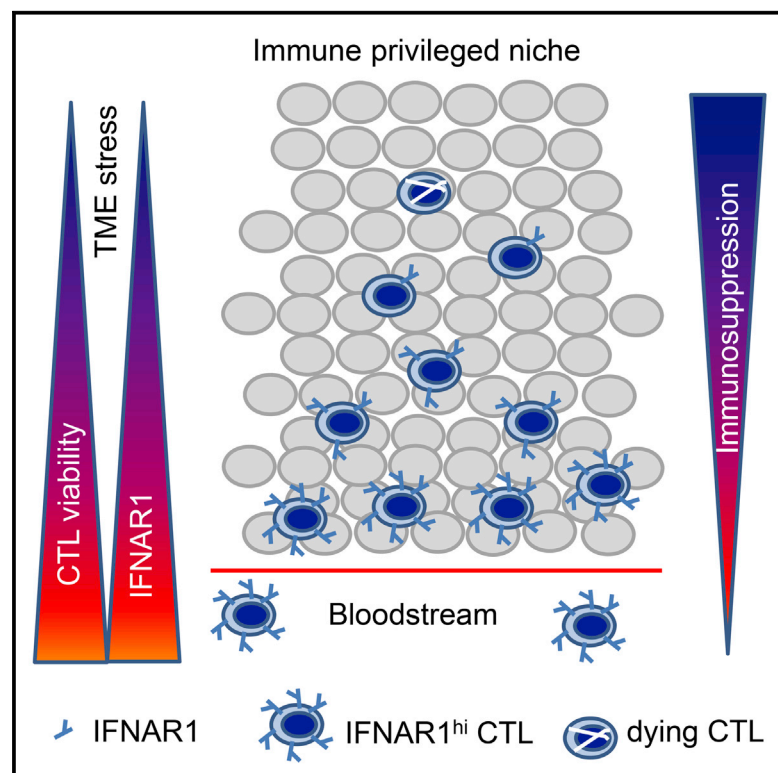


Inactivation of Interferon Receptor Promotes the Establishment of Immune Privileged Tumor Microenvironment

Graphical Abstract



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In Brief

Katlinski et al. show reduced type I interferon receptor chain IFNAR1 in colorectal cancer (CRC) stroma, which is important in forming the immune-privileged niche to support CRC development and growth. Stabilization of IFNAR1 improves cytotoxic T lymphocyte survival and suppresses tumor growth.

Highlights

- Colorectal tumors downregulate interferon receptor IFNAR1
- Loss of IFNAR1 promotes generation of immune-privileged niche
- IFNAR1 regulates viability of cytotoxic lymphocytes and efficacy of immunotherapies
- Pharmacologic stabilization of IFNAR1 suppresses tumor growth



Inactivation of Interferon Receptor Promotes the Establishment of Immune Privileged Tumor Microenvironment

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SUMMARY

Refractoriness of solid tumors, including colorectal cancers (CRCs), to immunotherapies is attributed to the immunosuppressive tumor microenvironment that protects malignant cells from cytotoxic T lymphocytes (CTLs). We found that downregulation of the type I interferon receptor chain IFNAR1 occurs in human CRC and mouse models of CRC. Downregulation of IFNAR1 in tumor stroma stimulated CRC development and growth, played a key role in formation of the immune-privileged niche, and predicted poor prognosis in human CRC patients. Genetic stabilization of IFNAR1 improved CTL survival and increased the efficacy of the chimeric antigen receptor T cell transfer and PD-1 inhibition. Likewise, pharmacologic stabilization of IFNAR1 suppressed tumor growth providing the rationale for upregulating IFNAR1 to improve anti-cancer therapies.

INTRODUCTION

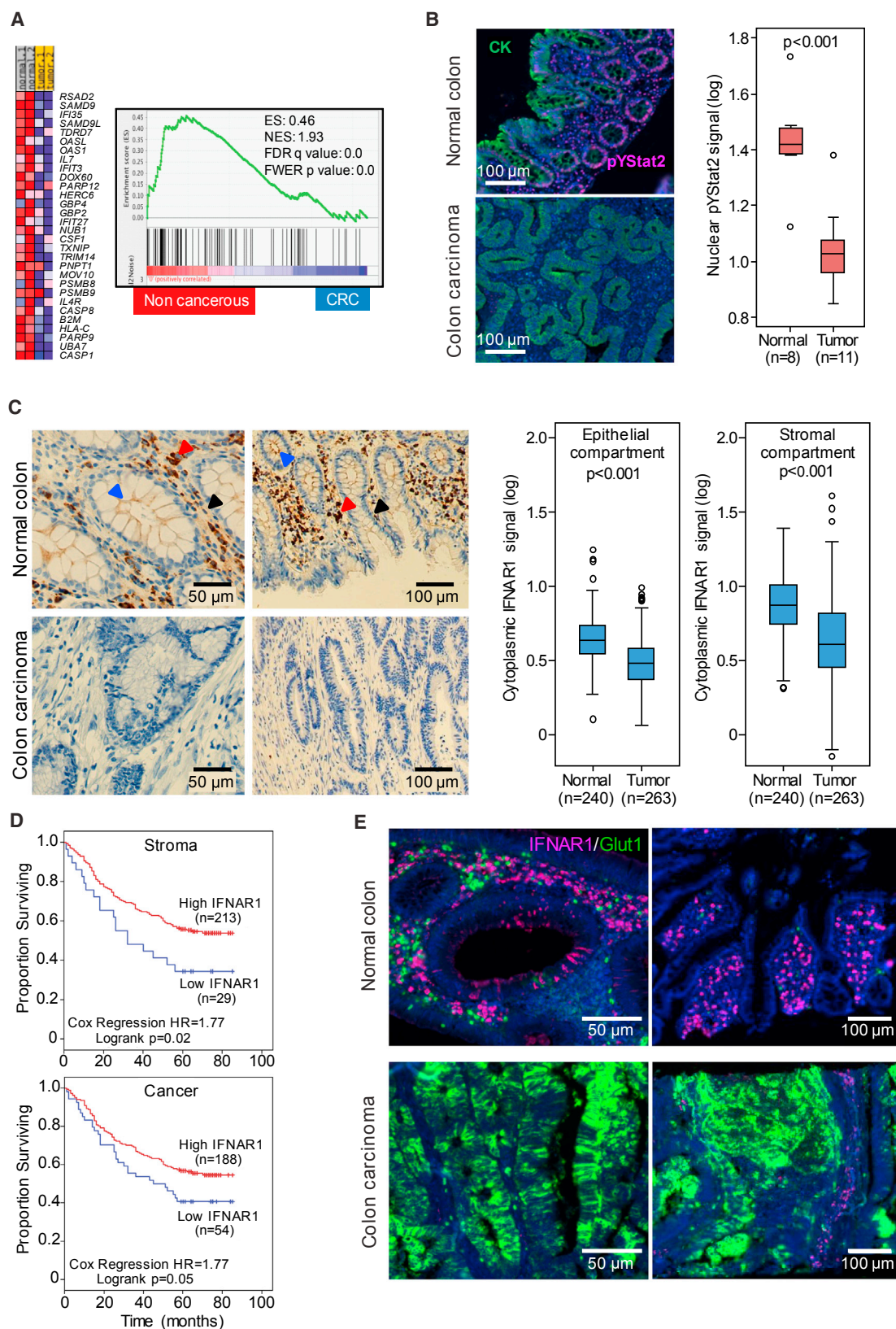
The presence of tumor-specific T cells in the blood of cancer patients, the “Hellström paradox” (Hellström et al., 1968), suggests that, although developing cancers are able to induce a comprehensive immune response, tumors can progress by hindering anti-tumor effector cells (Klemm and Joyce, 2015; Quail and Joyce, 2013). However, recent success in cancer immunotherapy indicates that augmentation of the immune response can improve prognosis. As such, current approaches

to cancer immunotherapy focus on increasing either tumor antigen presentation (via vaccines) or the number of tumor-specific CD8⁺ cytotoxic T lymphocytes (CTLs) (via chimeric antigen receptors [CARs] therapy and other types of adoptive transfer) or enhancing CTL activities (via checkpoint inhibition; reviewed in Rosenberg and Restifo, 2015; Sharma and Allison, 2015).

Regrettably, the majority of patients with solid tumors, including colorectal cancers (CRCs), are refractory to these treatments (Brahmer et al., 2012; Gilham et al., 2012; Topalian

Significance

Understanding the mechanisms by which solid tumors suppress anti-tumor immunity is critical for success of immune therapies. Here we demonstrate that tumor microenvironment factors-induced downregulation of type I interferon receptor IFNAR1 is a central mechanism underlying the ability of the tumor microenvironment to undermine the viability of cytotoxic T cells and to generate intra-tumoral immune-privileged niches devoid of these cells. Means to prevent the loss of IFNAR1 eliminate these niches, inhibit tumor growth, and increase the efficacy of immunotherapies utilizing checkpoint inhibitors or chimeric antigen receptor T cells. These findings delineate a mechanism of localized intra-tumoral immune suppression and prompt the development of IFNAR1-stabilizing agents for use in anti-cancer immune therapies.



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et al., 2012). Solid tumors evade anti-cancer immune control by establishing immune-privileged niches within the tumor micro-environment (TME). Diverse cellular and acellular (e.g., deficit of oxygen and nutrients) TME elements reduce proliferation, viability, or activity of intra-tumoral CTLs thereby inhibiting their anti-tumor effector function (Fearon, 2014; Joyce and Fearon, 2015; Zhou et al., 2014). Indeed, the apparent exclusion of CTLs from CRC is associated with a poor prognosis (Chiba et al., 2004; Galon et al., 2006; Naito et al., 1998) while, conversely, increased accumulation of CTLs within tumors is associated with a favorable outcome (Talmadge et al., 2007). Delineating the mechanisms that prevent CTL accumulation within the TME remains a major challenge in understanding the immunosuppressive properties of the TME and increase the efficacy of immunotherapies (Joyce and Fearon, 2015).

Studies modeling sarcomas and melanomas in mice lacking the IFNAR1 chain of type I interferon (IFN) receptor suggest that endogenous IFNs contribute to anti-tumor immunity via stimulating specific CD8 α^+ lineage dendritic cells (DCs) to cross-present antigen to CTLs (Diamond et al., 2011; Fuertes et al., 2011; Hildner et al., 2008). IFNs also provide a “third signal” to stimulate the clonal expansion of CD8 $^+$ T cells (Aichele et al., 2006; Curtsinger et al., 2005; Hervás-Stubbs et al., 2010) and increase the viability of activated anti-viral CD8 $^+$ T lymphocytes (Crouse et al., 2014; Kolumam et al., 2005; Wang et al., 2012; Xu et al., 2014) and tumor-specific CTLs (Hiroishi et al., 2000). These reports are consistent with the long-known anti-tumorigenic effects of IFN (Platanias, 2005; Trinchieri, 2010; Zitvogel et al., 2015). Nevertheless, given that tumorigenesis readily proceeds in IFNAR1-competent mice and humans, it is apparent that cancers manage to overcome the effects of endogenous IFN through a poorly understood mechanism.

Cell surface IFNAR1 levels are critical for all IFN effects (Fuchs, 2013; Uze et al., 2007). These levels are controlled by IFNAR1 ubiquitination and degradation facilitated by the SCF- β Trcp E3 ligase, which binds to IFNAR1 phosphorylated on Ser535 (Ser526) in mouse IFNAR1; (Kumar et al., 2003). Phosphorylation of these serine residues can be triggered in vitro by stimuli characteristic for the TME such as unfolded protein response (Liu et al., 2009), oxygen or nutrient deficit (Bhattacharya et al., 2013), vascular endothelial growth factor (Zheng et al., 2011), and inflammatory cytokines (Huangfu et al., 2012). Here we aimed to characterize the status of IFNAR1 and IFN signaling in CRC tumors and to determine the importance of IFNAR1 downregulation in establishing the intra-tumoral immune-privileged niche.

RESULTS

IFNAR1 Levels and Signaling Are Reduced in the TME

Global expression profiling within hypoxic areas of transplanted tumors revealed a decrease in expression of the immune response genes (Marotta et al., 2011). We also noted a suppression of the IFN-signaling signature in hypoxic tumor areas characterized by TME stress (Figures S1A and S1B). Importantly, mining of datasets from patients with CRC (Rohr et al., 2013) revealed a decrease in IFN-induced gene expression in tumors compared with benign colorectal tissues from the same patients (Figure 1A). In addition, compared with normal colorectal tissues, tumors exhibited markedly decreased levels of nuclear phosphorylated STAT2 (Figure 1B), which is a downstream effector of IFN signaling (Platanias, 2005). These results suggest that IFN signaling is inhibited in human CRC tumors.

TME-associated stress stimuli such as nutrient/oxygen deficit can cause a loss of IFNAR1 protein in vitro (Bhattacharya et al., 2013). Although comparable *IFNAR1* mRNA expression was reported in CRC and normal colorectal tissues (Rohr et al., 2013), we noted dramatic differences in IFNAR1 protein levels. IFNAR1 was detected in all normal human colon cell types including epithelial cells (especially at their apical surface), stromal fibroblasts, and infiltrating immune cells. However, all cell types within human colorectal adenocarcinomas exhibited partial or complete loss of IFNAR1 (Figures 1C and S1C). For these samples, IFNAR1 levels in the cancer cell compartment and in the stromal compartment positively correlated ($r = 0.700$, $p < 0.001$; $n = 263$). Importantly, downregulation of IFNAR1 in either stromal or cancer cell compartments of human CRC tumors were associated with poor prognosis (Figure 1D). Furthermore, whereas many cells expressed high levels of IFNAR1 in normal human colon, those few IFNAR1-positive cells found in colon carcinomas were spatially segregated from the tumor areas, which were positive for GLUT1, a marker of TME stress (Figures 1E and S1D). These data collectively suggest that TME conditions in human CRC prompt IFNAR1 downregulation and suppress IFN signaling.

Downregulation of IFNAR1 in the Stromal Compartment Stimulates Colorectal Tumorigenesis

Guided by these data in human patients, we sought to determine the role of partial loss of IFNAR1 using murine CRC models. Notably, downregulation of IFNAR1 protein observed in human CRC (Figure 1C) was faithfully recapitulated in the mouse model of inflammatory colorectal carcinogenesis induced by treatment with azoxymethane and dextran sodium sulfate (AOM-DSS). The

Figure 1. IFNAR1 Levels and Signaling Are Reduced in Colorectal Adenocarcinomas

(A) Heatmap and gene set enrichment analysis (GSEA) of IFN signaling pathway genes of the transcriptome profiles of human normal colon and matched CRC tissues (from Rohr et al., 2013). ES, enrichment score; NES, non-enrichment score; FDR, false discovery rate; FWER, family-wise error rate.

(B) Representative immunofluorescent analysis of phospho-Tyr-STAT2 (red) and pan-cytokeratin (CK, green) in normal and malignant colon tissues counterstained with DAPI (blue). Boxplot showing nuclear pTyr-Stat2 levels in representative normal ($n = 8$) and cancer ($n = 11$) cases indicates median (dark line), 25%–75% range (box), minimum and maximum values (whiskers), and individual scatterplot values (circles) overlaying the boxplot.

(C) Representative chromogen immunohistochemistry analysis of IFNAR1 in normal and malignant colon. Arrows point to IFNAR1-positive fibroblasts (black), epithelial cells (blue), and immune cells (red). Boxplots as in (B) show cytoplasmic IFNAR1 expression levels in the epithelial (left) or stromal (right) compartments of malignant colon and adjacent normal tissue.

(D) Kaplan-Meier plot of cases of colorectal adenocarcinomas based on levels of cytoplasmic IFNAR1.

(E) IFNAR1 (red)- and GLUT1 (green)-positive cells in normal and malignant human colon tissues. See also Figure S1.

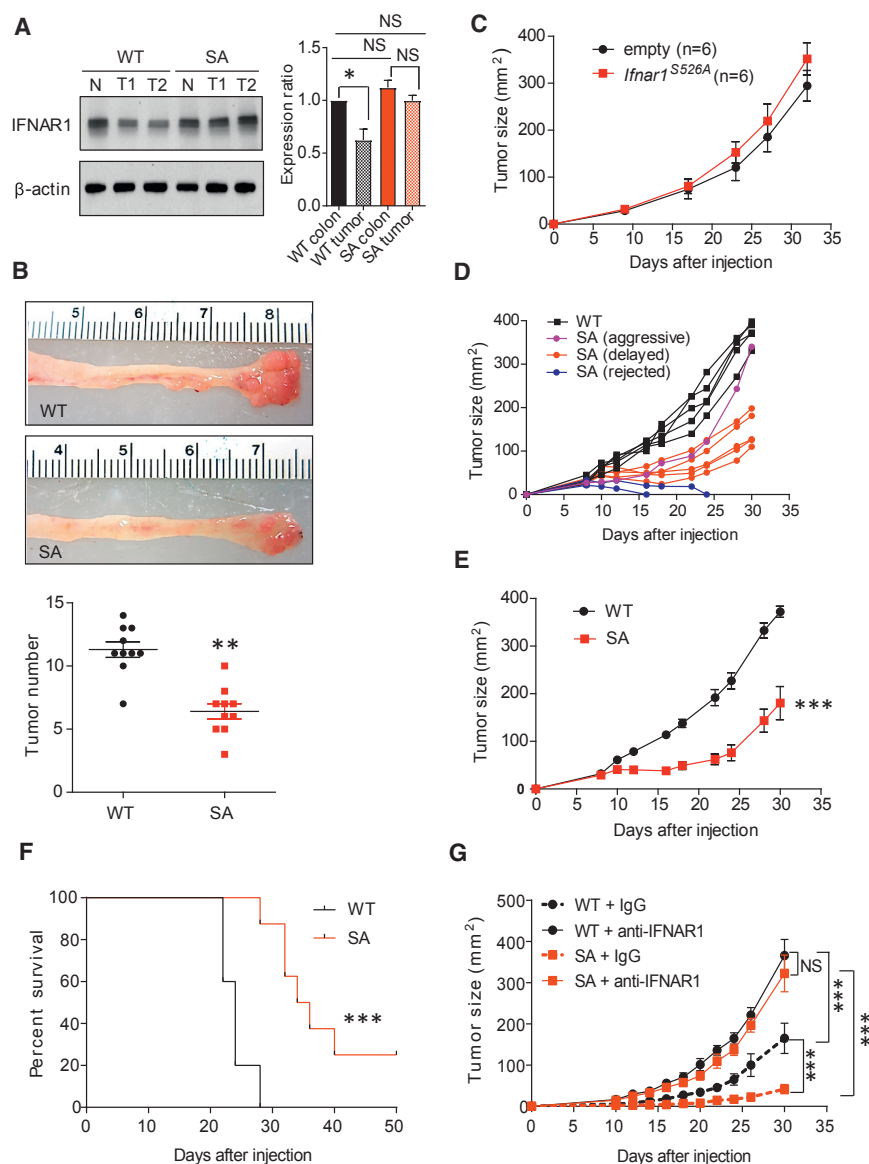


Figure 2. Downregulation of IFNAR1 in the Stromal Compartment Stimulates Colorectal Tumorigenesis

(A) Immunoblot analysis of IFNAR1 immunoprecipitated from the whole-tissue lysates prepared from normal colon or AOM-DSS-induced tumors from WT and SA mice. The IFNAR1/β-actin (loading control) signal relative ratios calculated from six mice for each group (WT colon taken as 1.0 and shown as mean ± SD) are depicted on the right. Henceforth asterisks: *p < 0.05; **p < 0.01; ***p < 0.001. NS, not significant.

(B) Representative images and quantification of colorectal tumors in mice of indicated genotypes at day 70 after treatment with AOM-DSS.

(C) Growth of MC38mRFP cells that received GFP or IFNAR1^{S526A}-GFP constructs after subcutaneous injection into WT mice (mean ± SEM, n = 6).

(D) Subcutaneous growth of individual MC38 tumors in WT and SA mice.

(E) A representative experiment demonstrating the average size of MC38 tumors growing in WT (n = 5) and SA (n = 8) mice (mean ± SEM).

(F) Kaplan-Meier analysis of the survival of MC38 tumor-bearing WT and SA mice from (E).

(G) Effect of anti-IFNAR1 neutralizing antibodies on MC38 tumor growth in WT and SA mice (mean ± SEM, n = 5–6 for each of two experiments).

See also Figure S2.

observed decrease in levels of IFNAR1 protein (Figures 2A and S2A) but not *Ifnar1* mRNA (Figure S2B) in AOM-DSS-induced colorectal tumors suggested an increased IFNAR1 degradation within tumors. Therefore, we next used *Ifnar1*^{S526A} mice (henceforth “SA”), previously shown to be deficient in IFNAR1 ubiquitination and degradation (Bhattacharya et al., 2014). SA mice treated with AOM-DSS sustained high levels of IFNAR1 protein (Figures 2A and S2A) and mRNA for IFN-stimulated and inflammatory genes (Figure S2C) relative to wild-type (WT) mice. Importantly, AOM-DSS treatment induced fewer tumors in SA mice (Figure 2B), indicating that downregulation of IFNAR1 contributes to efficient colorectal tumorigenesis.

In a transplantation model, tumors formed in WT mice by MC38 colon adenocarcinoma cells expressed lower levels of IFNAR1 compared with these cells cultured in vitro (Figure S2D), demonstrating that the MC38 tumor model re-capitulates the IFNAR1 loss observed in human CRC. To determine the impor-

tance of IFNAR1 downregulation in the malignant cell compartment, we next aimed to restore IFNAR1 levels in MC38 cancer cells. Previous studies in fibrosarcomas and mammary adenocarcinomas demonstrated a tumor-suppressive effect of the IFN signaling in malignant cells (Bidwell et al., 2012; Sistigu et al., 2014). We also reported that expression of the *Ifnar1*^{S526A} allele in *Ifnar1*-null mouse melanoma cell line delays growth of these tumors (Katlinskaya et al., 2016). However, expression of the *Ifnar1*^{S526A} allele

in MC38mRFP cells did not affect their ability to form tumors in WT mice (Figures 2C, S2E, and S2F), indicating that the cell-autonomous anti-tumorigenic effects of IFNAR1 expression and IFN signaling might be cell or tumor type specific.

To determine the role of IFNAR1 downregulation in the stromal compartment, we inoculated WT or SA mice with MC38 cells (Figure S2G). While WT mice readily supported tumor growth, very few of MC38 tumors grew aggressively in SA mice (Figure 2D). Notably, most of these tumors were either rejected or exhibited a delayed growth (Figures 2D, 2E, and S2H) resulting in a prolonged survival (Figure 2F) in SA mice, suggesting an important role of downregulation of stromal IFNAR1 in tumorigenesis. Indeed, injection of IFNAR1-neutralizing antibodies further stimulated MC38 tumor growth in WT mice and dramatically rescued tumor growth in SA hosts (Figure 2G). These results indicate that efficient tumor growth requires downregulation of IFNAR1 levels primarily in the non-malignant cells.

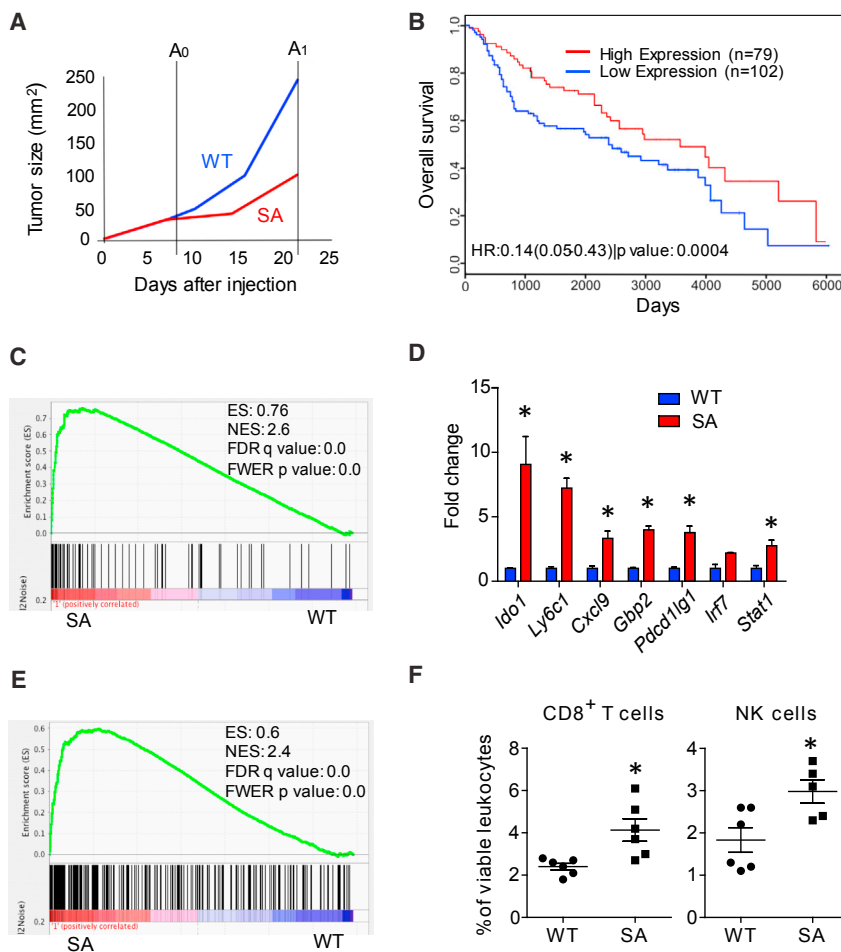


Figure 3. Alterations of Gene Expression Associated with IFNAR1 Downregulation Correlate with Local Immunosuppression and with a Poor Prognosis in CRC Patients

(A) Schematic representation of MC38 tumor growth in WT and SA mice. Time points of harvesting tumors of comparable (A0) and disparate (A1) size are indicated.

(B) Survival of adjusted for stage CRC patients (GEO: GSE41258) harboring the expression pattern of 30 selected genes similar to MC38 that grew either in WT (blue) or in SA (red) mice.

(C) GSEA results of the IFN- α/β signaling pathway in tumors harvested at day 9 (time point A0) and used for RNA isolation and microarray analysis.

(D) qPCR analysis of the indicated genes expressed in WT and SA tumors.

(E) GSEA results for the immune system process in tumors harvested at A0.

(F) Percent of NK and CD8⁺ T cells (relative to CD45⁺ cells) infiltrating MC38 tumors in WT and SA mice.

Data are shown as mean \pm SEM from five to six tumors.

See also [Figure S3](#) and [Table S1](#).

Alterations of Gene Expression Associated with IFNAR1 Downregulation Correlate with Local Immunosuppression and Poor Prognosis in CRC Patients

We next profiled the gene expression associated with IFNAR1 downregulation (in tumors with WT stroma) or stabilized IFNAR1 (in tumors with SA stroma). Notably, at an early time point after MC38 transplantation (A0, [Figure 3A](#)), the stromal compartments from tumors of comparable size that arose in WT or SA mice already differed in their gene expression patterns ([Table S1](#)). While most of differentially expressed genes (e.g., *Irf7*, *Ifit2*, *Mx2*, *Usp18*, etc.) are well known to be induced by IFN, others (*Clec7a*, *Sdc3*) have not been previously reported as bona fide IFN-regulated genes in global expression studies ([Mostafaei et al., 2016](#); [Rusinova et al., 2013](#)), suggesting that downregulation of IFNAR1 in the context of a growing tumor may elicit a more complex response than merely an IFN-signaling suppression. Specifically, the status of a set of 30 genes whose expression was increased in the stroma of early mouse SA tumors compared with WT ones ([Table S1](#)) was associated with impaired tumor progression in SA mice ([Figures 2D, 2E, and S2H](#)).

More importantly, this gene expression profile was also predictive of a better prognosis in two separate stage-adjusted co-

hort of human CRC patients ([Figures 3B and S3A](#)). Furthermore, dramatic suppression of the IFN-induced genes ([Figures 3C and 3D](#)) correlated with subsequent aggressive tumor growth ([Figure 2D](#)) was seen in early WT (but not SA) tumors. These data suggest that IFNAR1 downregulation and the ensuing alterations in gene expression contribute to CRC progression and appear to be predictive of disease outcome in human CRC patients.

Additional comparison of WT and SA gene expression patterns revealed a suppressed immune pathway in early WT tumors ([Figures 3D and 3E](#)) pointing to the immune system as a putative target of IFNAR1 downregulation. At later time points, when tumors in WT mice became larger than tumors in SA mice (A1, [Figure 3A](#)), we noted a similar suppression of IFN signaling and in the signature of immune genes in WT tumors ([Figures S3B and S3C](#)). These results indicate that decreased IFNAR1 levels in the stromal compartment may determine both the immunosuppressive capacity and growth potential of the tumor.

Prompted by gene expression data we assessed the levels of immune cells in WT and SA mice burdened with MC38 tumors. Compared with SA mice, spleens from WT mice contained somewhat greater overall levels of CD11b⁺Ly6G⁺ cells; however, we did not detect significant differences in the frequencies of splenic natural killer (NK) cells or T cells ([Figure S3D](#)) that would be characteristic of generalized immunosuppression in tumor-bearing WT animals. Conversely, analysis of tumor-infiltrating leukocyte subsets revealed significantly reduced frequencies of CD8⁺ T cells, NK cells, and Ly6C^{hi}Ly6G⁺ cells in tumors from WT animals compared with tumors from SA mice ([Figures 3F and S3E](#)). This result indicates that downregulation of IFNAR1 within WT tumors is associated with a localized intra-tumoral

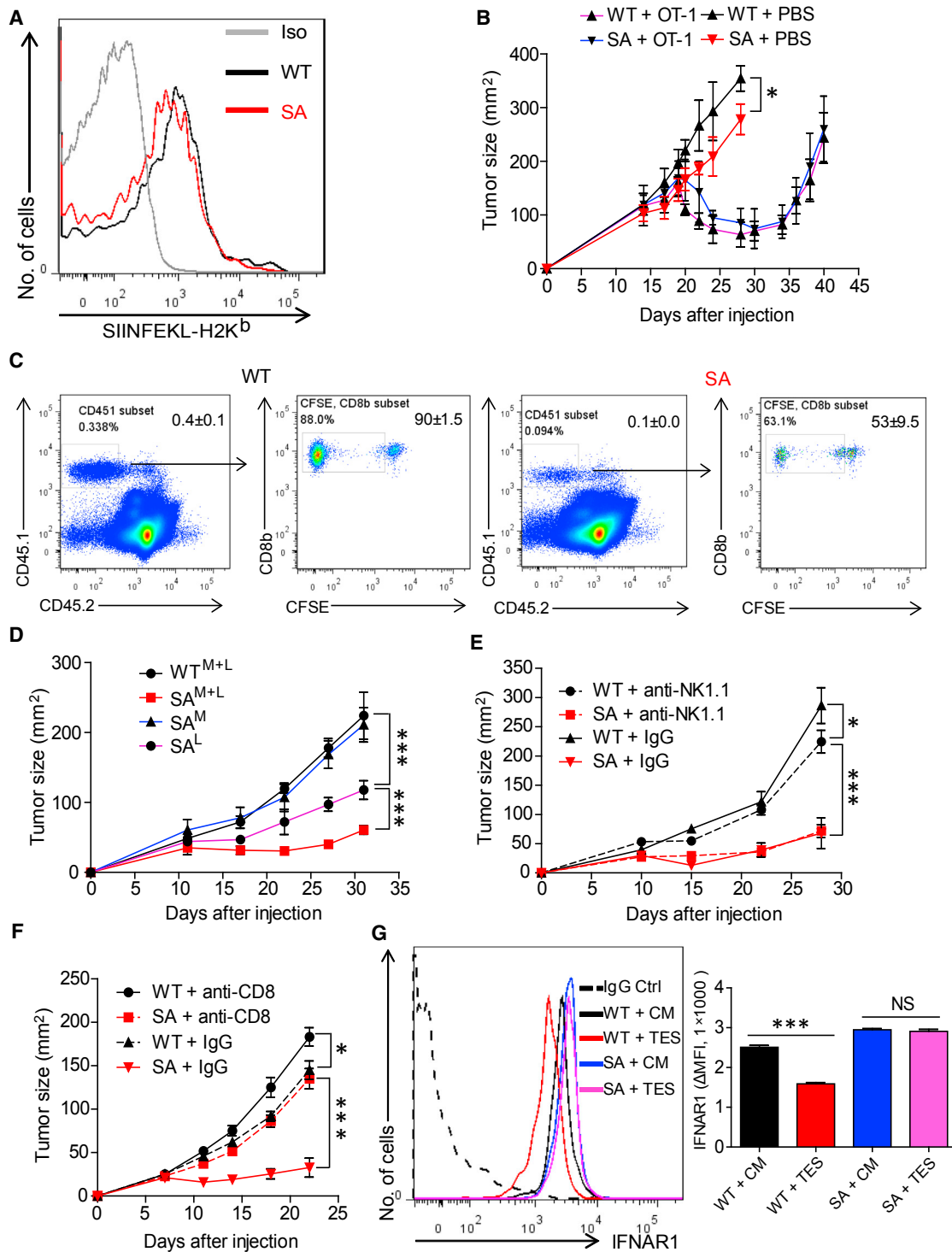


Figure 4. Downregulation of IFNAR1 on CTLs Promotes Tumor Growth

(A) Representative fluorescence-activated cell sorting (FACS) analysis of levels of MHC-I-complexed OVA peptide on the surface of intra-tumoral CD45⁺MHC-II⁺CD103⁺CD11c⁺ DCs isolated from the MC38OVA tumors grown in *Rag1*^{-/-} mice harboring WT or SA IFNAR1.

(B) Growth of MC38-OVA tumors in *Rag1*^{-/-} mice harboring WT or SA IFNAR1 after adoptive transfer of WT OT-1 T cells injected on day 18 after tumor inoculation.

(C) Representative flow cytometry analysis of the percentage (left panels) and proliferation (right panels) of CD8⁺CD45.1⁺ at day 7 in the spleens from WT and SA mice after adoptive transfer of naive carboxyfluorescein succinimidyl ester (CFSE)-labeled OT-1 T cells at day 0 and subsequent challenge with MC38OVA cells at day 1.

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immunosuppression, resulting in reduced CTL accumulation within the TME.

Downregulation of IFNAR1 in CTLs Contributes to Development of the Immunosuppressive TME in CRC

Previous studies of sarcomas and melanomas grown in WT or *Ifnar1* knockout mice suggested a critical role of IFN in the ability of specific CD8 α ⁺ lineage DCs to cross-present antigen to the CD8⁺ cytotoxic lymphocytes and highlighted the critical role of these DCs in anti-tumor immune protection (Diamond et al., 2011; Fuertes et al., 2011; Hildner et al., 2008). We did not observe changes in the overall frequency of intra-tumoral CD11b⁺ CD11c⁺ MHC-II⁺ CD103⁺, CD11c⁺ MHC-II⁺ CD8 α ⁺, or CD11c⁺ MHC-II⁺ CD103⁺ DCs between MC38 tumors that grew in WT and SA mice (Figure S3E). DCs isolated from WT or SA mice exhibited a similar efficacy in direct antigen presentation (Figure S4A) as well as in cross-presentation of tumor antigens (Figure S4B), indicating that reduced tumorigenesis in SA mice cannot be readily explained by an increased antigen presentation capacity.

We next transplanted MC38 tumor cells ectopically expressing ovalbumin (OVA) (MC38OVA) into immune-deficient *Rag1*^{-/-} mice that harbored either WT or SA IFNAR1. Dendritic cells (CD45⁺ MHC-II⁺CD103⁺ CD11c⁺) isolated from either WT or SA tumors presented comparable levels of MHC-I-complexed OVA peptide (Figure 4A). Consistent with these data, adoptive transfer of WT OVA-specific OT-1 CTLs into these *Rag1*^{-/-} mice resulted in an initial decrease in tumor volume (followed by subsequent re-growth of tumors) regardless of IFNAR1 status (Figure 4B).

We next injected WT or SA mice with naive OT-1 T cells followed by challenge with MC38OVA and subsequent assessment of numbers and proliferation of splenic OT-1 CD8⁺ T cells 6 days later. Under these conditions, even less CTL proliferation was seen in the SA hosts compared with WT mice (Figure 4C), further indicating that the tumor growth defect observed in SA mice is unlikely to depend on increased antigen presentation by SA DCs.

We next generated mixed bone marrow chimeras in which myeloid and/or lymphoid cells from either WT or SA animals were used to reconstitute bone marrow in lethally irradiated WT mice (Diamond et al., 2011). These chimeric mice harbored comparable numbers of myeloid and lymphoid cells and the expected IFNAR1 levels on these cells in the spleen (Figure S4C). A dramatic suppression of MC38 tumor growth observed in chimeras that received both lymphoid and myeloid cells from SA donors ("SA^{M+L}", Figures 4D and S4D) was indicative of the critical importance of IFNAR1 downregulation in the bone marrow-derived cells. Relative to this group, only a slight acceleration of tumorigenesis was seen in mice that received WT myeloid cells and SA lymphocytes ("SA^L"). This result indicates that, while

there is a role for IFNAR1 expressed on myeloid cells, maintaining the levels of IFNAR1 on lymphocytes appears to be critical for tumor growth suppression.

Indeed, while depletion of NK cells in SA mice did not alter growth of MC38 tumors (Figures 4E and S4E), depletion of CD8⁺ cells notably stimulated tumor growth in SA animals (Figures 4F and S4F). We next sought to determine whether IFNAR1 can be downregulated specifically on the intra-tumoral CD8⁺ T cells. Incubation of WT (but not SA) CD8⁺ T cells with the tumor explant supernatant (Figure 4G) or MC38 cell-conditioned medium (Figure S4G) robustly downregulated IFNAR1 cell surface levels. Together with notably lower levels of IFNAR1 on the surface of CD3⁺CD8⁺ WT (but not SA) cells isolated from tumors compared with those isolated from spleens (Figure S4H), these data suggest that tumor conditions trigger downregulation of IFNAR1 on the surface of CTLs and that this downregulation contributes to aggressive tumorigenesis.

The majority of cells expressing high levels of IFNAR1 in normal human colon were CD3⁺ cells (Figure S5A). Importantly, in human CRC tissues, most IFNAR1-positive T lymphocytes were peripheral to the tumor and very few of them were found inside human tumors (Figures 5A and S5B). Whereas these low numbers of CTLs found in human CRC could be recapitulated in MC38 tumors grown in WT mice, a greater absolute and relative number of the CD3⁺CD8⁺ CTLs was found within tumors developed in SA animals (Figures 3F and 5B). Consistent with this result, mouse SA tumors exhibited a prominent T cell gene expression signature (Figure 5C) and particularly high levels of mRNA of genes indicative of T cell effector function including *Ifng* and *Gzmb* (Figure S5C), as well as increased levels of IFN- γ and granzyme B proteins in MC38 tumor lysates (Figure S5D). Likewise, greater levels of *Ifng* mRNA expression were also observed in SA tumors induced by AOM-DSS treatment compared with WT tumors (Figure S2C). These results suggest that reduced accumulation of CTLs (indicative of immune-privileged niche) in CRC is associated with IFNAR1 downregulation.

Accordingly, studies involving adoptive transfer of CD8⁺ T cells into *Rag1*^{-/-} mice burdened with MC38OVA tumors revealed a greater intra-tumoral accumulation of CTLs derived from SA OT-1 mice compared with CTLs from WT OT-1 animals (Figure 5D). These results suggest that the status of IFNAR1 on CTLs determines their ability to accumulate within tumors. These findings in human and mouse tumors collectively indicate that the IFNAR1 downregulation on CTLs that occurs within CRC tumors prevents CTL accumulation, thereby establishing a local immune-privileged TME.

Given that little, if any, difference was detected in proliferation of SA and WT CD8⁺ T lymphocytes activated in vitro (Figure S5E) or isolated from tumor-bearing mice (Figure S5F), we next focused on other mechanisms that could explain preferential

(D) Growth of MC38 tumors in *Rag1*^{-/-} mice that received bone marrow containing WT or SA myeloid and lymphoid cells (WT^{M+L} and SA^{M+L}), WT myeloid and SA lymphoid cells (SA^L), or WT lymphoid and SA myeloid cells (SA^M).

(E) MC38 tumor growth in WT or SA mice treated with anti-NK.1.1 or immunoglobulin G (IgG) control antibodies.

(F) MC38 tumor growth in WT or SA mice treated with anti-CD8 or IgG control antibodies.

(G) Representative FACS analysis and quantification (n = 4, each in triplicate) of IFNAR1 levels on the surface of CD3⁺ CD8⁺ cells isolated from WT or SA spleens and incubated in vitro with control medium (CM) or tumor explant supernatant (TES) for 2 hr. NS, not significant.

Data depicted as mean \pm SEM (n = 4–6); similar results were obtained in at least two independent experiments.

See also Figure S4.

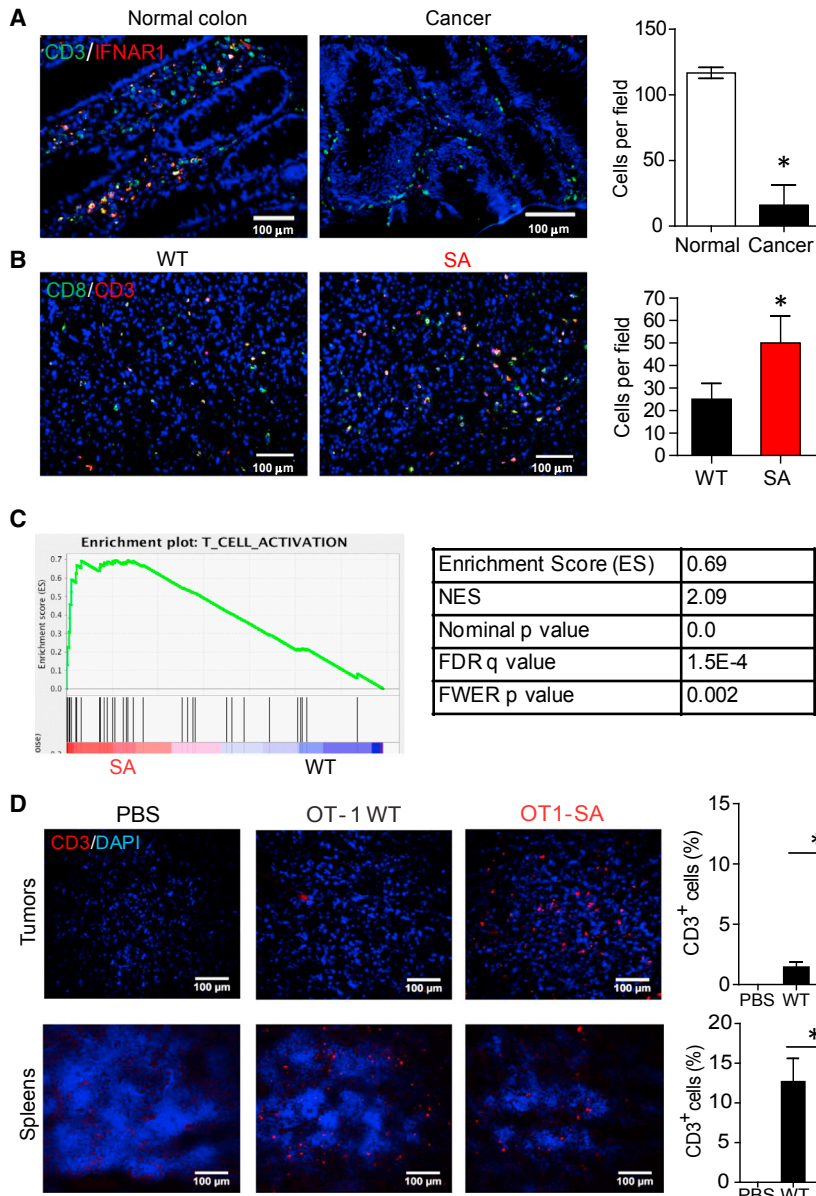


Figure 5. Downregulation of IFNAR1 in CTLs Contributes to Development of the Immunosuppressive Tumor Microenvironment in CRC

(A) Representative immunofluorescent analysis and quantification of IFNAR1^{hi}CD3⁺ T cell infiltration of human normal colon and CRC. Tissue sections were stained with antibodies against IFNAR1 (red) and CD3 (green) and contrasted with DAPI (blue). At least 20 randomly chosen fields from each of eight patient samples for each group were quantified.

(B) Representative immunofluorescent analysis and quantification of CD3⁺CD8⁺ T cell infiltration of MC38 tumors from WT and SA mice. Tumor sections were stained with anti-CD3 (red) and -CD8 (green) antibodies and contrasted with DAPI (blue). At least 20 randomly chosen fields from each of five tumor samples were quantified.

(C) GSEA results of the T cell activation gene signature in WT and SA tumors.

(D) Representative immunofluorescent analysis of T cells found in the spleens or MC38OVA tumors grown in *Rag1*^{-/-} mice after treating these mice with PBS or adoptive transfer of WT or SA OT-1 T cells (2×10^7 per mouse). Data are depicted as the percentage of CD3⁺ cells among all DAPI-stained cells and are representative of at least 20 random fields scored from tissues of four mice.

Data are shown as mean \pm SEM.

See also Figure S5.

CTL accumulation in tumors of SA mice. Cancer-associated fibroblasts positive for the fibroblast activation protein (FAP) produce CXCL12 chemokine that prevents intra-tumoral CTL buildup in a mouse pancreatic cancer model (Feig et al., 2013). Intriguingly, activated SA and WT CTLs exhibited a similar chemotaxis toward CXCL12 or CXCL9 (Figure S5G), suggesting that retaining IFN signaling may not necessarily increase the migratory abilities of CTLs.

Downregulation of IFNAR1 on CTLs Undermines Their Survival within the TME

IFN promotes survival of anti-viral CTLs by protecting them from killing by NK cells (Crouse et al., 2014; Xu et al., 2014). Depletion of NK cells in *Rag1*^{-/-} mice indeed increased the total number of transferred T cells but did not affect a greater viability of SA T cells compared with WT T cells (Figure S6). Given this result

and the observation that NK depletion did not alter tumorigenesis in either WT or SA mice (Figure 4E), we focused on other mechanisms by which downregulation of IFNAR1 may affect anti-tumor CTLs. Antigen-exposed SA CTLs cultured in vitro maintained greater levels of IFNAR1, the mRNA, and the protein levels for the anti-apoptotic regulator, B cell lymphoma-extra large (Bcl-XL), and lower levels of cleaved caspase-3 compared with WT CTLs (Figures 6A and S7A). Accordingly, a decrease in cell viability was more pronounced in WT cells than in SA cells under

these conditions (Figure 6B). Importantly, while pre-treatment with interleukin-2 (IL-2) increased the viability of WT CTLs, neutralizing the IL-2 receptor using anti-CD25 antibody undermined the survival of SA CTLs (Figure 6B). We further found that SA CTLs produced notably more IL-2 (Figure S7B) and expressed greater levels of IL-2R α mRNA and protein compared with WT cells (Figures S7C and S7D). Thus, it is likely that downregulation of IFNAR1 promotes death of activated CTLs by attenuating the pro-survival effects of the IL-2 pathway.

Activated SA OT-1 CTLs exhibited greater viability than WT OT-1 CTLs when these cells were simultaneously injected in a 1:1 ratio intravenously or directly into the MC38OVA tumors grown in *Rag1*^{-/-} mice (Figures 6C and 6D). This result suggests that stabilization of IFNAR1 on antigen-specific CTLs improves their survival within the tumors. To further corroborate this possibility, we used the CAR-based approach that involved the

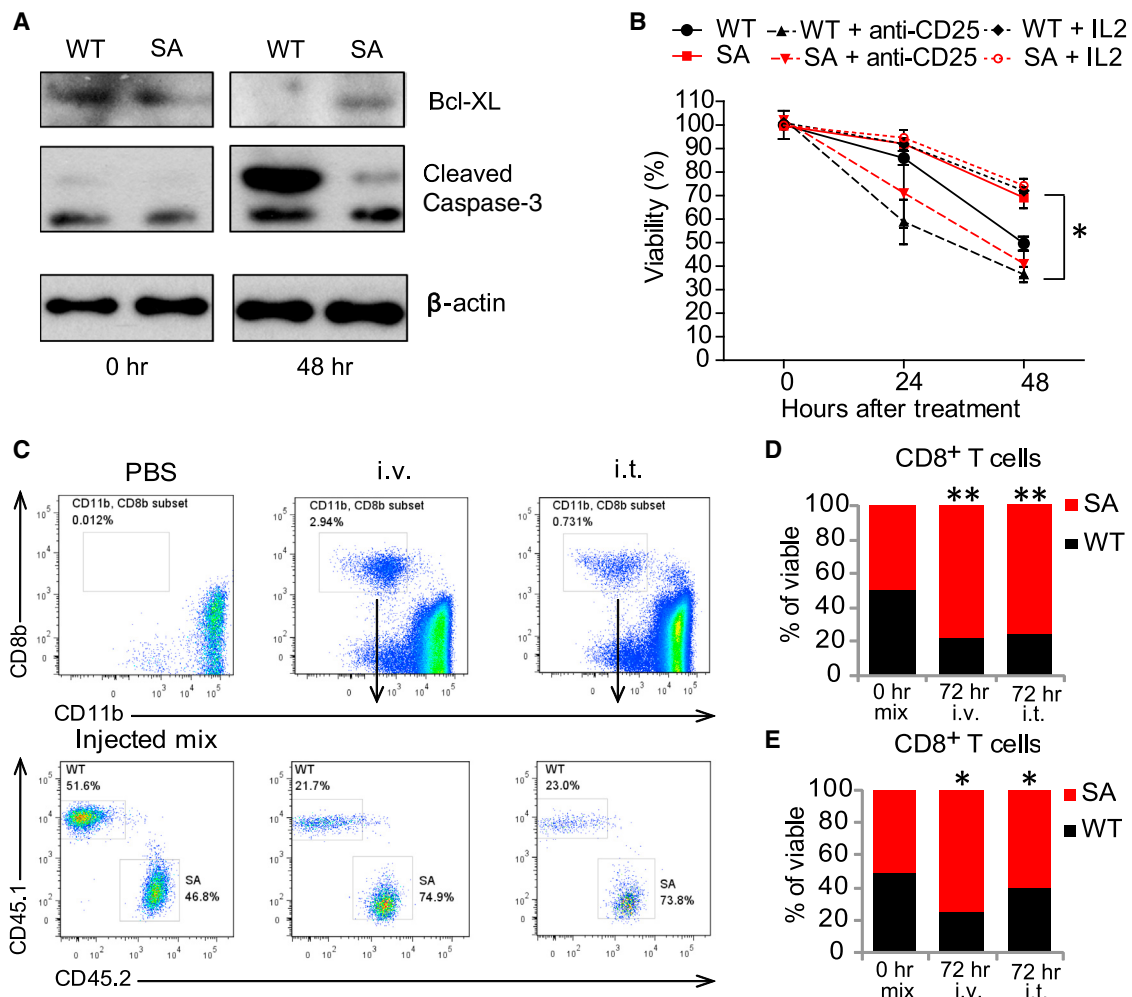


Figure 6. Downregulation of IFNAR1 on Cytotoxic T Lymphocytes Undermines their Survival within the TME

(A) Immunoblot analysis of Bcl-XL, cleaved caspase-3 levels, and β-actin (loading control) in splenocytes from WT and SA OT-1 mice activated with SIINFEKL peptide (0.5 μg/mL for 48 hr) and then cultured for indicated times.

(B) Viability of activated CD3⁺CD8⁺ cells in the presence of medium supplemented or not with either IL-2 (100 U/mL) or anti-CD25 antibody (100 μg/mL), as indicated, was determined by flow cytometry analysis after the indicated times. Mean ± SD (triplicates per mouse spleen, average from three mice) are shown. Asterisks denote statistical significance (p < 0.05) between WT and SA, between WT and WT treated with IL-2, and between SA and SA treated with anti-CD25 antibody.

(C) Flow cytometry analysis of the fraction of viable OT-1 WT (CD45.1) or SA (CD45.2) CTLs in the MC38OVA tumors 72 hr after intravenous (i.v.) injection (1:1 ratio) or directly into the tumors (i.t.) of *Rag1*^{-/-} mice bearing MC38OVA tumors.

(D) Quantitation of the experiments shown in (C) (mean percentage of viable cells from tumors from three to five mice). Similar results were obtained in at least two independent experiments.

(E) Quantitation of flow cytometry analysis of the fraction of viable FAP-CAR EGFP⁺ WT (CD45.1) or EGFP⁺ SA (CD45.2) CTLs in the MC38 tumors 72 hr after i.v. injection (1:1 ratio) or directly into the tumors (i.t.) of WT MC38 tumor-bearing mice. Data are shown as the mean percentage of viable cells (n = 5).

See also [Figures S6](#) and [S7](#).

introduction of the CAR against FAP (FAP-CAR [[Wang et al., 2014](#)]) into WT or SA CTLs. We generated FAP-CAR T cells separately from WT or SA lymphocytes, and then mixed these cells in equal parts prior to adoptive transfer (as a 1:1 mixture) into MC38 tumor-bearing WT mice ([Figure S7E](#)). Regardless of the route of administration (intra-tumoral or intravenous), a greater fraction of SA cells was found in the tumor 3 days later ([Figures 6E](#) and [S7F](#)). These results suggest that TME-induced downregulation of IFNAR1 on CTLs compromises the viability of these CTLs inside tumors.

Downregulation of IFNAR1 in CTLs Limits the Efficacy of Anti-cancer Therapies

We next examined the importance of IFNAR1 downregulation in modulating the efficacy of adoptive CTL transfer-based immunotherapy. Adoptive transfer of WT OT-1 lymphocytes into *Rag1*^{-/-} mice bearing MC38OVA tumors was much less efficient in sustained suppression of tumor growth and prolonging animal survival compared with SA OT-1 cells ([Figures 7A](#) and [7B](#)). Furthermore, FAP-CAR CTLs prepared from SA cells exhibited a substantially greater therapeutic effect against MC38 tumors

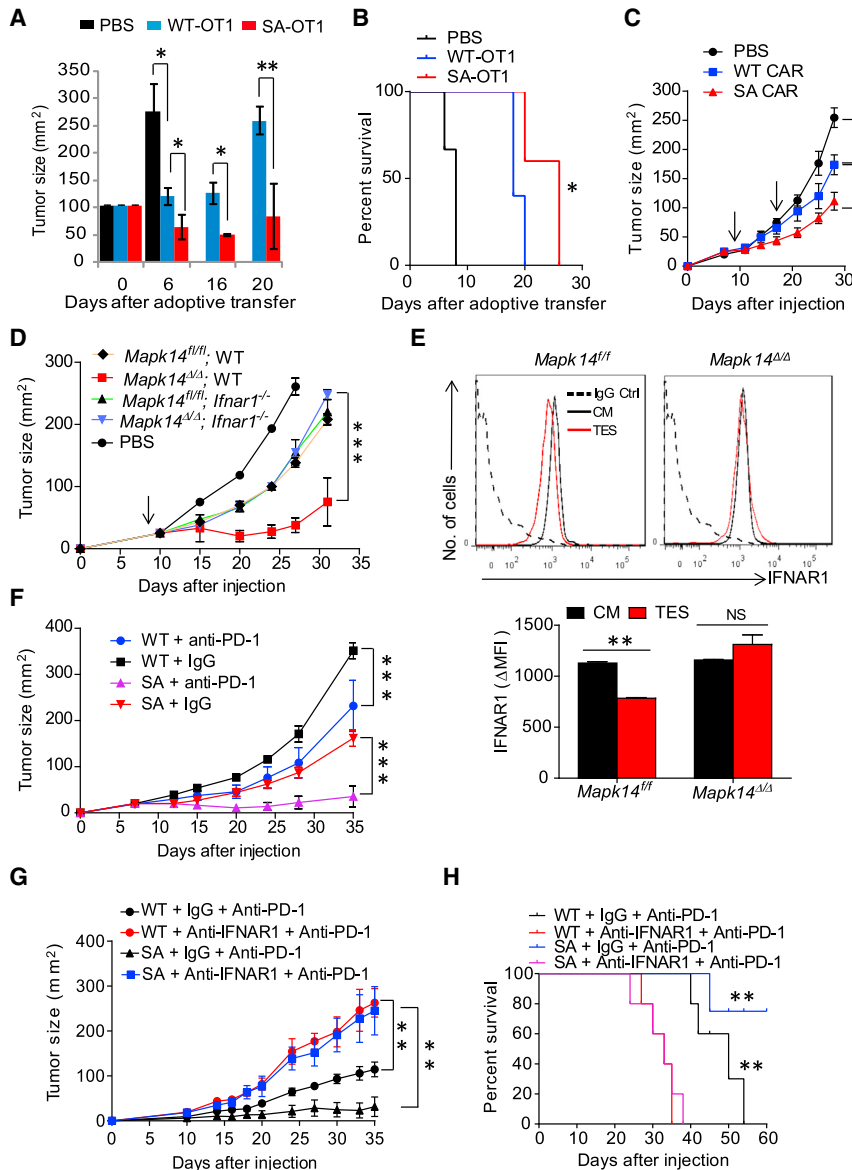


Figure 7. Downregulation of IFNAR1 in CTLs Limits the Efficacy of Immunotherapies

(A) Anti-tumor effects of adoptively transferred OT-1-SA and OT-1 WT T cells in MC38OVA tumor-bearing *Rag1*^{-/-} described in Figure 5D. (B) Kaplan-Meier analysis of survival of MC38 tumor-bearing mice from (A). (C) Anti-tumor effects of IFNAR1 WT and IFNAR1 SA FAP-CAR T cells (time of administration indicated by arrow) in MC38 tumor-bearing mice. (D) Growth of MC38 tumors in WT mice that received PBS or FAP-CAR T cells harboring indicated status of *Mapk14* and *Ifnar1* shown. (E) Cell surface IFNAR1 levels on CD3⁺CD8⁺ splenocytes of the indicated genotype treated with TES or CM for 2 hr. Representative FACS and quantitation (below) are shown. (F) Anti-tumor effect of anti-PD-1 antibody administration in WT mice and SA mice bearing MC38. (G) Effect of IFNAR1 neutralization on the efficacy of anti-PD-1 treatment of WT or SA mice bearing MC38 tumors. (H) Kaplan-Meier analysis of survival of MC38 tumor-bearing mice from (G). Data shown as mean \pm SEM (n = 3–5) from each of at least two to three independent experiments.

relative to FAP-CAR WT CTLs (Figure 7C). The magnitude of these effects is probably underappreciated because the SA allele may inhibit proliferation of CAR CTLs (data not shown), and FAP-CAR SA cells used in these experiments were likely to partially suppress IFN signaling downstream of the receptor.

To overcome this problem we sought to acutely stabilize IFNAR1 via inducible ablation of p38 α , a kinase potentially involved in the ligand-independent downregulation of IFNAR1 (Bhattacharya et al., 2011). We prepared FAP-CAR T cells from the splenocytes of mice harboring floxed *Mapk14* (a gene that encodes p38 α), either *Ifnar1*^{+/-} (WT) or *Ifnar1*^{-/-} alleles, and either no Cre or inducible *Ubc9-Cre*^{ERT2}. These FAP-CAR T cells were treated with 4-hydroxytamoxifen and then injected into WT mice burdened with MC38 tumors. As seen from Figure 7D, inactivation of p38 α in IFNAR1-expressing FAP-CAR CTLs (*Mapk14* ^{Δ/Δ} WT) dramatically increased the anti-tumor efficacy of these cells. Importantly, this increased effect could be negated by concurrent

ablation of *Ifnar1*, suggesting that most (if not all) effects of p38 α deletion depend on sustained IFNAR1 signaling within CTLs (Figure 7D). Together with the inability of CTLs lacking p38 α to downregulate IFNAR1 in response to an in vitro treatment with the tumor explant supernatant (Figure 7E), these data provide genetic evidence suggesting that tumor-derived factors-induced p38 α -dependent downregulation of IFNAR1 on the surface of CTLs limits the efficacy of CAR-based therapeutics in solid tumors.

Intriguingly, a fraction of MC38 tumors that did not get rejected in SA mice eventually reached a larger size (A1, Figure 3A). Whereas SA tissues retained a greater immune response and robust IFN signatures, there was also a notable increase in expression of the PD-L1/CD274 checkpoint molecule (Figure S3C). Accordingly, treatment with the anti-PD-1 antibody at a dose that only slightly delayed MC38 tumor growth in WT mice caused a robust therapeutic effect leading to a stable disease in SA mice (Figure 7F). Importantly, anti-PD-1 therapy was notably less efficient in suppressing tumor growth (Figure 7G) and improving animal survival (Figure 7H) in SA mice that also received anti-IFNAR1 neutralizing antibody. These data collectively suggest that downregulation of IFNAR1 undermines the efficacy of checkpoint-targeted immunotherapeutics against solid tumors.

We noted a greater phosphorylation of p38 α in lysates from MC38 tumors relative to cultured MC38 cells (Figure 8A), consistent with activation of p38 α by TME stress. Accordingly, we next examined whether downregulation of IFNAR1 can be reversed

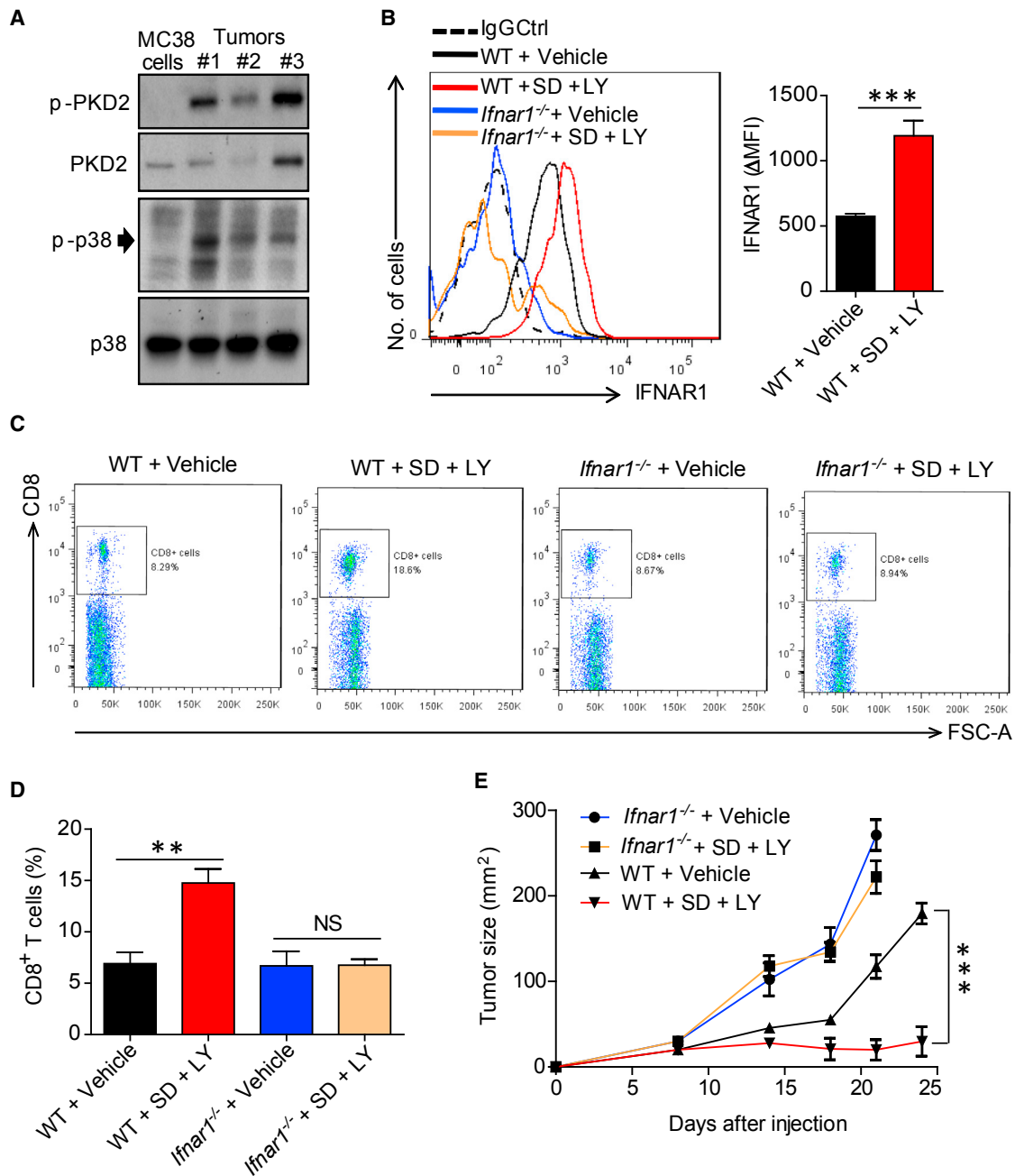


Figure 8. Pharmacologic Stabilization of IFNAR1 Disrupts Immune-Privileged Niche and Elicits a Therapeutic Effect against Tumors

(A) Immunoblot analysis of phosphorylation and levels of p38 α and PKD2 kinases in cultured MC38 cells and MC38 tumors.

(B) Levels of cell surface IFNAR1 on tumor-infiltrating CD3⁺CD8⁺ cells isolated from MC38 tumors grown in WT or *Ifnar1*-null mice treated with kinase inhibitors as indicated. Representative FACS and quantification are shown.

(C) Frequency of CD8⁺ T cells (% of CD45⁺ cells) isolated from MC38 tumors grown in WT or *Ifnar1*-null mice treated with kinase inhibitors as indicated.

(D) Quantification of results shown in (C).

(E) Anti-tumor effect of SD-208 and LY2228820 administered to *Ifnar1*^{-/-} and WT mice bearing MC38 tumors as described in the [Experimental Procedures](#).

Data are shown as mean \pm SEM (n = 5 from each of three independent experiments).

See also [Figure S8](#).

by pharmacologic means. To this end, we attempted to stabilize IFNAR1 using the p38 inhibitor LY2228820 (LY). Given that some TME factors such as vascular endothelial growth factor can also downregulate IFNAR1 via activating protein kinase-2

(PKD2) (Zheng et al., 2011), and that PKD2 activity was indeed increased in MC38 tumors compared with cultured MC38 cells (Figure 8A), we combined the p38 inhibitor with SD-208 (SD), a PKD inhibitor.

The combination of these small molecules (LY + SD) prevented tumor explant supernatant-induced downregulation of IFNAR1 on CTLs in vitro (Figure S8A). Furthermore, in vivo treatment with this combination (which was well tolerated by tumor-bearing mice, Figure S8B), led to a notable increase in the overall levels of IFNAR1 within tumors (Figure S8C) and specifically of cell surface IFNAR1 levels on intra-tumoral CD3⁺CD8⁺ cells (Figure 8B). Remarkably, administration of LY + SD robustly increased numbers of CTLs found inside MC38 tumors that grew in WT but not in *Ifnar1*^{-/-} mice (Figures 8C, 8D, S8D, and S8E), suggesting that inhibition of p38 and PKD disrupts the immune-privileged niche within the TME in an IFNAR1-dependent manner.

Consistent with an important role of IFNAR1 downregulation in the stimulation of tumorigenesis, treatment with these kinase inhibitors dramatically suppressed growth of MC38 tumors in WT mice, but not in mice lacking *Ifnar1* (Figure 8E), indicating that stimulation of IFNAR1 signaling is a major mechanism underlying the immune-reactivating and anti-tumorigenic effects of these agents. In all, these results provide a proof of principle for pharmacologic stabilization of IFNAR1 as the means to attenuate local immunosuppression within tumors and to suppress tumor growth.

DISCUSSION

Delineating the mechanisms that impose localized immune suppression within the TME is essential for improving the efficacy of immunotherapeutics in solid tumors. Here we present evidence that links the TME stress-driven downregulation of IFNAR1 to the reduced viability of intra-tumoral CTLs and the ensuing establishment of an immune-privileged niche in CRC tumors. A decrease in IFNAR1 levels and expression of IFN-inducible genes found in human CRC tumors and recapitulated in mouse tumors is associated with the establishment of a localized niche virtually void of CTLs, as well as with robust tumor growth and poor prognosis. Downregulation of IFNAR1 specifically in CTLs induced by tumor-associated factors inhibits CTL viability and undermines the efficacy of immune therapies. Conversely, genetic or pharmacologic stabilization of IFNAR1 disrupts the immune-privileged niche, suppresses tumor growth, and increases the efficacy of CAR T cell therapy and immune checkpoint inhibitors.

These findings suggest that IFNAR1 downregulation contributes to the development and progression of CRC. While not arguing against additional role of IFNAR1 in IFN-modulated regulation of antigen presentation and activation of DCs (Diamond et al., 2011; Fuertes et al., 2011; Hildner et al., 2008), stromal resistance to tumor-induced angiogenesis (Zheng et al., 2011) and other processes, our current results strongly indicate that IFNAR1 downregulation on intra-tumoral CTLs contributes to the establishment of immune-privileged TME by undermining CTL survival. These results are consistent with an important role of IFN as an activation signal for T cells (Curtsinger et al., 2005) and the observation that insufficient “third signal” contributes to the inhibition of CTLs in solid tumors (Curtsinger et al., 2007). It appears that downregulation of IFNAR1 on CTL negatively affects responses of these CTLs to IL-2 pro-survival signals and, accordingly, stimulates pro-apoptotic pathways, although other mechanisms cannot be ruled out. Regardless of the exact

mechanisms, the data presented here argue for the development of therapeutic strategies aimed to stabilize IFNAR1 and improve CTL viability within solid tumors.

Our results specifically emphasize the importance of IFNAR1 downregulation on CTLs. Given the importance of these cells in anti-tumor immunity against diverse malignant lesions, it is likely that downregulation of IFNAR1 in the stromal compartment may stimulate growth and progression of other cancer types. Indeed, we have recently demonstrated that loss of IFNAR1 stimulates growth of transplanted melanomas (Katlinskaya et al., 2016). Overall, our data are consistent with intra-tumoral IFN production being linked with CTL generation and viability (Hiroishi et al., 2000), observations that IFN may act to improve the effect of adoptive transfer of CTLs (Hervas-Stubbs et al., 2012), and with recent finding that a specific CAR design, which serendipitously increased IFN signaling in CTLs, evoked an augmented therapeutic effect (Zhao et al., 2015). Nevertheless, our results do not rule out additional putative cellular targets (e.g., interleukin-10-expressing Treg cells; Stewart et al., 2013) and additional mechanisms by which elimination of IFNAR1 and suppression of IFN signaling can further contribute to localized immunosuppression and stimulation of solid tumors growth.

Previous studies utilizing chemically induced and transplantable sarcomas and melanomas in IFNAR1 knockouts have identified specific CD8 α ⁺ DCs as targets of protective role of IFN against tumors (Diamond et al., 2011; Fuertes et al., 2011; Hildner et al., 2008). Functional defects of *Ifnar1*-null DCs reported in these studies are consistent with an important role of IFNAR1 in the maturation of DCs (Le Bon and Tough, 2002; Santini et al., 2009). We did not observe an increase in antigen presentation in SA mice. Furthermore, SA DCs might have a survival disadvantage given that elimination of IFNAR1 plays an important role in preserving the viability of IFN-expressing DCs exposed to inducers of pathogen recognition receptor signaling (Qian et al., 2011). Future use of SA animals in sarcoma and melanoma models is likely to reveal additional information on the relative contribution of IFNAR1 status in DCs and other leukocytes to anti-tumor immunity.

Genetic and pharmacologic studies described here provide a proof of principle for a focus on stabilization of IFNAR1 to increase the efficacy of immunotherapies against CRC and possibly other solid tumors. Whereas the mechanisms underlying the therapeutic effect of p38/PKD inhibitors are likely to be mediated by many cell types (in addition to CTLs), it is noteworthy that these inhibitors still act in an IFNAR1-dependent manner. In addition to targeting p38 and PKD kinases responsible for phosphorylation of IFNAR1 leading to recruitment of the SCF- β Trcp E3 ligase, it might be possible to inhibit this class of ligases. Cullin-dependent ligases (including SCF- β Trcp) can be targeted by inhibiting the NEDD9-activating enzyme; its selective inhibitor, MLN4924, is currently under clinical trials in solid tumors (Sarantopoulos et al., 2015). Additional studies on combining IFNAR1-stabilizing regimens with diverse immunotherapeutic approaches are currently in progress.

EXPERIMENTAL PROCEDURES

A detailed description of the procedures utilized in this work can be found in the Supplemental Experimental Procedures. Use of pre-existing human

archival de-codified and de-identified CRC tissue arrays, previously collected under informed consent, and samples that could not be directly or indirectly linked to individual human subjects was exempt from institutional review. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Pennsylvania and were carried out in accordance with the IACUC guidelines. All mice were on the C57BL/6 background and had water ad libitum and were fed regular chow. Mice were maintained in a specific pathogen-free facility in accordance with American Association for Laboratory Animal Science guidelines. Littermate animals from different cages were randomly assigned into the experimental groups. These randomized experimental cohorts were either co-housed or systematically exposed to the bedding of other groups to ensure equal exposure to the microbiota of all groups. Statistical analysis was performed using Microsoft or GraphPad Prism 7 software. Unpaired Student's *t* test was used for the comparison between two groups. One-way ANOVA or two-way ANOVA analysis followed by the Bonferroni post hoc test were used for the multiple comparisons. Repeated-measure two-way ANOVA (mixed-model) followed by the Bonferroni post hoc test was used for the analysis of tumor growth curve. A value of *p* < 0.05 was considered significant.

Data from the global expression profiling studies were collected with Illumina BeadStudio 3.1.1.0 software, and statistical analyses were conducted on the IlluminaGUI R-package. Gene sets from microarray data were analyzed for overlap with curated datasets (C5, H) in the MSigDB using the web interface available at <http://www.broadinstitute.org/gsea/msigdb/index.jsp>. The raw data have been deposited at NCBI (GEO: GSE76889).

For AOM-DSS colorectal carcinogenesis, co-housed experimental mice were intraperitoneally injected with 10 mg/kg azoxymethane (Sigma). A week later, they were supplied with tap water containing 2.5% dextran sodium sulfate (TdB Consultancy) for 7 days followed by 14 days of regular water. This cycle was repeated three times and mice were killed 2 weeks after the end of the last DSS cycle or at the end of 10 weeks. Colons were harvested, washed of feces with Dulbecco's PBS, and slit open longitudinally to count tumors. Tumors were flash frozen in liquid nitrogen or embedded into optimal cutting temperature medium for subsequent analysis.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, eight figures, and one table and can be found with this article online at <http://dx.doi.org/10.1016/j.ccell.2017.01.004>.

AUTHOR CONTRIBUTIONS

S.Y.F., K.V.K., J.G., E.P., H.R., C.K., and J.A.D. designed the research; K.V.K., J.G., A.O., Y.V.K., R.C., C.J.C., S.B., D.P.B., M.G., A.R.P., P.C., and A.K.R. performed the experiments and interpreted the data; S.Y.F., K.V.K., J.G., E.P., H.R., C.K., and J.A.D. wrote the manuscript with the help of all authors.

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