Cancer-Specific Loss of p53 Leads to a Modulation of Myeloid and T Cell Responses

Highlights

- Tumor-specific loss of p53 delays tumor rejection in immune-competent hosts
- p53 loss increases myeloid infiltration through enhanced cytokine secretion
- The increase in Treg cells in response to loss of p53 is independent of Kras mutation
- Kras mutations coordinate with p53 loss to regulate myeloid recruitment

In Brief

TP53 is one of the most frequently mutated genes in cancer; however, its significance in controlling tumor-immune crosstalk is not fully understood. Blagih et al. highlight a key role for tumor-associated loss of p53, a common oncogenic event, in regulating myeloid and T cell responses.
Cancer-Specific Loss of p53 Leads to a Modulation of Myeloid and T Cell Responses

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SUMMARY

Loss of p53 function contributes to the development of many cancers. While cell-autonomous consequences of p53 mutation have been studied extensively, the role of p53 in regulating the anti-tumor immune response is still poorly understood. Here, we show that loss of p53 in cancer cells modulates the tumor-immune landscape to circumvent immune destruction. Deletion of p53 promotes the recruitment and instruction of suppressive myeloid CD11b+ cells, in part through increased expression of CXCR3/CCR2-associated chemokines and macrophage colony-stimulating factor (M-CSF), and attenuates the CD4+ T helper 1 (Th1) and CD8+ T cell responses in vivo. p53-null tumors also show an accumulation of suppressive regulatory T (Treg) cells. Finally, we show that two key drivers of tumorigenesis, activation of KRAS and deletion of p53, cooperate to promote immune tolerance.

INTRODUCTION

There is strong evidence that cancer cells have the potential to be recognized by the immune system but that they can mobilize various mechanisms of immune evasion and escape, such as upregulation of immune checkpoint proteins to dampen T cell effector responses (Ribas and Wolchok, 2018). Current human studies have shown durable and complete responses to immune checkpoint blockade in a number of tumors; however, for reasons that are not completely clear, a sizable proportion of cancers fail to respond. It is apparent that the constellation of oncogenic events that leads to full neoplastic transformation can influence the effector function of the immune response in several ways. Oncogenic RAS can promote expression of various cytokines (Ancrile et al., 2008) that result in an inflammatory response, which is thought to promote cancer progression. RAS signaling also increases tumor cell expression of PD-L1 (Coelho et al., 2017), thereby suppressing activated T cells, and increases secretion of granulocyte-macrophage colony-stimulating factor (GM-CSF) to promote pancreatic neoplasia (Bayne et al., 2012; Pylayeva-Gupta et al., 2012). The co-activation of KRAS and MYC in lung tumors restructures macrophage and T cell responses in a CCL9 and interleukin-23 (IL-23)-dependent manner (Kortlever et al., 2017). Loss of PTEN, another common oncogenic event, results in resistance to PD-1 blockade in both melanoma and uterine leiomyosarcoma (George et al., 2017; Peng et al., 2016), while β-catenin signaling in melanoma was shown to limit T cell infiltration (Spranger et al., 2015).

p53 is best understood as a tumor suppressor (Hollstein et al., 1991). However, in the immune compartment, p53 also functions as a negative regulator of autoimmunity by supporting regulatory T (Treg) cells, through directly upregulating Foxp3 and promoting STAT5 activity, and restricting STAT3 in the pro-inflammatory helper T cells (T helper 17 [Th17] cells) (Kawashima et al., 2017; Peng et al., 2016), while β-catenin signaling in melanoma was shown to limit T cell infiltration (Spranger et al., 2015). In the context of cancer, activation of p53 in the tumor stromal compartment has been shown to promote a tumor-restricting immune response. Induction of p53 in hepatic stellate cells (HSCs) results in senescence and the senescent-associated-secretory phenotype (SASP) that drives M1-macrophage polarization and limits cancer progression (Lujambio et al., 2013). Conversely, HSCs lacking p53 induce the differentiation of macrophages toward the tumor-promoting M2 state (Lujambio et al., 2013). Stromal loss of p53 changes the cytokine secretion pattern to promote myeloid-derived suppressor cells (MDSCs), thereby accelerating tumor growth (Guo et al., 2013). Interestingly, activation of p53 in the tumor microenvironment using local injection of the MDM2 inhibitor Nutlin selectively eradicated tumors that were rich in leukocytes. This response was dependent on stromal-p53 expression (Guo et al., 2017). These studies show that p53 levels in the stroma shape the inflammatory responses that influence tumor progression.
Despite the clear role of p53 in immune regulation, relatively few studies have examined how p53 status of the cancer cells affects the immune response in vivo. Reactivation of p53 in established liver cancers induced senescence and SASP, which promoted polymorphonuclear (PMN) infiltration and tumor regression (Xue et al., 2007). Further studies showed that the induction of p53-dependent senescence in hepatocellular carcinomas was accompanied by the elimination of the tumor cells through a mechanism dependent on natural killer (NK) cells (Iannello et al., 2013). *In silico* correlations between the retention of wild-type (WT) p53 expression and immune infiltration in breast and head and neck cancers have also been noted (Siemers et al., 2017). However, a recent study of a PTEN-driven prostate cancer model indicated that concomitant loss of p53 enhanced tumor infiltration of CD11b+Gr1+ PMN cells. The recruitment of this myeloid population was through increased CXCL17 secretion by p53-null prostate cancer cells, and their role in promoting tumor development was associated with the expansion of immunosuppressive Treg cells (Bezzi et al., 2018). Similar findings were observed in mouse models of breast cancers, where loss of p53 increased frequencies of circulating and tumor neutrophils through unchecked WNT signaling, resulting in enhanced metastasis (Wellenstein et al., 2019).

In this study, we show that tumor-specific loss of p53 expression in both autochthonous lung and pancreatic tumor models correlates with changes in the tumor microenvironment. Using KRAS-driven pancreatic-tumor-derived cancer cells as a model of p53 loss, we demonstrate that p53 deletion can promote immune tolerance through the recruitment of both myeloid cells and Treg cells. The enrichment of these suppressive populations results in enhanced protection of p53-null cancer cells from immune-mediated elimination. Furthermore, concomitant activation of KRAS and loss of p53 coordinate to promote immune tolerance.

**RESULTS**

**Loss of Trp53 Promotes Myeloid Recruitment in the Tumor Microenvironment**

Tumor growth involves a complex interaction between stromal cells (of mesenchymal and immune origin) and cancer cells. Numerous studies have shown a role for macrophages in supporting cancer progression (Cassetta and Pollard, 2018; Noy and Pollard, 2014; Prenen and Mazzone, 2019; Gian and Pollard, 2010), and we examined whether loss of p53 in autochthonous mouse models of pancreatic and lung cancers could influence myeloid cell recruitment to the tumor microenvironment (TME). Immunohistochemistry (IHC) sections were analyzed for F4/80+ immune cells in pancreatic tumors derived at equivalent endpoints from a pancreatic ductal adenocarcinoma cell (PDAC) model driven by pancreas-specific mutations in KRAS(G12D) with either wild-type p53 (KC model; Pdx1-cre; LSL-KrasG12D/+), or one floxed Trp53 allele (KFC model; Pdx1-cre; LSL-KrasG12D/+; Trp53fl/fl) (Tan et al., 2014) (Figure 1A, left). Tumors derived from KRAS pancreatic tumors revealed increased macrophage F4/80+ staining ([Figure 1A](#)), while we examined an epidermal growth factor receptor (EGFR)-driven model of lung cancer with ROSA26tTA-LSL-EGFRL858R; Trp53fl/fl, EFL). Tumors excised at similar endpoints were digested and stained for flow cytometry to detect CD11b+ and F4/80+ tumor immune infiltrates. Frequencies of CD11b+ myeloid-derived cells were significantly increased in EGFR-driven tumors null for p53, which were also enriched in CD11b+F4/80+ macrophages ([Figure 1B](#)).

To gain insight into the interplay between macrophages and the p53 status of cancer cells, we isolated primary cancer cells from three independent KC and KFC tumors. KC-tumor-derived cells lines retained p53 expression and activity, showing growth inhibition in response to the p53 activator Nutlin, while cell lines derived from KFC tumors did not express p53 and were resistant to Nutlin treatment (Figures S1A and S1B). Previous studies have also shown that KFC tumors undergo loss of heterozygosity and become p53 null during tumor development (Tan et al., 2014). Reexpression of p53 in tumors can induce their ability to produce various inflammatory cytokines (Iannello et al., 2013), prompting us to examine whether conditioned media from the KC- and KFC-tumor derived cells could impact macrophage surface activation markers: major histocompatibility complex (MHC) class I, MHC class II, PD-L1, and CD80. In comparison to untreated bone-marrow-derived macrophages (BMDMs), conditioned media from all PDAC cell lines induced expression of all the activation markers to similar levels ([Figure S1C](#)), although this was not affected by p53 status. As both our autochthonous models (pancreas and lung) displayed a p53-associated change in macrophage infiltration, we examined the effect of conditioned medium on BMDM migration and chemotaxis. Interestingly, BMDMs exposed to conditioned media from KFC-derived cell lines displayed increased migration, demonstrated by enhanced wound healing and chemotaxis, compared to conditioned media from KC cell lines ([Figures 1C, 1D, S1D, and S1E](#)).

To explore the basis of this difference in the activity of the conditioned media, we analyzed cytokine secretion from three independently derived p53WT KC and p53-null KFC tumor-derived cell lines. Out of the 35 cytokines tested by a cytokine Luminex array, 20 were detectable and only 5 (CCL11, CXCL1, CXCL5, CCL3, and macrophage colony-stimulating factor (M-CSF)) were elevated in the three KFC cell lines ([Figure 1E](#)). While these myeloid-attracting and differentiating cytokines were elevated, T-cell-associated cytokines were not dependent on p53 expression ([Figure S1F](#)). Taken together, these results indicate that the KFC tumors secrete cytokines that have a myeloid-macrophage stimulating effect.

To understand the consequences of the p53 status of our tumor-derived cell lines on the myeloid compartment in vivo, we used a T-cell-deficient CD1+nu/nu subcutaneous tumor model. In order to track accurately and in an unbiased fashion in vivo tumor growth, KC and KFC cells were engineered to express near-infrared fluorescent protein (iRFP) (Hock et al., 2014; Scherbakova and Verkhuza, 2013) ([Figure 1F](#)). While the initial GEMM KC and KFC tumors arise at different rates, cells derived from these tumors grew at similar rates in CD1+nu/nu recipients, as assessed by *in vivo* real-time imaging of the tumors ([Figures 1G, S1G, and S1H](#)). Flow cytometry analysis of tumor digests at endpoint revealed no changes in total frequency of CD11b+ myeloid infiltration; however, CD11b+F4/80+ macrophages were enhanced in KFC tumors, as seen in the autochthonous
Figure 1. Cancer-Specific p53 Loss Increases Macrophage Infiltration, Chemokine Secretion, and Migration

(A) Immunohistochemical stain for F4/80 expression in pancreatic tumor sections from Pdx1-Cre;Kras<sup>LSL-G12D</sup>/+ (KC) (left) and Pdx1-Cre;Kras<sup>LSL-G12D</sup>/+;Trp53<sup>fl/+</sup> (KFC) (right) mice. F4/80<sup>+</sup> expression was evaluated based on color intensity per section. Scale bar at 1 μm. Each point on the graphs represents one mouse; cohort size n = 5, the means are represented as ± SEM.

(B) Lung tumors induced by adenoviral Cre were assessed by flow cytometry for CD11b<sup>+</sup> and F4/80<sup>+</sup> cell infiltrates from mice bearing the following genotypes: ROSA26tTA-LSLEGFRL858R (EL) (gray) and Rosa26tTA-LSLEGFRL858R;Trp53<sup>fl/fl</sup> (EFL) (red). Cohort sizes n = 8–9; the means are represented as ± SEM.

(C and D) Migration and chemotaxis assays using IncuCyte technology with bone-marrow-derived macrophages (BMDMs) cultured in the presence of conditioned media from PDAC-derived cell lines from KC1 (black) and KFC1 (red) tumors. The means are represented as ± SD of technical replicates (n = 6–8).

(C) Scratch-wound assay performed on BMDMs to measure wound closure.

(D) Chemotaxis assay of BMDMs migrating toward conditioned media of KC1 or KFC1 cancer cells.

(E) Luminex cytokine array performed on three independent KC and KFC cell lines derived from mouse PDACs. Values are represented as fold change in concentration compared to one of the PDAC-derived cell lines (KC1), and the means are represented as ± SEM.

(F) Schematic representation of the experimental design. Pancreatic derived cancer cell lines (KC1 and KFC1) were transduced with a near-infrared plasmid (iRFP) and subcutaneously injected into CD1<sup>nu/nu</sup> recipient mice.

(G) Growth curve represented as fold increase in iRFP fluorescence measured in real time by the Pearl imager over 12 days for KC1 (black circle) and KFC1 (red circle) cell lines injected into CD1<sup>nu/nu</sup> recipient mice (cohort size n = 5 per genotype). Two-way ANOVA was used for statistical analysis and the means are represented as ± SEM.

(H) Flow cytometry analysis of individual tumor digests for immune infiltrates expressing CD11b<sup>+</sup> and F4/80<sup>+</sup> surface markers. The means are represented as ± SEM, with cohort sizes n = 7–9.

Student’s unpaired t test was performed when not otherwise indicated, and p values are *p < 0.05 and **p < 0.01. See also Figure S1.
mouse models (Figures 1H and S1). These results demonstrate that p53 ablation in the tumor can influence the TME independently of tumor growth.

p53 Deletion Delays Tumor Rejection and Promotes Myeloid-Associated Cytokines

Loss of p53 in the tumor-derived cell lines did not accelerate subcutaneous tumor growth in athymic recipients, despite the increase of CD11b+ F4/80+ infiltrates in the TME. We therefore turned to examine the effect of p53 loss in an immunocompetent model. Since our tumor-derived cells originated from mice of a mixed background, we used a tumor rejection model that has been previously employed to explore contributions of tumor-associated genetic alterations on a complete immune response (Chang et al., 2015; Dunn et al., 2005). Mixed strain KC- and KFC-derived cells were injected subcutaneously into pure FVB recipient mice, an immunocompetent MHC-mismatched strain (Figure 2A). The p53WT KC1 cells followed the expected growth kinetics of initial expansion until day 7, followed by progressive rejection. Surprisingly, the p53-null KFC1 cells continued to grow until the point where ethical considerations determined the termination of the study (Figure 2B). A similar pattern of delayed rejection in p53-null cells was seen in two further independently derived KFC cells (Figures S2A and S2B). Further examination of the tumor digests taken from these mice at day 7 showed an increase in F4/80+ macrophages in p53-null (KFC1) tumors (Figure 2C). In order to confirm that the p53-dependent effect on tumor-associated myeloid cells was not site specific, we performed pancreatic orthotopic injections of KC1 and KFC1 cell lines in FVB mice. In line with the genetic tumor models and the subcutaneous models (Figures 1A, 1H, and 2C), p53-null orthotopic tumors displayed increased myeloid infiltration (Figure 2D).

One potential caveat with tumors arising in genetically engineered mice is that additional genetic alterations acquired during tumor development may also contribute to tumor-immune interactions. To test directly the effect of p53 loss, we used gene editing to delete p53 from two KC-p53WT cell lines (KC1 and KC2), generating matched isogenic cell lines named KFC1-p53KO and KFC2-p53KO (Figure 2E). BMDM chemotaxis assays using conditioned media from KC1-p53WT and KC1-p53KO PDAC cell lines confirmed our previous findings that KFC-p53KO conditioned media enhanced BMDM migration (Figure 2F). ELISAs for CCL11, CXCL1, CXCL5, CCL3, M-CSF, and MCP1 (Figure 2G) showed that as seen in the tumor-derived KFC cell lines, p53 loss increased production of these cytokines (Figures 2G and S2C). Additionally, we performed an unbiased analysis of 62 cytokines in conditioned media from KC1-p53WT and KC1-p53KO cells, which further revealed increased CXCL10 and 11, and decreased tumor necrosis factor α (TNF-α) secretion (Figure 2H). Of note, CXCL10 and 11 uniquely bind to the CXCR3 receptor. Our results show that cancer cells defective for p53 increase the production of chemokines involved in myeloid recruitment and macrophage differentiation.

Turning back to the in vivo rejection model, we found that the isogenic p53KO cells displayed delayed rejection kinetics, similar to...
Figure 3. Tumoral Loss of p53 Promotes Suppressive Myeloid Lineages and Reduces T Cell Activation

(A) CD11b+ cells isolated from individual KC1-p53WT (gray shade) and KC1-p53KO (black line) tumors 3 days post-injection and co-cultured with pre-activated CD8+ T cells stained with V450 proliferation dye. Cells were analyzed after 48 h. T cell proliferation in the absence of CD11b+ cells is denoted in green.

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to PDAC cells that lost p53 during tumor development (Figures 2I and S2D). At an early time point (3 days post-injection), CD11b+ myeloid cells were enriched in p53-deleted tumors in comparison to their parental p53WT controls (Figure 2J), although this difference was lost by day 7 (Figure S2E). Next, we looked more closely for potential in vivo consequences of the changes in cytokine secretion detected in vitro. M-CSF is a key driver of myeloid differentiation and proliferation, and we detected a 2-fold increase of proliferating CD11b+Ki67+ myeloid cells in p53KO tumors (Figures 2K and S2F), correlating with enhanced M-CSF production by these cells. We also probed for myeloid cells expressing CXCR3 (the receptor for CXCL9-11) and CCR2 (one of the receptors for CCL11 and uniquely a receptor for MCP1) (Martini et al., 2001) within our tumor digest. Interestingly, both CXCR3+ and CCR2+ infiltrating CD11b+ myeloid cells were significantly enhanced within KC1-p53KO tumors (Figures 2L, 2M, and S2G), correlating with increased production of the cytokines for these receptors by the p53-deleted tumor cells. We further confirmed an increased in intra-tumoral CD11b+ cells expressing CXCR3 in the second isogenic tumor pair at day 3 (Figure S2H). Systemic cytokine changes were also observed in tumor-bearing mice. At endpoint (day 14 post-tumor challenge), mice harboring p53-null tumors harbored significantly increased serum levels of MCP1 (Figure 2N). In conclusion, data from both GEMM-derived cells and the isogenic pairs show that loss of p53 in tumor cells promotes the production of cytokines involved in myeloid recruitment and homeostasis.

**p53-Null Tumors Reduce Myeloid Cells to Attenuate T Cell Responses**

Our observation that loss of p53 affects tumor growth in immunocompetent, but not athymic, mice indicated that an intact T cell response is required for the rejection of p53-expressing cells. To test whether the CD11b+ cells found in the KC1-p53KO tumors were immune suppressive, we isolated CD11b+ cells from the tumors at day 3 and co-cultured them with preactivated T cells stained with the v450 proliferation dye. While the myeloid cells derived from both tumors attenuated T cell proliferation, the myeloid cells isolated from KC1-p53KO tumors more robustly inhibited CD4+ and CD8+ T cell proliferation (Figures 3A, 3B, S3A, and S3B). These observations were confirmed in the second pair of isogenic cells (Figures S3C and S3D).

Cancer cells have been shown to influence the crosstalk between myeloid cells and T lymphocytes (Cook et al., 2018), so we examined the effect of conditioned media from KC1-p53WT and KC1-p53KO cells on BMDM-dependent T-cell-activating functions (Figure 3C). Focusing on cytokines involved in CD4+ T helper and CD8+ cytotoxic T lymphocyte (CTL) differentiation, we found only two of the seven cytokines showed significantly changed mRNA expression in response to KC1-p53KO conditioned media; namely, a strong increase in expression of Il6, a Th1 helper 1 (Th1) cell antagonist, and a subtler decrease in expression of Il12a, a Th1 cell- and CTL-promoting cytokine (Figure 3D). These changes predict that BMDMs conditioned by KC1-p53KO media would show impaired polarization of CD4+ Th1 cells and CTL activation (Anhe-Morales et al., 2004; Dodge et al., 2003; Wu et al., 2015). To verify this hypothesis, we co-cultured BMDMs pulsed with the model antigen ovalbumin (OVA) with T cell receptor (TCR)-transgenic CD4+ or CD8+ T cells recognizing the OVA peptides (OTI [CD4+] or OT1 [CD8+] T cells). BMDMs educated by KC1-p53KO conditioned media and loaded with OVA peptide were less effective in differentiating Th1 cells, as demonstrated by reduced interferon-γ (IFN-γ) TNF-α+ producing OTI CD4+ T cells (Figure 3E). Furthermore, CTL activation was compromised by KC1-p53KO-instructed BMDMs, as shown by the weaker expression of the activation marker
Figure 4. CSF1R Blockade Increases the Activation of Intratumoral T Cells in p53-Null Tumors but Depends on Treg Cell Depletion for Regression

(A) Scheme of CSF1R blockade administration and subcutaneous growth in FVB mice.
CD44 on CD8+ OTI cells (Figure 3F). Led by our mouse data, we stratified pancreatic cancer patients from The Cancer Genome Atlas (TCGA) dataset according to a designed gene list based on classical MDSC markers, including ITGAM (CD11b) and CXCL10 (Figure S3E). Patients with a high MDSC signature displayed statistically lower overall survival (Figure S3F). Interestingly, patients with a high MDSC gene signature trended toward an enrichment of TP53 mutations compared to the low-MDSC-signature population (Figure S3G). Taken together, these data demonstrate that p53 deletion in cancer cells creates a tumor-promoting environment through remodeling myeloid-T cell crosstalk.

Encouraged by our in vitro and ex vivo results, we assessed T cell activity in mice bearing GEMM-derived or isogenic p53WT and p53-null tumors. Ex vivo restimulation of the spleen and tumor draining lymph node (dLN) on day 7, when tumors were of equivalent sizes, revealed a reduction in anti-tumor T cell responses (as measured by CD4+ Th1 TNF-α and IFN-γ+ cells) in mice bearing p53-null tumors from either isogenic (Figures 3G, 3H, and S3H) or the GEMM-derived lines (Figures S3I and S3J). Furthermore, sorted CD4+CD25+ T cells from mice bearing KC1-p53KO tumors expressed less IL2 and Ifng mRNA than CD4+CD25+ T cells from KC1-p53WT recipients (Figure 3I), suggesting an overall lack of CD4+ T cell activation. Impaired CD8+ CTL IFN-γ production further demonstrated weakened T cell responses in mice carrying p53-null tumors (Figures 3J and S3K–S3M). Overall, our data suggest that p53 deletion in cancer cells underlines T cell effector responses, at least in part through co-opting myeloid cell functions.

Combination Therapy of CSF1R and CD25 Blockade Attenuates p53-Null Cancer Cells

Our in vitro data suggested that the dampened T cell responses seen in mice bearing p53KO tumors may be a consequence of pro-tumorigenic myeloid cells responding to elevated M-CSF from p53-ablated cancer cells (Figure 2G). To test the contribution of M-CSF production by p53-null cells to the modulation of the T cell response in vivo, we treated immunocompetent mice challenged with either p53WT or p53-null tumor cells with neutralizing antibodies against CSF1R, the receptor for M-CSF (Figure 4A). CSF1R specificity was confirmed by showing the expected compensatory increase in serum M-CSF levels in treated mice (Bartocci et al., 1987), with no change in serum granulocyte colony-stimulating factor (G-CSF) (Figures S4A and S4B). Subcutaneous tumors were harvested at day 7, and effector function of infiltrating CD4+ and CD8+ T cells was assessed. Consistent with results from spleen and dLN of tumor-bearing mice, vehicle-treated, p53-null tumor-bearing mice contained less activated effector CD4+ and CD8+ T cells secreting IFN-γ and TNF-α (Figures 4B, 4C, and S4C–S4E). CSF1R blockade restored T cell function in p53-null tumors to levels seen in p53-WT tumors (Figures 4B, 4C, and S4C–S4E) but did not change the rate of rejection of tumors of either genotype (Figure 4D).

Furthermore, T cell reactivation in p53-null tumors was irrespective of absolute tumor size, as confirmed by in vivo imaging of the tumors (Figure S4F). These data suggest that inhibition of M-CSF signaling that is induced by p53-deleted cells can reactivate T cell function within the tumor but fails to promote rejection when used as a monotherapy.

The failure of CSF1R blockade to promote tumor rejection prompted us to investigate other mechanisms of immune suppression that may reflect additional effects of tumor cells on T cell function. One essential immunosuppressive population that is at the epicenter of T cell immune tolerance are Treg cells (Sakaguchi et al., 2008), which are currently being targeted for cancer immunotherapies (Tanaka and Sakaguchi, 2017). Flow cytometry analysis of the original untreated tumors showed an accumulation of Treg cells within p53-null tumors with no changes in CD4+ T cell frequencies (Figure 4E). Tumors deleted for p53 were biased toward Treg cells, as assessed by the Treg cell/non-Treg cell ratio of CD4+ T cell infiltrates (Figures 4E and 4F). The inability of CSF1R blockade to induce intra-tumoral Treg cell frequencies (Figure 4G) led us to perform a double blockade targeting both CSF1R and Treg cells using αCD25 to eradicate Treg cells. This method is well established to effectively deplete Treg cells (Onizuka et al., 1999; Setiady et al., 2010; Shimizu et al., 1999), although various studies have shown
CD25 blockade alone does not effectively block the growth of established tumors (Arce Vargas et al., 2017; Onizuka et al., 1999). Using a combined treatment of αCD25 for 3 days prior to tumor challenge followed by a αCSF1R regime, we were able to attenuate the growth of the p53-null tumors in FVB recipients (Figure 4H). These intervention data suggest that immunotherapy for p53-compromised tumors requires targeting both the myeloid and Treg cell compartments for a positive outcome.

Finally, we examined the consequences of direct interactions between CD8+ cytotoxic T lymphocytes (CTLs) and cancer cells. CTL cytotoxic function is elicited by IFN-γ and granzyme B (GzmB) production and can be affected by cancer cells (Fischer et al., 2007). CTLs co-cultured with KC-p53WT and KFC-p53KO cells were equally capable of secreting IFN-γ (Figure 4I). However, co-culture with KFC-p53KO cells clearly impaired CTL GzmB production upon restimulation (Figure 4J). Our observation that targeting Treg cells in parallel with CSF1R was critical in reducing tumor growth of p53-null cells prompted us to examine the effect of the p53 status of cancer cells on regulatory T cells more closely. Treg cells have different degrees of suppressive abilities, which can be phenotypically identified through surface expression of CD25, GITR, and KLRG1 (Arpaia et al., 2015). Mice bearing KFC1-p53-null tumors displayed increased systemic presence of CD4+FOXP3+ CTLA-4+ T cells in the spleen and dLN (Figures S5A and S5B), suggesting a greater suppressive immune response. To determine whether the skewing toward more suppressive Treg cells depended on the site of the tumor, we orthotopically implanted KC1 and the KFC1 cell lines into the pancreas of FVB recipients. Tumors of both genotypes were topically implanted KC1 and the KFC1 cell lines into the pancreas of FVB recipients. Tumors of both genotypes were topically implanted KC1 and the KFC1 cell lines into the pancreas of FVB recipients. Tumors of both genotypes were topically implanted KC1 and the KFC1 cell lines into the pancreas of FVB recipients. Tumors of both genotypes were topically implanted KC1 and the KFC1 cell lines into the pancreas of FVB recipients. Tumors of both genotypes were topically implanted KC1 and the KFC1 cell lines into the pancreas of FVB recipients. Tumors of both genotypes were topically implanted KC1 and the KFC1 cell lines into the pancreas of FVB recipients. Tumors of both genotypes were topically implanted KC1 and the KFC1 cell lines into the pancreas of FVB recipients. Tumors of both genotypes were topically implanted KC1 and the KFC1 cell lines into the pancreas of FVB recipients. Tumors of both genotypes were topically implanted KC1 and the KFC1 cell lines into the pancreas of FVB recipients. Tumors of both genotypes were topically implanted KC1 and the KFC1 cell lines into the pancreas of FVB recipients. Tumors of both genotypes were topically implanted KC1 and the KFC1 cell lines into the pancreas of FVB recipients. Tumors of both genotypes were topically implanted KC1 and the KFC1 cell lines into the pancreas of FVB recipients. Tumors of both genotypes were topically implanted KC1 and the KFC1 cell lines into the pancreas of FVB recipients. Tumors of both genotypes were topically implanted KC1 and the KFC1 cell lines into the pancreas of FVB recipients. Tumors of both genotypes were topically implante

CancerCell-Associated Loss of p53 Selects for Suppressive Regulatory T Cell Lineages

Our observation that targeting Treg cells in parallel with CSF1R was critical in reducing tumor growth of p53-null cells prompted us to validate the p53 specificity of these Treg cell responses, we turned again to the isogenic KC1-p53WT and KC1-p53KO cells. Identical CD4+ T cell frequencies were present in the tumor dLN of both KC1-p53WT and KC1-p53KO recipients (Figure 5A). However, the proportion of FOXP3+ Treg cells present within the CD4+ population was enhanced in mice bearing KC1-p53KO cancer cells (Figure 5B). Phenotypic characterization of these Treg cells revealed an increase in the proportion expressing markers of suppression (high CD25, GITR, and KLRG1 expression) (Figures 5C and S5E). In addition, while p53 status did not affect total CD4+ T cell frequency in the tumor (Figure 5D), KC1-p53KO tumors accumulated more FOXP3+ Treg cells, especially those with KLRG1 surface expression (Figure 5E).
Figure 6. KRAS Coordinates with p53 Loss for the Recruitment of Myeloid, but Not Treg, Cells

(A) Schematic representation of the inducible iKRAS^{G12D}, p53^{lo} PDAC cell model (iKRAS^{G12D}).

(B) Growth kinetics of the iKRAS^{G12D} cell line (red circles) expressing iRFP subcutaneously injected into FVB recipients. Doxycycline (Dox) was maintained (red circles) or removed at day 7 (open circles) to modulate mutant KRAS expression. Tumor growth was measured using in vivo imaging using the Pearl imager and expressed relative to initial iRFP fluorescence at day 1 post-injection. Cohort sizes n = 10 and the means are represented as ±SEM.

(C–E) Analysis of individual tumor infiltrates of myeloid and T cell subsets 48 h post-Dox withdrawal. Cohort sizes n = 8–12 and the means are represented as ±SEM.

(C) Analysis of CD11b+, CD11b+F4/80+, and CD11b+CXCR3+ tumor-associated myeloid cells analyzed by flow cytometry (open circles Dox OFF and red circles Dox ON).

(D) Flow cytometry analysis of CD4+ and CD8+ T cell infiltrate frequencies.

(E) Analysis of intra-tumoral CD4+ T cell post ex vivo restimulation for expression of TNF-α (left) and CD4+FOXP3+KLRG1+ (right).

(F) Schematic representation of experimental design. C57Bl6/J-derived ID8 ovarian cancer cell lines were deleted of p53 using CRISPR/Cas9 and injected intraperitoneally into syngeneic recipients.

(G–I) Flow cytometry analysis of ascites and omentum at equivalent endpoints from mice bearing ID8-p53WT (black circles) and ID8-p53KO (open circles) tumors.

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Consistent with these data, ex vivo Treg cell suppression assays of CD4+/CD25+ Treg cells sorted from tumor-bearing mice showed that Treg cells isolated from KC1-p53KO recipients were more efficient at inhibiting the proliferation of CD4+/CD25+ T cells in vitro (Figures 5F and S5F). Both our in vitro characterization and ex vivo functional assays highlight the relationship between tumor p53 loss and the local and systemic accumulation of regulatory T cell responses.

**KRAS Mutations Coordinate with p53 Loss for Myeloid, but Not Treg, Cell Recruitment**

In humans, nearly 90% of pancreatic cancer patients show mutations in KRAS (cBioportal) (Cerami et al., 2012), and our autochthonous models were based on mutations in KRAS or in upstream signaling receptors (EGFR). Recent work has shown that activating KRAS mutations can drive an immunosuppressive response in cancer cells through increased PD-L1 expression (Coelho et al., 2017). In order to examine the role of KRAS activation in promoting tumor tolerance in the context of p53 loss, we used previously described doxycycline (Dox)-inducible KRASG12D-driven p53-null PDAC mouse cells (Ying et al., 2012) (Figure 6A). Tumors were initiated for 7 days in FVB recipient mice treated with Dox to drive mutant KRAS expression before either maintaining Dox treatment (iKRASG12D-ON) or removing Dox (iKRASG12D-OFF). Despite the lack of p53 in all of these tumor cells, removal of Dox resulted in complete eradication of the tumor within 7 days (Figure 6B). We evaluated early and late tumor-associated immune infiltrates at 48 h and 5 days post-Dox withdrawal. Within 48 h of Dox removal, iKRASG12D-OFF tumors displayed reduced frequencies of CD11b+ cells, especially those positive for F4/80 and CXCR3 expression (Figure 6C), with a modest influx of CD4+ T cells but no changes in CD8+ T cell frequencies (Figure 6D). At this time point, iKRASG12D-ON and iKRASG12D-OFF tumors showed no difference in CD4+ T effector cells or CD4+FOXP3+KLRG1+ suppressive Treg cells (Figure 6E).

Evaluation of peripheral T cells in recipient mice 5 days after Dox removal showed a significant increase of pro-inflammatory CD4+ and CD8+ T cells in the spleen and a trend toward an increase in the dLNs under iKRASG12D-OFF conditions (Figures S6A and S6B). There was no overall change in suppressive Treg cell populations (Figure S6C). These data suggest that KRAS inactivation in part reactivates T effector immune responses but has no impact on the accumulation of regulatory T cells. To further explore the cooperation between p53 loss and KRAS activation, we turned to a previously described model of isogenic C57Bl6/J ID8 ovarian cancer cells with and without p53, which are WT for KRAS (Walton et al., 2016). These cells were injected into the peritoneum of syngeneic C57Bl6/J mice (Figure 6F), and as we previously published, there was a skewing toward PMN cells present in the ascites of mice carrying p53KO tumors (Walton et al., 2016). Interestingly, while there was no change in the total frequency of CD4+ T cells (Figures S6D and S6E), the ascites from mice injected with ID8 p53KO cells showed a 2-fold increase of regulatory T cells (Figures S6G and S6H) and a preferential skewing toward Treg cells, as illustrated by the FOXP3+ to FOXP3− ratio (Figure S6F). A major site of ovarian cancer spread is to the omental fat or the omentum (Worzfeld et al., 2017). Processing and staining of omental tumors for flow cytometry revealed enriched populations of regulatory T cells present in p53-ablated lesions (Figure 6I). These results further support a model in which p53 loss in tumors promotes Treg cell infiltration.

**DISCUSSION**

In this study, we showed that p53-deficient tumors can re-orchestrate the innate immune response through suppression of effector CD4+ and CD8+ T cells, which reflected an increase in the inhibitory actions of myeloid suppressor cells and regulatory T cells, accompanied by a direct impairment of GzmB in CD8+ T cells. Other studies have shown that mutations in p53 correlate with reduced GzmB in gastric cancers (Jiang et al., 2019) and a CSF1 (M-CSF) response signature in breast cancers (Beck et al., 2009). Somatic changes in the tumor can also induce systemic changes beyond the tumor microenvironment, and we showed dampened local and systemic T cell effector function in mice harboring p53-deficient tumors, seen as decreased IFN-γ and TNF-α production (Figure 3). We propose that these weakened T cell responses are partially mediated by suppressive myeloid cells derived from the tumor as well as through Treg cell suppressive functions. Conditioned media from p53-null cancer cells alters the cytokine profile of BMDMs, which in turn influences CTL and Th1 cell differentiation (Figure 3). These observations are consistent with previous publications showing that tumor-derived CD11bGr+ cells promote the de novo differentiation of Treg cells in prostate cancer models and reduce circulating IFN-γ-producing T cells in B cell malignancies (Bezzi et al., 2018; Christopoulos et al., 2011). Systemic changes were also seen in p53-deficient breast cancer models, which also showed an increase in MCP1 serum levels similar to those seen in our p53-null tumor-bearing mice (Wellenstein et al., 2019).

It is important to note that unlike the response to reexpression of p53 in p53-null cancers, the p53-expressing cells in our study did not undergo senescence and maintained proliferative ability that was similar to the p53-null cells both in vitro and in vivo (Figure 1). Recent studies have attributed changes in cytokine profiles to oncogenic drivers, such as mutation in KRAS, MYC, and loss of PTEN (Coelho et al., 2017; Kortlever et al., 2017; Peng et al., 2016; Pylayeva-Gupta et al., 2012). Our data suggest that one consequence of p53 loss is enhanced secretion of cytokines involved in myeloid recruitment and proliferation. Our work suggests the loss of p53 in tumors has a complex effect.

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(G) CD4+FOXP3+ Treg cells in the ascites. Cohort size n = 4; means are represented as ±SEM.
(H) Representative plot of CD4+ T cells expressing FOXP3 and CD44.
(I) CD4+FOXP3+ T cells in tumor-immune infiltrates in the omentum Cohort size n = 9–10; means are represented as ±SEM. A two-way ANOVA was used to analyze in vivo growth kinetics, and an unpaired Student’s t test was used for all bar graphs. p values are *p < 0.05, **p < 0.01, and ***p < 0.001. See also Figure S6.
on cytokine secretion of both tumor and macrophage populations, 
ultimately affecting the T cell response. These observations 
complement recent work showing that an increase in 
WNT secretion in response to p53 loss in breast cancer cells 
can stimulate systemic inflammation and drive metastasis (Weil-
enstein et al., 2019). The complexity of the immune response to 
p53 loss is further highlighted by a recent study showing that loss 
of p53 in a prostate cancer model increases expression of 
CXCL17 and CXCL5 and consequent attraction of tumor-promo-
ting Gr-1+CD11b+ cells (Bezzi et al., 2018). Consistent with 
our study, loss of p53 in the prostate model also led to an 
increased expression of CXCL9 and CXCL10 (Bezzi et al., 
2018). Although we were unable to identify any changes in the 
intrinsic ability of p53-null cancer cells to present antigen, previ-
ous studies have shown p53-dependent alterations in antigen 
presentation (Wang et al., 2013; Zhu et al., 1999), and it remains 
possible that cell-intrinsic changes also play a role in the immune 
response to p53 loss.

Although blockade of the M-CSF receptor, CSF1R, produced 
a positive effect in allowing infiltration of T cell effector function in 
the p53-null tumors, this was not sufficient for rejection. This 
observation dovetails with a previous study targeting CSF1R 
with PD-L1 in an orthotopic pancreatic model (with mutations 
in KRAS and loss of INK) to increase the efficacy of PD-L1 
blockade (Zhu et al., 2014). Blocking CSF1R by chemical target-
ing in the p53R172H mutant KRAS pancreatic mouse model has shown a 
2-week increase in survival and, as we also showed, an increase 
in T cell effector function (Candido et al., 2018). In addition, 
macrophage depletion with clodronate and CSF1R inhibition 
did not provide therapeutic benefit to primary tumors in the 
KPC pancreatic cancer model, similar to our results (Griesmann 
et al., 2017). Given the complexity of the immune modulatory 
response to loss of p53, it is not surprising that reversal of only 
one part of the response is insufficient to fully regain tumor rejec-
tion, since CD11b+ and Treg cell populations remained enriched 
in p53-null tumors. By targeting both suppressive populations 
through double blockade with anti-CSF1-R and anti-CD25, we 
were able to diminish the growth of p53-null tumors in an 
MHC-mismatched recipient. Indeed, our data show that the 
enhanced Treg cell infiltration into p53-null tumors is not pre-
vented by CSF1R blockade, highlighting the importance of a 
combination immunotherapy.

MSDCs and Treg cells are two major immune-suppressive 
populations hijacked by many cancer cells. MDSCs arise and 
expand under pathological conditions, especially during cancer. 
Naturally occurring Treg cells are a heterogeneous population, 
with different surface markers predicting suppressive capacity 
(Cheng et al., 2012). In both our subcutaneous and orthotopic 
models, we detect an enrichment of KLREG1+ and CLTA4+ Treg 
cells in p53-null tumor-bearing mice. KLREG1+ Treg cells are 
considered a highly suppressive and terminally differentiated 
population of Treg cells that arise during tissue repair, such as 
influenza-induced lung injury, and help in tissue regeneration 
(Arpaia et al., 2015). Consistently, Treg cells isolated from recip-
ients harboring p53-deficient tumors displayed increased sup-
pressive capacity compared to their WT counterparts, dovetail-
ing with a similar observation in PTEN- and p53-null prostate 
tumors (Bezzi et al., 2018).

Pancreatic and lung tumors harboring mutations in KRAS 
also frequently acquire mutations in p53. While loss of p53 per-
mits the proliferation of mutant-KRAS-expressing cells, which 
would otherwise undergo senescence, our data also demon-
strate cooperation between KRAS and p53 mutations in allow-
ing cancer cells to evade the immune response. Previous 
studies on pancreatic cancer have focused on the effects of 
mutant KRAS in pancreatic duct epithelial cells (PDECs) and 
in combination with mutant p53 in PDAC models, where GM-
CSF was a contributing factor for neutrophil recruitment to the 
tumor (Bayne et al., 2012; Pylayeva-Gupta et al., 2012). Inter-
estingly, while neutrophils were enriched in orthotopically im-
planted KRASG12D PDECs, there was no change in Treg cell 
filtration (Pylayeva-Gupta et al., 2012), supporting our obser-
vation that p53 plays a more important role in modulating Treg 
cells in cancer. The selection for suppressive Treg cells seems 
to depend predominantly on p53 loss rather than the coordi-
nated overexpression of mutant KRAS, as demonstrated by 
the iKRAS system and the ID8 ovarian cancer model. Moreover, 
a recent study analyzing lung cancer patients treated with PD-
L1 blockade reported that patients harboring both KRAS and 
TP53 mutations were resistant to immunotherapy through a 
mechanism that was independent of PD-L1 expression levels 
(Skoulidis et al., 2018). Hence, understanding how different 
oncogenic drivers interact to promote immune tolerance will 
be required to understand how best to apply immunomodula-
tory therapies in cancer.

STAR METHODS

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Supplemental Information can be found online at https://doi.org/10.1016/j.celrep.2019.12.028.

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AUTHOR CONTRIBUTIONS


REFERENCES


# STAR Methods

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<tr>
<td>ID8</td>
<td>Walton et al., 2016</td>
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<td>iKRAS;p53f/+</td>
<td>Ying et al., 2012</td>
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**Experimental Models: Organisms/Strains**

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**Oligonucleotides**

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<td>Primers for qPCR T cell cytokines See Table 1</td>
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<tr>
<td>Trp53 gRNA/Cas9</td>
<td>Walton et al., 2016</td>
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LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact Karen H Vousden (karen.vousden@crick.ac.uk). All unique/stable reagents generated in this study are available from the Lead Contact with a completed MTA.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice

The mouse models of pancreatic ductal adenocarcinomas were generated using Pdx1-Cre; LSL-Kras<sup>G12D</sup>/+; Trp53<sup>+/+</sup> and Pdx1-Cre; LSL-Kras<sup>G12D</sup>/+; Trp53<sup>fl/fl</sup> as previously published (Morton et al., 2010). The mouse model of non-small cell lung carcinomas was based on the tetracycline inducible EGFR-L858R [Tg(tet-O-EGFR*L858R)56Hev] from the Mouse Repository of the National Cancer Institute. The R26tTA and Trp<sup>53</sup>fl/fl mice were obtained from the Jackson laboratory. Mice were crossed to generate Rosa26tTaLSLtet(O)EGFRL858R and Rosa26tTaLSLtet(O)EGFRL858RTp53<sup>fox/fox</sup> mice and all backcrossed to C57Bl6/J background. Adenoviral Cre (Viral Vector Core, University of Iowa, USA) was delivered via intratracheal intubation (single dose, 2.5x10<sup>7</sup> virus particles in 50 μl).

FVB (male and females) and CD1nu/nu (females) were purchased from Charles River, and OTI and OTII mice were purchased from Jax and maintained at the Beatson Institute for Cancer Research and the Francis Crick Institute animal facilities. All animals used ranged from 10-30 weeks of age and littermates of the same sex were randomly assigned to experimental groups.

Animal experiments were subject to ethical review by the Francis Crick Animal Welfare and Ethical Review Body and regulation by the UK Home Office project license P8AA77917 and P319AE968 or at the BICR reviewed and approved by the University of Glasgow and UK Home Office for the project license (70/8645). All mice were housed under conditions in line with the Home Office guidelines (UK). Mice were housed from 3-5 per cage and were kept in a 12-hour day/night cycle starting at 7:00 until 19:00. Food and water were available <i>ad libitum</i> and rooms were kept at 21°C at 55% humidity. All procedures were performed following the Animals (scientific procedures) Act 1986 and the EU Directive 2010.

Cell lines and transfection

Phoenix-ECO (ATCC® CRL-3214™) were purchased from the ATCC. PDAC cells from Pdx1-Cre; LSL-Kras<sup>G12D</sup>/+; Trp53<sup>+/+</sup> and Pdx1-Cre; LSL-Kras<sup>G12D</sup>/+; Trp53<sup>fl/fl</sup> mice were derived as previously described (Tan et al., 2014). Cell lines used (KCs and KFCs) were tested for genetic background purity by the Charles River Genetic Testing Services. After a 384 SNP panel batch analysis (MB-160318AJ), cell lines derived from tumors were deemed mismatched (e.g., B6N 73%–78.9%, FVB 46.7%–59.8%, 129S4SvJae 60.3%–71.7%). ID8 isogenic ovarian cancer cells were previously described (Walton et al., 2016). iKRAS cell line was gifted by RA DePinho and were maintained in DMEM, 10% FBS and 2 μg/ml of doxycycline (Ying et al., 2012). All other cell lines were maintained in DMEM, 10% FBS and penicillin-streptomycin and in 37°C, 5% CO<sub>2</sub> humidified incubators.

Primary T cells and bone marrow were derived from C57Bl6/J mice (either male or female) held at the BICR or Francis Crick institute and aged between 10-30 weeks.

Continued
Mouse Cancer Orthotopic Models

The pancreatic orthotopic model protocol was described by Kim et al. (2009). Briefly, pancreatic cancer cells were surgically implanted into the pancreas of recipient mice (500,000 cells in 25μl of Matrigel, BD-Biosciences). For the ovarian ID8 cancer model, 5x10⁶ cells were injected intraperitoneally (IP) in 6-8-week-old female C57Bl6/J mice (Charles River Laboratories, UK). The development of ascites and other symptoms were diagnosed as previously described (Walton et al., 2016).

Tumor Challenge and Rejection Models

PDAC derived cells expressing iRFP were subcutaneously injected into the left flank of FVB mice at 1x10⁶ cells/mouse. Growth was monitored by in vivo imaging and mice were taken at humane endpoint as dictated by the UK Home Office and the animal license. Allograft growth in CD1™/MT™ mice was performed by unilateral flank injections of 1x10⁷ per mouse. Growth was measured by in vivo imaging and humane endpoints were respected. All in vivo antibodies were purchased from Bio-X-Cell. Anti-CSF1R (clone AFS98) was used at a concentration of 300μg/mouse and administered twice a week. Anti-CD25 (PC-61.5.3) was used three days prior to tumor challenge and used at 400μg/mouse. Isotype control used was IgG2a (C1.18.4) and 200μg/mouse was used as a control.

METHOD DETAILS

T cell and CD11b⁺ cell purification, macrophage differentiation, and cell culture

CD8⁺ T cells and CD4⁺ T cells were isolated from spleens and peripheral lymph nodes, prepared into single cell suspensions and lysed for red blood cells (10x RBC lysis buffer, Biolegend). Negative isolation kits and positive isolation kits (CD11b⁺ cells) were purchased from StemCell Technologies and isolation was performed following manufacturer’s procedures. T cells were activated and cultured as previously described (Jones et al., 2007) using plate-bound anti-CD3 and anti-CD28 antibodies.

Isolation of intratumoral CD11b⁺ cells was performed on tumors digested using the tumor preparation protocol (see below). CD11b⁺ cells were isolated from tumor single cell suspensions using positive isolation kits from StemCell Technologies, following the manufacturer’s protocol.

Macrophages were differentiated from bone marrow flushed using PBS, a 1mL syringe and a 25G needle. Bone marrow was collected, lysed for red blood cells (10x RBC lysis buffer, Biolegend), and plated on non-TC treated plates at 5x10⁶ cells/10cm dish and 20ng/ml of M-CSF (Peprotech).

Cell culture of pancreatic ductal adenocarcinoma cells (PDACs) and ID8 cell lines were maintained in DMEM supplemented with 10% FBS and Pen/Strep.

Treg and CD11b suppression assays

CD4⁺ CD25⁺ and CD4⁺ CD25⁻ T cells were isolated by performing CD4⁺ T cell negative isolation by STEMCELL technology kits and sorted by flow cytometry (FACS Aria Sorter) (CD4-FITC and CD25-APC). Briefly, ratios of 1:1 (Treg:Teffector) to 1:32 were generated in the presence of irradiated splenocytes and activated with 1μg/ml of a-CD3 for 3 days (Collison and Vignali, 2011).

CD11b⁺ cells were isolated by positive selection from digested tumors (see above). T cells were stained with V450 dye and plate-bound activated with a-CD3 (5μg/ml) and a-CD28 (2μg/ml) for 24 hours. After 24 hours, T cells were co-cultured with isolated tumor-associated CD11b⁺ cells at a ratio of 1:4 in a 24 well plate for a further 2 days and proliferation was assessed using a the eBioscience live/dead fixable viability dye, APC-Cy7, and flow cytometry. Isolated CD11b⁺ intratumoral cells were plated 8 hours prior to co-culture at 0.5x10⁶ cells per 24 well plate (Coming). Plate-bound activated T cells were plated 24 hours post-activation at 0.75x10⁶ cells per 24 well plate.

Flow cytometry

Single cell suspensions were stained for surface markers in PBS for 20 minutes at 4°C. Intracellular proteins (i.e., cytokines, FOXP3, and Ki67) were assessed using the FOXP3/Transcription staining buffer set (eBioscience, San Diego, CA) and following manufacturer’s instructions. Cells were permeabilized for 30 minutes and stained for intracellular proteins for 1 hour at 4°C. All fluorochromes were purchased from Biolegend and eBioscienes. Ex vivo re-stimulation was performed using PMA (Sigma-Aldrich), ionomycin (Sigma-Aldrich), and Golgi Stop (BD Biosciences) for 4 hours as previously described (Kornete et al., 2012). For OTI and OTII peptide re-stimulation, peptides OVA257-264 and OVA323-339, were incubated with transgenic T cells at 10μg/ml with Golgi Stop (BD Biosciences) for 6 hours followed by surface staining and ICS. Dead cells were distinguished using the fixable viability dye efluor780 from eBioscience. Single cell suspensions were fixed and permeabilized using the FOXP3 Transcription staining buffer set. Samples were acquired on the BD LSRFortessa™ and on the BD FACSymphony™. Flow cytometry data was analyzed using FlowJo (TreeStar).

Extracellular Cytokine Measurements

ELISA kits were purchased from R&D systems for M-CSF, CXCL1, CCL11, and G-CSF. ELISAs for MCP1, CCL3 and CXCL5 were purchased from Life Technologies, Invitrogen. All ELISAs and cytokine arrays were performed on conditioned media (cells were plated at 1x10⁶ cells/10cm dish) harvested after 24 hours. Cytokine arrays were purchased from R&D systems (Mouse cytokine panel array A) and performed on conditioned media from cells prepared as for ELISAs. Cytokines were detected following the
manufacture’s procedures and using chemiluminescence (read at 450nm). Pixels from cytokine array data were analyzed using ImageJ software. The Luminex cytokine array used in this study was the Invitrogen eBioscience ProcartaPlex Mouse Cytokine & Chemokine Panel 1 (26 plex) with 4 additional cytokines.

**Immunoblotting**

Cells were lysed using RIPA buffer supplemented the 1%SDS and phosphatase inhibitors (La Roche Ltd), denatured at 95 °C, and resolved on NuPAGE polyacrylamide pre-cast gels (ThermoFischer Scientific). Transfer of gels onto nitrocellulose membranes was performed using the iBlot2 (Invitrogen). Cells were probed for p53 with monoclonal anti-mouse p53 antibody from Cell Signaling Technologies (clone 1C12). Vinculin (H10, Santa Cruz Biotecnologies) was detected as a loading control. Secondary antibodies were purchased from LiCOR IRDye 800CW and 700CW.

**In Vivo Imaging**

Mice were anesthetized with isoflurane and imaged using the Pearl Imager by LiCOR. iRFP fluorescence was excited using the 685nm laser and emission was detected in the 700nm channel (730nm). Fluorescence was analyzed using the Image Studio v5 from LiCOR.

**Co-Culture Assays**

**Activated T cells and PDACS**

Primary T cells were activated with plate-bound anti-CD3 (5μg/ml) and anti-CD28 (2μg/ml) for 24 hours. Activated T cells were then co-cultured with adherent PDAC cells in 24 well plates or 96 well plates. PDACs were plated at 20 000 cells/well with 1x10^6 T cells (24 well plate) or 10,000 PDAC cells/well with 200,000 T cells (96 well plate) and co-incubated for 2 days. T cells were re-stimulated with PMA, ionomycin and GolgiStop (BD Biosciences) for 4 hours prior to ICS. Intracellular cytokines probed by ICS and flow cytometry were Granzyme B, IFNγ and TNFα.

**BMDMs and Transgenic T cells**

0.3x10^6 BMDMs pulsed with 10μg/ml of ovalbumin (Sigma-Aldrich) for 1 hour, washed and naive OTI or OTII cells were added to the culture at 1x10^5 cells/well. T cells were kept in co-culture for 3–4 days followed by OVA257-264 and OVA323-339 re-stimulation at 10μg/ml in the presence of a Golgi blocke (GolgiStop, BD Biosciences), surface staining, ICS and acquired by flow cytometry.

**OTI and PDAC-mOVA cell viability**

Splenocytes from OT1 TCR transgenic mice were stimulated in vitro with 10μg/ml of SIINFEKL (OVA257-264) for 48 hours. Cells were spun down using Lympholyte M (Cedarlane) following the manufacturer’s instructions. Activated CD8+OT1 cells were co-cultured in a 24 well plate (Corning) at 0.5x10^6 cells to 200,000 PDAC-mOVA isogenic cells. Cells were stained with propidium iodide for cell viability and acquired on the BDLSRFortessa™.

**Tissue collection, Immunohistochemistry (IHC) and scoring**

Tissue was collected and fixed in 10% neutral buffered formalin (NBF). Samples were replaced with 70% ethanol after 48hours, embedded in paraffin blocks, and processed by standard histological techniques. Sections were cut at 5μM. IHC and H&E was performed as previously published (Myant et al., 2013). F4/80 antibody was purchased from eBioscience (rat anti-mouse F4/80 Cat. No 14-4801-82; RRID:AB_467558). Scoring of tumor sections for individual markers was performed by counting 30 fields using QuPath open source digital software (Bankhead et al., 2017) and set as an average per field.

**Tumor Preparation and Serum Collection**

**Tumors**

Tumors were carefully excised from the animals and kept in ice cold medium till processing. Tumors were minced into small (about 1mm) pieces and digested in digestion buffer containing: collagenase 0.012%, dispase, 0.1mg/ml DNase I, 1% FBS in Krebs Ringer Bicarbonate Buffer (KRB) for 45-60 min at 37°C with gentle oscillation. The digestion was stopped by the addition of at least 10 volume of ice cold DMEM supplemented with 10% FBS. The solution was filtered through a 100 μm cell strainer and the isolated cells were precipitated by centrifugation at 300 X g for 5 min, washed with PBS before further processing.

**Serum**

Serum was collected by cardiac puncture into EDTA coated tubes. Blood was spun in 1.5mL Eppendorf tubes at 2000xg for 15 minutes at 4°C. Serum was collected in upper phase and stored at –80°C.

**RNA extraction and QPCR**

Total RNA was extracted using RNeasy® columns (QIAGEN) from at least 3 technical replicates per sample according to manufacturer instructions. Genomic DNA was removed using on column DNA digestion (QIAGEN). cDNA was generated using the High-Capacity cDNA reverse transcription kit (Thermofisher) according to manufacturer’s instructions. Power up™ SYBR® Green Master MIX (Applied Biosystem) was used to perform QPCR with the following primers:
Table 1

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<th>Gene and accession number</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<td>Trnf-α</td>
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<td>cyc</td>
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Gene expression values were calculated according to Pfaffl method (Pfaffl, 2001) and expressed as relative units compared to the control group.

**Scratch-Wound and Migration Assays**

**Scratch-wound Assay**

BMDMs were differentiated with 10ng/ml M-CSF and re-plated onto Imagelock 96 well plates. Scratches were made using the Essenbio scratch making tool. Wound closure was monitored in real-time using IncuCyte™ Live-Cell Analysis System and analyzed using IncuCyte S3 software.

**Chemotraction assays**

BMDMs at 5000 cells per chamber were plated in the upper chamber of an IncuCyte™ ClearView 96-well Cell Migration plate (Essen BioScience). Conditioned medium from different PDAC cell lines was placed in the bottom chamber. The Incucyte ZOOM® live-cell imaging system was used to measure cell migration.

**Plasmids, stable expression and CRISPR/CAS9**

Plasmids for IRFP (Hock et al., 2014) were transfected into Phoenix-ECO (ATCC® CRL-3214) using GeneJuice® Transfection reagents (Merk Milipore). PDAC cell lines were transduced with viral media and pBABE-iRFP selected for by puromycin (Sigma-Aldrich) at a concentration of 2.5μg/mL. Plasmid positive cells were cultured in selection media for 7 days. CRISPR/CAS9 methods were employed for genetic deletion of the Trp53 gene as previously described (Walton et al., 2016). Retroviral introduction of ovalbumin sequence for membrane bound ovalbumin (mOVA) was cloned out of the pCI-neo-mOVA plasmid (Addgene No 25099) (Yang et al., 2010) and into the MSCV-based MIGR1 (Addgene 27490) vector (Pear et al., 1998). Phoenix-EC (ATCC® CRL-3214) were used for virus production and PDAC cells were virally transduced three consecutive days. mOVA expressing cells were selected by cell sorting for GFP expression.

**Bioinformatics Analysis of TCGA datasets**

Survival curves & gene expression: SEM normalized data was downloaded from the TCGA firehose website [https://gdac.broadinstitute.org/]. Top (high group) and bottom (low group) quartiles pancreatic adenocarcinoma samples for MDSC-signature ranked expression were compared using the survival package version 2.41-3 (https://cran.r-project.org/web/packages/survival/index.html) in R version 3.4.3 (http://www.R-project.org) and the level of statistical significance determined by the log rank test. Expression data from PAAD patients with a mutation in TP53 was compared to those without a mutation using DESEQ2 (Love et al., 2014). Boxplots show the log2 expression values of selected genes grouped by their TP53 mutation status, with the adjusted p value determined by the Wald test.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Data are presented as mean ± SD for technical replicates, or mean ± SEM for biological replicates. Data was analyzed using the unpaired Student t test when comparing two conditions. One-way ANOVA with a Tukey’s multiple comparisons test was performed on comparisons of more than two conditions as well as in vivo growth studies. Two-way Anova was performed on the in vivo tumor growth studies with treatments and different p53 alterations. Statistical significance is indicated in all figures by the following annotations: * p < 0.05; **p < 0.01; ***p < 0.005. GraphPad Prism 7 was used for statistical analysis and graph generation.

**DATA AND CODE AVAILABILITY**

This study did not generate any unique datasets or code.