Inhibition of HCK in myeloid cells restricts pancreatic tumor growth and metastasis

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SUMMARY

Pancreatic ductal adenocarcinoma (PDAC) is an aggressive disease with a low 5-year survival rate and is associated with poor response to therapy. Elevated expression of the myeloid-specific hematopoietic cell kinase (HCK) is observed in PDAC and correlates with reduced patient survival. To determine whether aberrant HCK signaling in myeloid cells is involved in PDAC growth and metastasis, we established orthotopic and intrasplenic PDAC tumors in wild-type and HCK knockout mice. Genetic ablation of HCK impaired PDAC growth and metastasis by inducing an immune-stimulatory endotype in myeloid cells, which in turn reduced the desmoplastic microenvironment and enhanced cytotoxic effector cell infiltration. Consequently, genetic ablation or therapeutic inhibition of HCK minimized metastatic spread, enhanced the efficacy of chemotherapy, and overcame resistance to anti-PD1, anti-CTLA4, or stimulatory anti-CD40 immunotherapy. Our results provide strong rationale for HCK to be developed as a therapeutic target to improve the response of PDAC to chemo- and immunotherapy.

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is an aggressive disease with a 5-year survival rate of less than 10% (Raimondi et al., 2009). While chemotherapy confers transient tumor regression in 30% of patients, 9 out of 10 patients that undergo surgery still die of the disease due to local recurrence and/or metastasis (Conroy et al., 2011). Likewise, immune checkpoint therapies including anti-(α)PD1 or αCTLA4 have failed to translate into meaningful improvements in a majority of PDAC patients (Diamond et al., 2021; Galon and Bruni, 2019).

The poor response of PDAC to immune cell-related therapies can be accounted for by two major obstacles. The first tumor-intrinsic barrier relates to insufficient immune activation due to limited immunogenic mutations and presentation of cancer neo-epitopes, resulting in the current clinical recommendation for αPD1 to be limited as the second-line therapy for <1% of PDAC patients with DNA-mismatch repair deficient disease (Bailey et al., 2016; Rojas and Balachandran, 2021; Tempero et al., 2019). The second tumor-extrinsic barrier arises from an immunosuppressive and desmoplastic microenvironment characterized by an influx of cancer-associated fibroblasts (CAFs), myeloid-derived suppressor cells (MDSCs), and tumor-associated macrophages (TAMs), which collectively promote the exclusion of cytotoxic T cells and natural killer (NK) effector cells from tumors (Ho et al., 2020). Thus, therapies that can simultaneously activate tumor immunity and relieve immune suppression represent promising adjuvant strategies to better control PDAC progression and metastasis.

Elevated expression of the myeloid SRC family kinase hematopoietic cell kinase (HCK) is observed in most human solid malignancies including PDAC, where more than 95% of all HCK expression occurs in immune cells and correlates with poor patient survival (Bailey et al., 2016; Crnogorac-Jurcevic et al., 2005; Heidenblad et al., 2004; Isella et al., 2015; Poh et al., 2015; Zhu et al., 2021). We have previously demonstrated a tumor-extrinsic role for myeloid HCK signaling in gastric and colon cancer by promoting an immunosuppressive tumor microenvironment (Poh et al., 2017, 2020). Conversely, genetic ablation or pharmacologic inhibition of HCK reduced tumor growth...
Figure 1. Genetic ablation of HCK in myeloid cells impairs PDAC tumor growth and metastasis
(A) HCK gene expression in tumors and matched normal tissue samples of human pancreatic adenocarcinoma patients (n = 179) using the GEPIA online tool (Tang et al., 2017).
(B) tSNE plot depicting HCK gene expression in human PDAC tumors using primary data from Elyada et al. (2019).
(C) tSNE plot depicting Hck gene expression in mouse KPC PDAC tumors using primary data from Elyada et al. (2019).
(D) Mass of primary PDAC tumors from WT and HckKO hosts 5 weeks following orthotopic injection of KPC tumor cells. Each symbol represents an individual mouse. n ≥ 11 mice per group.

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Here, we establish the therapeutic benefit of targeting HCK in PDAC to reduce immune suppression, attenuate the desmoplastic response, and re-invigorate adaptive anti-tumor immunity.

**RESULTS**

**Genetic ablation of HCK in hosts reduces PDAC growth and metastasis**

To explore a potential tumor-promoting role for HCK in pancreatic cancer, we interrogated the expression level of HCK in PDAC patients (Tang et al., 2017) and observed elevated expression in tumor samples compared with matched normal tissues (Figure 1A). We also analyzed single-cell RNA sequencing (scRNA-seq) datasets of human and mouse PDAC tumors (Elyada et al., 2019), and we confirmed that HCK was most prominently expressed in tumor-associated myeloid cells (Figures 1B and 1C).

To determine whether aberrant myeloid HCK signaling in the host is involved in PDAC growth and metastasis, we orthotopically engrafted syngeneic KPC pancreatic tumor cells into the distal pancreas of wild-type (WT) and Hck cKO mice (Poh et al., 2017, 2020). Here, we establish the therapeutic benefit of targeting HCK in PDAC to reduce immune suppression, attenuate the desmoplastic response, and re-invigorate adaptive anti-tumor immunity.

Given that myeloid cells are the primary source of IL12 and CXCL9/CXCL10 (Arnold et al., 2019; Chow et al., 2019; Garris et al., 2018; House et al., 2020; Reschke and Gajewski, 2022), we next determined the contribution of these molecules to HCK-dependent suppression of anti-tumor immunity using neutralizing antibodies against either IL12 or CXCR3 (cognate receptor for CXCL9/CXCL10). Blockade of IL12 or CXCR3 abrogated the survival benefit of Hck cKO hosts, while a difference in survival was not observed in WT hosts treated with these antibodies (Figure S2B and Table S2). Together, our findings suggest that genetic ablation of HCK promotes a shift of DCs and TAMs toward an activated endotype.

We next assessed the contribution of cDC1s and TAMs to the enhanced anti-tumor response in Hck cKO hosts. Following reconstitution of lethally irradiated WT hosts with bone marrow from either cDC1-deficient (Itgax<sup>-/-</sup>Il2r<sup>-/-</sup>, Caton et al., 2007; Chopin et al., 2013; Feng et al., 2011; referred to as cDC1 KO) or cDC1-proficient (WT) mice, we treated half of each cohort with a neutralizing antibody against CSF1R to also deplete TAMs prior to intrasplenic injection of KPC tumor cells. Following establishment of intrasplenic KPC tumors, mice were treated with the small molecule HCK inhibitor RK20449 or Captisol vehicle until clinical endpoint. While neither cDC1 nor TAM depletion affected the overall survival of vehicle-treated hosts compared with their immune cell-proficient controls, cDC1 depletion reduced the overall survival of RK20449-treated hosts, which was further reduced when TAMs were also simultaneously depleted (Figure 2C and Table S3).

We then clarified the contribution of adaptive immunity to the enhanced anti-tumor response observed in Hck cKO hosts by exploiting the intrasplenic KPC model to establish liver metastasis in WT, Hck cKO, and lymphocyte-deficient Rag1<sup>-/-</sup> and Hck cKO, Rag1<sup>-/-</sup> compound mutant hosts. We observed enhanced liver tumor burden and reduced overall survival in Hck cKO,Rag1<sup>-/-</sup> compound mutant hosts.

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**Genetic ablation of HCK enhances the immune-stimulatory endotype of myeloid cells and promotes an influx of activated cytotoxic effector cells into tumors**

To functionally link reduced Hck expression in myeloid cells to an improved anti-tumor immune response, we performed Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) pathway analysis on bulk RNA sequenced KPC liver metastases recovered from WT and Hck cKO hosts. In tumors of Hck cKO hosts, we observed a significant enrichment of pathways associated with innate and adaptive immune responses, including myeloid cell activation and effector cell-mediated cytotoxicity (Figure 2A).

Given the contribution of immunosuppressive myeloid cells to PDAC growth and metastasis (Poh and Ernst, 2021), we next profiled myeloid cells in KPC liver metastases of WT and Hck cKO hosts and found that HCK deficiency did not affect the overall abundance of TAMs. Instead, we observed a reduction of both CD206<sup>+</sup> alternatively activated macrophages and MDSCs, and an increase in CD103<sup>+</sup> conventional type 1 dendritic cells (cDC1s) in tumors of Hck cKO hosts (Figure 2B). Importantly, DCs and TAMs from tumors of Hck cKO hosts displayed increased expression of immune-stimulatory factors (i.e., Il12a, Il13a, Tnf, Cxcl9, and Cxcl10) and a concomitant downregulation of genes associated with immune suppression (i.e., Il4, Il10, Il13, Tgfb3, Argal) and matrix remodeling (i.e., Mmp7, Mmp9) (Figure S2A).

**Genetic ablation of HCK in hosts reduces PDAC growth and metastasis**

To explore a potential tumor-promoting role for HCK in pancreatic cancer, we interrogated the expression level of HCK in PDAC patients (Tang et al., 2017) and observed elevated expression in tumor samples compared with matched normal tissues (Figure 1A). We also analyzed single-cell RNA sequencing (scRNA-seq) datasets of human and mouse PDAC tumors (Elyada et al., 2019), and we confirmed that HCK was most prominently expressed in tumor-associated myeloid cells (Figures 1B and 1C).

To determine whether aberrant myeloid HCK signaling in the host is involved in PDAC growth and metastasis, we orthotopically engrafted syngeneic KPC pancreatic tumor cells into the distal pancreas of wild-type (WT) and Hck cKO hosts, and we observed significantly smaller pancreatic tumors in Hck cKO hosts compared with their WT counterparts (Figures 1D and S1A). Moreover, Hck cKO hosts did not develop metastatic lesions, which we consistently observed in WT mice, including in the liver (n = 15/15), spleen (15/15), intestine (7/15), peritoneum (4/15), and kidneys (4/15) (Figure S1B). To obtain better insights into the contribution of HCK during PDAC metastasis, we injected KPC tumor cells into the spleen followed by splenectomy to protect hosts against premature death arising from overgrowth of the primary tumor. Again, we observed reduced incidence of liver metastasis in Hck cKO hosts (Figures 1E and S1A), and we confirmed in both orthotopic and intrasplenic PDAC models that Hck expression was restricted to the myeloid cell compartment of KPC tumors (Figure S1C). To formally prove that the enhanced anti-tumor response in Hck cKO hosts was an intrinsic consequence of hematopoietic cells lacking Hck expression, we generated reciprocal bone marrow chimeras and subjected these mice to the intrasplenic tumor model. We observed improved survival of WT<sup>−/−</sup> KO (Recipient<sup>−/−</sup> Donor) bone marrow chimeras compared with WT<sup>−/−</sup> WT hosts, and reduced survival of KO<sup>−/−</sup>WT hosts compared with KO<sup>−/−</sup> KO hosts (Figure S1D and Table S1). We also extended these observations to a therapeutic setting by treating WT mice with the HCK-specific small molecule inhibitor RK20449 (Saito et al., 2013) after orthotopic or intrasplenic injection of KPC tumor cells. Compared with vehicle-treated mice, RK20449 treatment impaired the growth of primary tumors and liver metastases (Figures S1E and S1F).

Genetic ablation of HCK in hosts reduces PDAC growth and metastasis. We next assessed the contribution of cDC1s and TAMs to the enhanced anti-tumor response in Hck cKO hosts. Following reconstitution of lethally irradiated WT hosts with bone marrow from either cDC1-deficient (Itgax<sup>−/−</sup>Il2r<sup>−/−</sup>, Caton et al., 2007; Chopin et al., 2013; Feng et al., 2011; referred to as cDC1 KO) or cDC1-proficient (WT) mice, we treated half of each cohort with a neutralizing antibody against CSF1R to also deplete TAMs prior to intrasplenic injection of KPC tumor cells. Following establishment of intrasplenic KPC tumors, mice were treated with the small molecule HCK inhibitor RK20449 or Captisol vehicle until clinical endpoint. While neither cDC1 nor TAM depletion affected the overall survival of vehicle-treated hosts compared with their immune cell-proficient controls, cDC1 depletion reduced the overall survival of RK20449-treated hosts, which was further reduced when TAMs were also simultaneously depleted (Figure 2C and Table S3).

We then clarified the contribution of adaptive immunity to the enhanced anti-tumor response observed in Hck cKO hosts by exploiting the intrasplenic KPC model to establish liver metastasis in WT, Hck cKO, and lymphocyte-deficient Rag1<sup>−/−</sup> and Hck cKO, Rag1<sup>−/−</sup> compound mutant hosts. We observed enhanced liver tumor burden and reduced overall survival in Hck cKO,Rag1<sup>−/−</sup> compound mutant hosts.

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(E) Representative whole mounts and corresponding liver weights of WT and Hck cKO hosts 3 weeks after intrasplenic injection of KPC tumor cells. Scale bar: 1 cm. Each symbol represents an individual mouse. n ≥ 34 mice per group. Data represent mean ± SEM; **p < 0.001, with statistical significance determined by an unpaired Student’s t test for comparison between two means. See also Figure S1.
A
Positive regulation of immune response
Lymphocyte activation
Regulation of lymphocyte activation
Leukocyte activation
Response to interferon-gamma
Response to external stimulus
Regulation of immune response
Adaptive immune response
Positive regulation of immune system process
Immune effector process
Regulation of immune system process
Innate immune response
Defense response
Immune system process
Immune response

B

TAMs

AAMs

m-MDSCs

g-MDSCs

cDC1s

% F4/80^+CD11b^+ of CD45^+ cells

% CD206^+ of TAMs

% Ly6C^+Ly6G^+ of CD45^+CD11b^+

% Ly6C^+ of TAMs

% CD11b^+ of CD45^+CD11c^+

% Ly6C^+Ly6G^+ of CD45^+CD11c^+

% Ly6C^+ of CD45^+CD11c^+

% CD11b^+ of CD45^+CD11c^+

% F4/80^+CD11b^+ of CD45^+

% CD206^+ of AAMs

% Ly6C^+Ly6G^+ of CD45^+CD11b^+

% Ly6C^+ of AAMs

% CD11b^+ of CD45^+CD11c^+

% Ly6C^+Ly6G^+ of CD45^+CD11c^+

% Ly6C^+ of CD45^+CD11c^+

% CD11b^+ of CD45^+CD11c^+

% F4/80^+CD11b^+ of CD45^+

C

Days post tumor cell injection

% Survival

WT + Vehicle
WT + αCSF1R
WT + αCSF1R + Vehicle
WT + αCSF1R + αCSF1R
WT + αCSF1R + αCSF1R + Vehicle

D

Days post tumor cell injection

% Survival

WT
WT + Vehicle
WT + αCD4
WT + αCD8
WT + αNK.1.1

E

CD8^+ T-cells

NK cells

% CD8^+ T-cells of CD45^+

% CD4^+T-cells of CD45^+

% NK1.1 of CD45^+

% CD62L^+ of CD45^+

% NK1.1 of CD45^+

F

Granzyme B

Perforin

% positive per μm²

WT
WT + Vehicle
WT + αCD4
WT + αCD8
WT + αNK.1.1

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hosts compared with HckKO mice, as well as extended survival in HckKO hosts compared with all other cohorts (Figure S2C and Table S4). To delineate the role of NK and T cells in HckKO hosts, we individually depleted NK cells, CD4+ T cells, or CD8+ T cells, and we observed that CD8+ T cell depletion abrogated the tumor-suppressive effects conferred in the absence of HCK expression more effectively than either NK or CD4+ T cell depletion (Figure 2D and Table S5). These observations were consistent with an increased proportion of CD8+ T cells and NK cells in tumors of HckKO hosts compared with WT mice (Figures 2E and S2D). Furthermore, CD8+ T cells and NK cells isolated from tumors of HckKO hosts also showed elevated expression of genes encoding cytotoxic activities (i.e., Ifng, Tnf, GzmB, Prf1) (Figure S2E), and this in turn correlated with more abundant staining for granzyme B and perforin in tumors of HckKO hosts (Figure 2F). We surmised that these effects are primarily mediated by IL12 and CXCR3 signaling by myeloid cells, since administration of neutralizing antibodies against IL12 or CXCR3 significantly reduced CD8+ T cell recruitment and cytotoxicity in tumors of HckKO hosts (Figures S2F and S2G). Collectively, our findings suggest that CD8+ T cells are a major effector cell population that mediates the enhanced anti-tumor response in HckKO hosts.

Genetic ablation of HCK in myeloid cells reduces the desmoplastic response of PDAC

CAFs produce and remodel most of the extracellular matrix (ECM) in PDAC (Geng et al., 2021) and interact with myeloid cells to collectively amplify and sustain the immune-suppressive and fibrotic tumor microenvironment (Poh and Ernst, 2021). Consistent with these observations, KPC liver metastases of HckKO hosts displayed significantly less ECM including collagen and fibronectin compared with their WT counterparts (Figure 3A).

We therefore examined whether CAFs associated with tumors of HckKO hosts differed quantitatively and qualitatively from those of tumors from WT hosts. Immunohistochemistry and flow cytometry revealed that tumors of HckKO hosts contained fewer inflammatory, myofibroblastic, and antigen-presenting CAFs (Figures 3B and 3C). Likewise, CAFs from HckKO hosts also displayed reduced expression of genes associated with immune suppression (i.e., Tgfb, Il10), fibrosis (i.e., Il11), and ECM remodeling (i.e., Mmp3, Mmp7, Mmp9, Col1a1) than CAFs from tumors of WT hosts (Figure 3D).

Inhibition of HCK improves therapeutic response to chemotherapy and immunotherapy

The immune cell-excluded and fibrotic transcriptional profile of human PDAC correlates with poor clinical outcomes and resistance to immunotherapy (Bagaev et al., 2021). Given that durable responses to αPD1 therapy are limited by the exhaustion of cytotoxic effector cells and an immunosuppressive and desmoplastic stroma, we first examined whether HCK deficiency sensitizes treatment-refractory PDAC tumors to αPD1. We treated tumor-bearing WT or HckKO hosts with αPD1, and we observed that genetic ablation of HCK in hosts enabled response to αPD1 immunotherapy and blocked the outgrowth of liver metastases (Figures 4A and S3A). This correlated with prolonged survival of all corresponding αPD1-treated HckKO mice well beyond that of all other treatment cohorts (Figure 4B and Table S6).

To determine whether HCK inhibition not only alleviated αPD1-mediated local immunosuppression but also stimulated antigenic priming, we next assessed the contribution of HCK ablation in the host to therapeutic CTLA4 inhibition or CD40 stimulation, respectively. Unlike the PD1 checkpoint, which suppresses T cell activation in the tumor microenvironment, CTLA4 signaling controls T cell priming in lymph nodes (Mellman et al., 2011). Meanwhile, stimulation of CD40 enhances cross-presentation by cDC1s and broadens T cell response through epitope spreading (Diamond et al., 2021). We observed that treatment with either antagonistic αCTLA4 or agonistic αCD40 antibodies protected HckKO hosts from liver metastases and extended their survival over that of WT hosts treated with these immunotherapies (Figures 4C–4F and Tables S7–S8). Moreover, we observed that the standard-of-care chemotherapy gemcitabine extended the survival of HckKO hosts with no histological evidence of liver metastases in tissue sections (Figures 4G, 4H, and S3B and Table S9).

Finally, we expanded our findings to a therapeutic setting by treating tumor-bearing WT hosts with RK20449 in combination with immunotherapy or chemotheray. RK20449 treatment of WT hosts reduced tumor growth by enabling response of PDAC tumors to αPD1 therapy (Figure S4A), and it enhanced CD8+ T cell recruitment and activation (Figures S4B and S4C). RK20449 treatment also improved the efficacy of αCTLA4, αCD40, and gemcitabine (Figures S4D–S4F) and significantly extended survival in tumor-bearing WT hosts compared with hosts from monotherapy-treated groups (Figures S4G and S4H and Tables S10 and S11). Collectively, our data suggest that...
inhibition of HCK activity in hosts with established tumors licenses immune checkpoint-dependent anti-tumor immunity and provides a compelling rationale for targeting HCK as an adjuvant therapy to boost response of PDAC to chemo- and immunotherapy.

**DISCUSSION**

Myeloid cells are one of the earliest infiltrating cells in PDAC tumors and are associated with disease progression, recurrence, metastasis, and reduced overall survival (Poh and Ernst, 2021).

Accordingly, therapeutic strategies aimed at eliminating myeloid cells, inhibiting their infiltration, and/or reprogramming them toward an immune-stimulatory endotype have shown potential in both pre-clinical PDAC models (Kaneda et al., 2016; Stromnes et al., 2014; Zhu et al., 2014) and clinical trials (NCT01413022, NCT02345408, NCT00711191, NCT03214250).

Here, we have identified HCK as a critical promotor of myeloid-mediated immune suppression, metastasis, and desmoplasia in PDAC. Mechanistically, genetic ablation of HCK in myeloid cells suppressed the growth of primary and metastatic tumors by...
A  

**WT + IgG**  

**HckKO + IgG**  

**WT + aPD1**  

**HckKO + aPD1**  

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B  

**WT + IgG (n=10)**  

**HckKO + IgG (n=12)**  

**WT + aPD1 (n=10)**  

**HckKO + aPD1 (n=12)**  

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C  

Liver mass (g per mouse)  

WT + IgG  

HckKO + IgG  

WT + aCTLA4  

HckKO + aCTLA4  

---

D  

**WT + IgG (n=10)**  

**HckKO + IgG (n=11)**  

**WT + aCTLA4 (n=10)**  

**HckKO + aCTLA4 (n=10)**  

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E  

Liver mass (g per mouse)  

WT + IgG  

HckKO + IgG  

WT + aCD40  

HckKO + aCD40  

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F  

**WT + IgG (n=10)**  

**HckKO + IgG (n=11)**  

**WT + aCD40 (n=10)**  

**HckKO + aCD40 (n=12)**  

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G  

Liver mass (g per mouse)  

WT + Vehicle  

HckKO + Vehicle  

WT + Gemcitabine  

HckKO + Gemcitabine  

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H  

**WT + Vehicle (n=10)**  

**HckKO + Vehicle (n=12)**  

**WT + Gem (n=10)**  

**HckKO + Gem (n=12)**  

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skewing DCs and TAMs toward an immune-stimulatory endotype and by reducing the abundance of MDSCs. These changes were associated with increased NK and CD8+ T cell recruitment into tumors and a dramatic shift toward a tumor microenvironment enriched for non-exhausted effector cells. While our observations demonstrate that excessive HCK activity in myeloid cells promotes tumor progression in part through a T cell-dependent mechanism, our findings here also expand on our previously described T cell-independent mechanisms (Poh et al., 2017, 2020). Here, we attribute the latter to increased immune suppression and matrix remodeling associated with alternative macrophage polarization as a consequence of excessive HCK activity. Meanwhile, high HCK expression is associated with increased abundance of immunosuppressive macrophage and exhausted T cell signatures that correlate with reduced overall survival in pancreatic (Bailey et al., 2016), gastric (Poh et al., 2020), and colon cancer patients (Poh et al., 2017). Thus, we surmise that inhibition of HCK signaling offers an opportunity to simultaneously reprogram immune-suppressive myeloid cells and stimulate anti-tumor immunity.

Our findings reinforce a pivotal role for myeloid cells in promoting and sustaining a desmoplastic tumor microenvironment. During PDAC progression, TAMs undergo significant expansion and directly shape fibrotic responses through the production of signaling molecules that stimulate ECM deposition and remodeling (Xue et al., 2015; Zhu et al., 2017). TAMs also indirectly contribute to desmoplasia through the secretion of immunosuppressive growth factors and cytokines that activate CAFs and perpetuate a feed-forward loop to sustain fibrosis (Candido et al., 2018; Kaneda et al., 2016; Nielsen et al., 2016). Our observations imply that the concerted reduction of TGFβ, IL11 (Cook and Schafer, 2020), and other promoters of tissue fibrosis observed in tumors of Hck KO hosts may reduce the ECM remodeling and the immunosuppressive endotype of both TAMs and CAFs. Indeed, studies in pancreatic cancer (Creeden et al., 2020), atherosclerosis (Smolinska et al., 2011), and renal and pulmonary fibrosis (Ernst et al., 2002; Smolinska et al., 2011) suggest a link between excessive HCK activity and desmoplasia. However, our observation that tumors of Hck KO mice contained fewer antigen-presenting CAFs suggests their limited contribution to the improved anti-tumor immune response elicited by inhibition of HCK signaling.

Our observations suggest that the limited response of PDAC to immunotherapy may arise from an immune-suppressive tumor environment rather than insufficient de novo antigenicity of tumor cells, supporting findings that high tumor antigenicity is insufficient to elicit T cell-mediated tumor control in the pancreas (Diamond et al., 2021; Galon and Bruni, 2019). Indeed, our data complements observations that CD40 activation enables priming of T cells through DC activation independent of pattern recognition receptors (Morrison et al., 2020) and that escape of antigenic tumors can be antagonized by enhancing DC function through administration of FLT3 ligands (Regde et al., 2020; Vonderheide, 2018). Thus, targeting the catalytic activity of HCK not only overcomes major immunological barriers that limit therapeutic response of fibrotic tumors with minimal infiltration of effector cells, but it also enables durable anti-tumor responses when combined with chemo- or immunotherapy.

Limitations of the study
Limitations of the results presented here are that the KPC model does not evaluate which subtypes of human PDAC likely to benefit most from HCK inhibition, and whether HCK is an effective therapeutic target in PDACs that harbor mutations in genes other than KRAS and TP53. The latter include PDACs with loss of function mutations in SMAD4 that occur in approximately 30% of human pancreatic cancers. Likewise, we are aware that insights from the orthotopic KPC allograft model have provided limited prediction for the outcomes of clinical trials targeting CSF1R, CCR2, IDO, and some other immune modulatory molecules.

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

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- EXPERIMENTAL MODEL AND SUBJECT DETAILS

Figure 4. Genetic ablation of HCK in myeloid cells sensitizes PDAC tumors to immunotherapy and augments the efficacy of chemotherapy

(A) Representative whole mounts and corresponding liver weights of WT and Hck KO hosts treated once every 3 days with sPD1 or a matched IgG isotype control. Treatment commenced on the 5th day after intrasplenic KPC tumor cell injection and continued for 2 weeks. Scale bar: 1 cm. Each symbol represents an individual mouse. n ≥ 15 mice per treatment group, n = 6 mice per control naive group.

(B) Kaplan-Meier survival analysis of WT and Hck KO hosts treated as described in Figure 4A until clinical endpoint. Shaded area indicates treatment period. n ≥ 10 mice per group. A Mantel-Cox log rank test was used to evaluate statistical significance (see Table S6).

(C and D) Liver weights of WT and Hck KO mice treated once every 3 days with (C) sCTLA4, (E) sCD40, or a matched IgG isotype control. Treatment commenced on the 5th day after intrasplenic KPC tumor cell injection and continued for 2 weeks. Each symbol represents an individual mouse. n ≥ 11 mice per group.

(D and E) Kaplan-Meier survival analysis of WT and Hck KO hosts treated as described above with (D) sCTLA4, (F) sCD40, or a matched IgG isotype control until clinical endpoint. Shaded area indicates treatment period. n ≥ 10 mice per group. A Mantel-Cox log rank test was used to evaluate statistical significance (see Tables S7 and S8).

(G) Liver weights of WT and Hck KO hosts treated weekly with gemcitabine or PBS vehicle. Treatment commenced on the 5th day after intrasplenic KPC tumor cell injection and continued for 2 weeks. Each symbol represents an individual mouse. n ≥ 11 mice group.

(H) Kaplan-Meier survival analysis of WT and Hck KO mice treated as described in Figure 4G until clinical endpoint. Shaded area indicates treatment period. n ≥ 10 mice per group. A Mantel-Cox log rank test was used to evaluate statistical significance (see Table S9). Data represent mean ± SEM; ***p < 0.001, with statistical significance determined by one-way ANOVA followed by Tukey’s multiple comparison test or Mantel-Cox log rank test for Kaplan-Meier analysis. See also Figures S3 and S4 and Tables S6–S9.
Cell Reports

Report

- Animals
- Cell line

**METHOD DETAILS**
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- Immunohistochemistry
- Bulk RNA sequencing and analysis
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**SUPPLEMENTAL INFORMATION**

Supplemental information can be found online at https://doi.org/10.1016/j.celrep.2022.111479.

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**REFERENCES**


## STAR★METHODS

### KEY RESOURCES TABLE

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### Experimental models: Cell lines

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### Experimental models: Organisms/strains

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### Oligonucleotides

See Table S12 for Taqman Probes

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RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Matthias Ernst (matthias.ernst@onjcri.org.au).

Materials availability
This study did not generate new unique reagents.

Data and code availability
- Bulk RNA sequencing data generated from KPC liver metastases of WT and HckΔKO mice are publicly available at the Gene Expression Omnibus (GEO) under accession number GSE185540.
- All data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals
C57BL/6 WT, HckΔKO (Lowell et al., 1994), Rag1ΔKO, HckΔKO·Rag1ΔKO and ItgaxCre·Irflfl/fl (Chopin et al., 2013) mice were bred and maintained in specific pathogen-free facilities at La Trobe University, The Austin Hospital, and The Walter and Eliza Hall Institute, Australia. 10-week-old male and female littermates were randomly assigned to experimental groups. No sex differences were observed. All animal studies were approved and conducted in accordance with the Animal Ethics Committee at La Trobe University, the Olivia Newton-John Cancer Research Institute/Austin Hospital and The Walter and Eliza Hall Institute.
Luciferase labelled KPC cells derived from pancreatic tumors of KRasG12D;Trp53R172H;Pdx-1Cre (KPC) mice (Vennin et al., 2017) were maintained in DMEM/F-12 media (Gibco Cat#11320033) supplemented with 10% FCS at 37°C with 10% CO₂. The cell line was confirmed mycoplasma negative.

METHOD DETAILS

Tumor models
To establish orthotopic PDAC tumors, KPC pancreatic cancer cells were re-suspended as a single cell solution in PBS. A cell viability of >95% was confirmed using trypan blue exclusion. Prior to surgery, Carprofen analgesic (Zoetis, 5 mg/kg) was administered subcutaneously. Mice were anesthetized using Ketamine (Baxter, 100 mg/kg i.p.) and Xylazine (llum, 10 mg/kg i.p.). A left subcostal incision was made to access the peritoneal cavity and expose the tail of the pancreas. 5×10⁵ KPC cells were drawn up into a pre-cooled Hamilton syringe (Sigma Aldrich Cat#20702) and injected directly into the pancreas tail using a 27-gauge needle over a period of 15 seconds (Nikfarjam et al., 2013). A cotton tip was applied to the injection site for an additional 15 seconds to minimize leakage. The abdominal muscle and skin were closed separately using 5-0 coated Vicryl sutures, and 0.12% Bupivacaine analgesic (AstraZeneca) was applied to the incision site. Normal saline was instilled into the peritoneal cavity by i.p. injection. Mice were sacrificed 5 weeks after tumor cell injection. To confirm the absence of micro-metastases in HckKO mice, we obtained at least six sections from each organ at a depth of 200 μM apart. Sections were stained with H&E and analyzed with Aperio ImageScope v11.2.0.780 software for the presence of metastases.

To establish PDAC liver metastasis, KPC pancreatic cancer cells (>95% viability) were re-suspended as a single cell solution in PBS. Prior to surgery, Carprofen analgesic (Zoetis, 5 mg/kg) was administered via subcutaneous injection, and mice were anesthetized via 2% isoflurane inhalation. A left subcostal incision was made to access the peritoneal cavity and expose the spleen. 8–10×10⁵ KPC tumor cells were drawn up into a pre-cooled Hamilton syringe (Sigma Aldrich Cat#20702) and injected into the spleen using a 27-gauge needle over a period of 40 seconds to allow cells to perfuse into the liver (Nielsen et al., 2016). Cotton gauze was applied to the injection site for an additional minute to minimize leakage before a splenectomy was performed by cautery. The abdominal muscle and skin were closed separately using 5-0 coated Vicryl sutures and wound clips, respectively. Bupivacaine analgesic (AstraZeneca, 0.12%) was applied to the incision site, and normal saline was instilled into the peritoneal cavity by i.p. injection.

Drug treatments
For the orthotopic PDAC model, mice were either treated with RK20449 (synthesized by Reagency, 30 mg/kg, dissolved in 12% Captisol, twice daily i.p.) orCaptisol vehicle control (Cat#RC-0C7-K01) commencing one week after KPC tumor cell injection for 4 consecutive weeks. For the intrasplenic PDAC model, mice were treated with αCD40 (Clone FGK45, JPP Biologics, 100 μg once every 3 days i.p.), αPD1 (Clone RMP1-14, JPP Biologics, 200 μg once every 3 days i.p.), α CTLA4 (Clone 4F10, JPP Biologics, 200 μg once every 3 days i.p.), isotype-matched IgG (JPP Biologics, 200 μg once every 3 days i.p.), Gemcitabine (Pfizer, 120 mg/kg, weekly, i.p.) or PBS vehicle (weekly, i.p.) on the 5th day after KPC tumor cell injection for 2 consecutive weeks. In some cases, mice were also treated with RK20449 (30 mg/kg, dissolved in 12% Captisol, twice daily i.p.) or Captisol vehicle control (Cat#RC-0C7-K01). For Kaplan-Meier survival analysis, treatment commenced on the 5th day following intrasplenic KPC tumor cell injection and continued until mice reached clinical endpoint (i.e., lost ≥15% of their body weight or developed jaundice or other clinical signs of metastasis). To confirm the absence of micro-metastases in chemo- or immunotherapy treated HckKO mice, we obtained at least six sections from the liver at a depth of 200 μM apart. Sections were stained with H&E and analyzed with Aperio ImageScope v11.2.0.780 software for the presence of metastases.

For antibody-mediated depletion/neutralization experiments, mice were pre-treated with αCD4 (Clone GK1.5, JPP Biologics, 200 μg), αCD8 (Clone YTS169, JPP Biologics, 200 μg), αNK1.1 (Clone PK136, JPP Biologics, 200 μg), αCSF1R (Clone AFS98, BioxCell Cat#BP0213, 500 μg), αIL12 (Clone R2-9A5, BioxCell Cat#BE0233, 500 μg), or αCXCR3 (Clone CXCR3-173, BioxCell #BE0249, 500 μg) once every 3 days (total 3 treatments, i.p.) prior to intrasplenic KPC tumor cell injection, and continued until mice reached clinical endpoint.

Generation of bone marrow chimeras
Bone marrow was harvested from the femurs and tibias of donor mice by flushing with sterile PBS (Poh et al., 2017). Cell suspensions were filtered, washed twice in PBS, and kept on ice in PBS until ready for use. Recipient mice were lethally irradiated with 2 doses of 5.5 Gy γ-irradiation 3 hours apart, before receiving 5×10⁶ donor bone marrow cells via tail-vein injection using a 27-gauge needle. Mice were maintained on neomycin-supplemented water (ThermoFisher Cat#21810031, 2 mg/mL) for 3 weeks, and aged for an additional 8 weeks to allow for complete bone marrow reconstitution.

Flow cytometry
Tumors were minced into 1 mm pieces and digested in collagenase/dispace (Roche Cat#11097113001) and DNase I (Roche Cat#10104159001) diluted in Ca²⁺/Mg²⁺-free HBSS media (Gibico Cat#14170112) plus 10% FCS (Poh et al., 2017, 2020). Samples...
were incubated at 37°C for 20 min under continuous rotation, then vortexed for 30 seconds to dissociate immune cells. Cell suspensions were filtered and washed in PBS plus 10% FCS, and incubated with Fc block (eBioscience Cat#14-9161-73) on ice for 10 min. Samples were then stained with fluorophore-conjugated primary antibodies for 20 min on ice in the dark, washed twice, and re-suspended in PBS plus 10% FCS.

Fluorophore-conjugated primary antibodies were directed against mouse CD45 (Clone 30-F11, BioLegend Cat#103116 or Cat#103131), NK.1.1 (Clone PK136, eBioscience Cat#17-5941-82), TCRb (Clone H57-597, BioLegend Cat#109208), CD8a (Clone 53-6.7, BioLegend #100722), F4/80 (Clone BM8, BioLegend Cat#123108 or eBioscience Cat#25-4801-82), CD11b (Clone M1/70, BD Biosciences Cat#553311), Ly6G (Clone 1A8, BD Biosciences Cat#560602), Ly6C (Clone HK1.4, eBioscience Cat#48-5932-82 or BioLegend Cat#128016), CD206 (Clone C068C2, BioLegend Cat#141708), DC11c (Clone 3.9, eBioscience Cat#11-0116-42), MHCI (Clone M5/114.15.2, eBioscience Cat#48-5321-82 or BioLegend Cat#107645), CD103 (Clone 2E7, eBioscience Cat#12-1031-82), PDGFRα (Clone APA5, BioLegend Cat#135906), CD31 (Clone 390, BioLegend Cat#102418), PDPN (Clone 8.1.1, BioLegend Cat#127418) and EpCAM (Clone G8.8, BioLegend Cat#118208).

Flow cytometry was performed and analyzed on the BD FACSCanto and Aria cell sorter. Background fluorescence was estimated by substituting the primary antibodies with their specific isotype controls, and/or fluorescent-minus-one controls, as well as unstained controls. Dead cells were identified by Sytox Blue (Invitrogen Cat#S34857) and excluded from analysis. Analysis was performed using compensated data with FlowJo software (Version 10).

RNA extraction and qPCR
RNA extraction on FACS purified cells was performed using the RN-easy Micro Plus kit (Qiagen Cat#74034) and cDNA was generated with the SuperScript™ IV First-Strand Synthesis System (Invitrogen Cat#18091050). RNA extraction on tumor samples was performed using the RN-easy Mini Plus kit (Qiagen Cat#74134).

qPCR analysis on each biological sample was performed using technical replicates with TaqMan Fast Universal PCR Master Mix (Applied Biosystems Cat#4352042) and probes (Table S12) using the Viia7 Real-Time PCR System for 40 cycles (95°C for 15 seconds, 60°C for 1 min) and following an initial holding stage (50°C for 2 min, 95°C for 10 min). The cDNA concentration of target genes was normalized by amplification of 18S rRNA or Gapdh and fold changes in gene expression were obtained using the 2-ΔΔCT method (Livak and Schmittgen, 2001).

Taqman probes used were mouse 18s (Mm04277571_s1), Gapdh (Mm99999915_g1), Hck (Mm01241463_m1), Il4 (Mm00445259_m1), Il6 (Mm00446190_m1), Il10 (Mm01288386_m1), Il11 (Mm00434162_m1), Il13 (Mm00434204_m1), Arg1 (Mm00475988_m1), Tgfβ1 (Mm0127699_m1), Il12α (Mm00434169_m1), Ilnγ (Mm01168134_m1), Cxcl9 (Mm00434946_m1), Cxcl10 (Mm00445235_m1), Tnf (Mm00443258_m1), Prf1 (Mm00812512_m1), GzmB (Mm00442837_m1), Mmp9 (Mm00440295_m1), Mmp7 (Mm00487724_m1), Mmp9 (Mm00442991_m1), and Col1a1 (Mm00801666_g1).

Immunohistochemistry
Paraffin-embedded formalin-fixed sections were dewaxed in xylene and rehydrated in ethanol. Antigen retrieval was performed by boiling slides in citrate buffer (pH 6) for 15 min using a microwavable pressure cooker (Poh et al., 2017, 2020). Sections were immersed in 3% H2O2 for 20 min at room temperature to inhibit endogenous peroxidase activity, washed in TBS, then blocked in 10% normal goat serum (Gibco Cat#PCN5000) for 1 hour at room temperature. Sections were stained with primary antibodies (diluted in 10% normal goat serum) at 4°C in a humidified chamber overnight.

Primary antibodies used were Fibronectin (Abcam Cat#ab2143), PDPN (Abcam Cat#ab11936), αSMA (Abcam Cat#ab6949), PDGFRα (Abcam Cat#ab32570), CD8a (Clone 4S15, eBioscience Cat#14-0808-82), Granzyme B (Clone D6E9W, Cell Signaling Technology Cat#44153), and Perforin (Clone E3W4I, Cell Signaling Technology Cat#31647). Biotinylated secondary antibodies from the Avidin Biotin Complex kit (Vector Laboratories Cat#BA-4001 or Cat#BA-1000) were used according to the manufacturer’s instructions.

Antigen visualization was achieved using 3,3-Diaminobenzine (DAKO). Sections were counterstained with Mayer’s haematoxylin, developed in Scott’s tap water, and dehydrated in ethanol and xylene before cover-slipping. Images were collected and analyzed with Aperio ImageScope v11.2.0.780 software. Quantification of positive staining per counter script in FIJI (ImageJ) (Schneider et al., 2012).

Bulk RNA sequencing and analysis
RNA extracted from whole tumors were submitted to the Australian Genome Research Facility (AGRF) for sequencing on the Illumina NovaSeq 6000 platform with v1 200 cycle chemistry (100 bp paired ends). The Illumina TruSeq Stranded Total RNA Library Prep workflow with Ribo-Zero Gold was used to process the samples as per the manufacturer’s instructions. Quality control was evaluated by the AGRF, and reads were also screened for the presence of any Illumina adapter/overrepresented sequences and cross-species contamination.

Paired-end RNA-seq reads were aligned to the mouse reference genome GRCm38/mm10 using the Subread aligner (Subread version 2.2.6) (Liao et al., 2013). Gene-level read counts were obtained by running featureCounts, (Liao et al., 2014) a read count summarisation program within the Subread package (Liao et al., 2019) and the inbuilt Subread annotation that is a modified version of NCBI RefSeq mouse (mm10) genome annotation build 38.1. Pseudo genes or genes that did not meet a CPM (counts per million) read
cut-off of 0.5 in at least 5 libraries were excluded from further analysis. Read counts were converted to log2-CPM, quantile normalized, and precision weighted with the voom function of the limma package (Law et al., 2014; Ritchie et al., 2015). A linear model was fitted to each gene, and empirical Bayes moderated t-statistic was used to assess differences in expression (McCarthy and Smyth, 2009; Smyth, 2004). Genes were called differentially expressed (DE) if they achieved a False Discovery Rate (FDR) of 5% or less. Gene Ontology (GO) terms enrichment analysis on the differentially expressed genes was performed using the goa function within the limma package (Young et al., 2010). Pathway enrichment against the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways for differentially expressed genes was performed using the kegga function also implemented in the limma package. A p-value cut-off of 0.001 was applied when determining enriched GO terms or KEGG pathways. RNA sequencing data were submitted to the GEO repository under the accession number GSE185540.

**Analysis of scRNA-seq datasets**

scRNA-seq gene expression data together with metadata of tumor specimens from human PDAC patients which has been previously published (Elyada et al., 2019) was obtained from the authors. Raw counts were imported into R and processed using the Seurat pipeline (version 4) (Hao et al., 2021). Briefly, cells with a mitochondrial content of >20%, or ribosomal content >40%, or <200 or >4,000 detected genes were excluded from analysis. Furthermore, genes that failed to express (an expressed gene has at least 1 UMI count) in at least 3 cells in at least 1 sample together with Mitochondrial and Ribosomal genes were excluded from analysis. Data was then normalized using a global-scaling normalization method “LogNormalize” that normalizes the feature expression measurements for each cell by the total expression, multiplies this by a scale factor of 10,000, and log-transforms the result. A subset of highly variable genes between cells were identified using the ‘FindVariableFeatures’ function within Seurat. A linear transformation was applied to the normalized data prior to dimensional reduction. Principal component analysis was performed using the 2000 most variable genes and dimension reduction to identify clusters was performed using 10 principal components and a resolution of 0.5. Cell type annotation of clusters was performed by computing the mean expression of marker genes identified in the paper (Elyada et al., 2019) and by manual curation. The gene expression profile of HCK in each cell type was computed and overlaid on tSNE plots.

Furthermore, raw scRNA-seq data of KPC mouse tumors consisting of viable cells were downloaded from Sequence Read Archive (SRA) with accession number SRP191615. The raw data was processed by cellCounts, a function within Rsubread (Liao et al., 2019) for quantifying 10x scRNA-seq data. Sequence reads were mapped to the mouse genome (GRCm38) based on the align function (Liao et al., 2013) and UMI counts were generated for each gene in each cell based on featureCounts (Liao et al., 2014). The inbuilt mouse (mm10) annotation in Rsubread was used for quantification. Similarly, the generated counts were processed, normalized and dimension reduction performed using the steps discussed above. Clusters were also annotated based on marker genes identified in the paper as above. The gene expression profile of Hck was computed and visualized on tSNE plots to highlight differences Hck expression across different cell types.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

All experiments were performed at least twice with a minimum of four age- and sex-matched mice per group. The specific n (number of animals) used per cohort is indicated in the respective figure legends and shown as individual data points. No data was excluded from analysis. Tumor and liver weights were recorded by an independent assessor who was blinded to the experimental conditions. Statistical analysis was conducted using GraphPad Prism Software (Version 8). For comparison between multiple groups, a one-way ANOVA followed by Tukey’s multiple comparison test was performed as appropriate. Comparisons between two mean values were performed with a 2-tailed Student’s t-test. For survival studies, a Mantel-Cox log-rank test was used to evaluate statistical significance in Kaplan-Meier analysis. A p value of less than 0.05 was considered statistically significant.