γδ T Cells Support Pancreatic Oncogenesis by Restraining αβ T Cell Activation

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Summary

Inflammation is paramount in pancreatic oncogenesis. We identified a uniquely-activated γδT cell population which constituted ~40% of tumor-infiltrating T cells in human pancreatic ductal adenocarcinoma (PDA). Recruitment and activation of γδT cells was contingent on diverse chemokine signals. Deletion, depletion, or blockade of γδT cell recruitment was protective against PDA and resulted in increased infiltration, activation, and Th1-polarization of αβT cells. Whereas αβT cells were dispensable to outcome in PDA, they became indispensable mediators of tumor-protection upon γδT cell ablation. PDA-infiltrating γδT cells expressed high levels of exhaustion ligands and thereby negated adaptive anti-tumor immunity. Blockade of PD-L1 in γδT cells enhanced $CD4^+$ and $CD8^+$ T cell infiltration and immunogenicity and induced tumor-protection suggesting that γδT cells are critical sources of immune-suppressive checkpoint ligands in PDA. We describe γδT cells as central regulators of effector T cell activation in cancer via novel cross-talk.

Introduction

Pancreatic ductal adenocarcinoma (PDA) is a devastating disease in which the mortality rate approaches the incidence rate (Yadav and Lowenfels, 2013). PDA is almost invariably associated with a robust inflammatory cell infiltrate which has considerable influence on disease progression (Andren-Sandberg et al., 1997; Clark et al., 2007). Peri-pancreatic leukocytic subsets can have divergent effects on tumorigenesis by either combating cancer growth via antigen-restricted tumoricidal immune responses or by promoting tumor progression via induction of immune suppression (Zheng et al., 2013). For example, $CD8^+$ T cells and Th1-polarized $CD4^+$ T cells mediate tumor-protection in murine models of PDA and are associated with prolonged survival in human disease (De Monte et al., 2011; Fukunaga et al., 2004). Similarly, we found that negating cytotoxic $CD8^+$ anti-tumor responses by myeloid-derived suppressor cells (MDSC) markedly accelerates PDA growth (Pylayeva-Gupta et al., 2012). Conversely, we recently reported that antigen-restricted Th2-deviated $CD4^+$ T cells strongly promote PDA progression in mice (Ochi et al., 2012b). Accordingly, intra-tumoral $CD4^+$ Th2 cell infiltrates correlate with reduced survival in human PDA (De Monte et al., 2011; Fukunaga et al., 2004).

γδT cells are a non-MHC-restricted lymphocyte subset closely aligned with innate immunity. *In vitro* activated PBMC-derived γδT cells have cytolytic efficacy against PDA (Oberg et al., 2014). Conversely, a recent study showed that γδT cells produce high levels of tumor-promoting IL-17 in PDA(McAllister et al., 2014). Nevertheless, intra-pancreatic γδT cells have not been well characterized. We found that a novel population of γδT cells with a uniquely activated phenotype infiltrates the pre-neoplastic pancreas and invasive PDA in mice. In human PDA, γδT cells are a dominant T cell population comprising up to 75% of all T lymphocytes. Deletion of intra-pancreatic γδT cells markedly protects against oncogenesis *in vivo* and results in

an influx of immunogenic Th1 cells and $CD8^+$ T cells to the TME. Based on these observations, we postulated that pancreas-infiltrating γδT cells promote PDA progression by inducing adaptive immune suppression. We discovered novel γδT cells cross-talk with $CD4^+$ and $CD8^+$ T cells implicating γδT cells as primary regulators of αβT cell activation in PDA.

Results

Activated γδT cells are ubiquitous in human PDA

Immunohistochemical analysis revealed that γδT cells are widely distributed within the human PDA tumor stroma but absent in normal pancreas (**Figure 1a**). Moreover, up to 75% of human PDA-infiltrating T cells were TCRγ/δ^+ compared with a much lower fraction in PBMC (**Figure 1b)**. On average, γδT cells had a similar prevalence to select myeloid-derived cellular subsets within the PDA TME (**Figure 1c**) and comprised a significantly higher percentage of tumor-infiltrating lymphocytes compared with $CD8^+$ T cells (**Figure 1d)**. Human T cell subsets, including γδT cells, can be broadly classified as central memory (T_{CM}) or effector memory (T_{EM}) based on their co-expression of CD45RA and CD27 (Sallusto et al., 2004). We found γδT cells in PBMC were predominantly T_{CM} whereas PDA-infiltrating γδT cells were mostly T_{EM} cells, indicative of a distinctly activated phenotype (**Figure 1e**). Accordingly, tumor-infiltrating γδT cells down-regulated CD62L compared with their counterparts in PBMC (**Figure 1f**). However, $V\gamma9^+$ γδT cells – associated with tumoricidal function (Izumi et al., 2013; Kunzmann et al., 2012) – were notably absent in PDA, suggestive of tumor-permissive properties (**Figure 1g**).

A distinctly activated γδT cell population is prominent in invasive and pre-invasive murine PDA

In vivo imaging of pancreata from C57BL/6-*Trdc*^{tm1Mal} mice harboring orthotopically implanted $Pdx1^{Cre}$;$Kras^{G12D}$;$Tp53^{R172H}$ (KPC)-derived invasive PDA suggested that γδT cells were highly prevalent in the interstitial space of murine PDA (**Figure 2a**). Flow cytometry suggested a higher frequency of γδT cells infiltrating orthotopic KPC tumors compared with the spleen of tumor-bearing mice (**Figure 2b)**. Similar to human disease, the population of PDA-infiltrating γδT cells in mice were distinctly activated expressing higher FasL, NK1.1, CD39, CD44, JAML, and OX40 compared with spleen γδT cells (**Figure 2c**). Further, in contrast to

spleen, PDA-infiltrating γδT cells contained a prominent $V\gamma4^+$ subset whereas $V\gamma1^+$ cells were rare (**Figure 2c**). Tumor-infiltrating γδT cells also expressed elevated levels of IL-10 and IL-17 (**Figure 2d, e**). Similarly, Th1- (TNFα, IFNγ), and additional Th2- (IL-13) cytokines were highly expressed in PDA-infiltrating γδT cells (not shown). Moreover, PDA-infiltrating γδT cells exhibited a substantial $FoxP3^+$ fraction which has been associated with immune suppressive function (Kang et al., 2009), compared with absent expression of $FoxP3^+$ in spleen γδT cells (**Figure S1a**). Conversely, T-bet was equally expressed in γδT cells in both compartments (**Figure S1b**). Further, PDA-infiltrating γδT cells expressed high levels of the NKG2D receptor (**Figure 2f**) as well as elevated TLR4, TLR7 and TLR9 (**Figure 2g)** which are potential avenues for cellular activation in PDA (Zambirinis et al., 2015). CCR2, CCR5, and CCR6 were also upregulated in PDA-infiltrating γδT cells (**Figure 2h).**

To determine whether γδT cells were similarly prominent in a slowly progressive model of PDA, we interrogated pancreata of 6 month-old $p48^{Cre}$;$Kras^{G12D}$ (KC) mice harboring pre-invasive tumor. γδT cells represented ~6-8% of $CD3^+$ T cells in the pancreas of KC mice compared with ~2% in the spleen and tumor-draining lymph nodes (**Figure S2a**). Further, similar to mice with invasive PDA, γδT cells expressed high levels of chemokine receptors (**Figure S2b**), TLRs (**Figure S2c**), and activation markers, and included a prominent $V\gamma4^+$ fraction (**Figure S2d**).

γδT cell recruitment and activation in PDA is contingent on diverse chemokine signaling

Since we found that PDA-infiltrating γδT cells express high CCR2, CCR5, and CCR6, we postulated that ligation of these receptors is critical in their recruitment to the TME. To test this, we challenged $CCR2^{-/-}$, $CCL2^{-/-}$, $CCR5^{-/-}$, and $CCR6^{-/-}$ mice with orthotopic KPC-derived tumor and measured γδT cell infiltration on day 21. Deletion of CCR2, CCL2, or CCR6

significantly reduced γδT cell infiltration to the TME (**Figure S3a**). Moreover, selective CCR2, CCR5, CCR6 or CCL2 deletion mitigated TNF-α and IL-13 expression from PDA-infiltrating γδT cells whereas γδT cell expression of IL-17 and IFN-γ were not affected (**Figure S3b-e**). γδT cell expression of FoxP3 or IL-10 were similarly not perturbed by blockade of chemokine signaling (not shown).

γδT cells promote pancreatic oncogenesis

Since γδT cells are a prominent lymphocytic subset within the pancreatic TME, we postulated that they play a critical role in oncogenesis. To test this, we crossed KC mice with Tcrδ$^{-/-}$ mice. Pancreata of KC;Tcrδ$^{-/-}$ mice were protected from progressive oncogenesis exhibiting a diminished rate of acinar replacement by dysplastic ducts and substantially slower PanIN progression at multiple time-points (**Figure 3a**). Analysis of pancreas weights confirmed the protective effects of γδT cell deletion (**Figure 3b**). γδT cell ablation was also associated with reduced peri-tumoral fibrosis (**Figure 3c**). Moreover, Kaplan-Meier analysis revealed a nearly 1 year increase in the median survival of γδT cell-deficient KC mice compared with controls (**Figure 3d**).

Since genetic deletion of γδT cells has limited translational applicability to human disease, we tested whether *in vivo* depletion of Vγ4^{+} γδT cells using a neutralizing mAb would offer similar protection (**Figure S3f**). We treated 6 week-old KC mice for 8 weeks with UC3-10A6 or isotype control and assessed their effects on tumorigenesis. γδT cell depletion protected against oncogenic progression based on histological analysis of ductal transformation (**Figure 3e**) and tumor mass (**Figure 3f**). To determine whether the presence of γδT cells are similarly associated with accelerated tumorigenesis in an invasive model of PDA, we orthotopically implanted KPC-derived tumor cells into the pancreatic body of WT and Tcrδ$^{-/-}$ mice. Consistent

with our data in KC mice, deletion of γδT cells impressively protected against tumor growth and extended survival in the orthotopic KPC model (**Figure S3g, h**). γδT cell depletion similarly extended survival in invasive PDA (**Figure S3g**). Moreover, blocking γδT cell recruitment and activation using mice deficient in selective chemokine signaling was also protective (**Figure S3i**). Notably, disease phenotype in caerulein-induced pancreatitis was not mitigated in Tcrδ$^{-/-}$ mice suggesting that the ability of γδT cells to modulate pancreatic disease is specific to PDA (**Figure S4a-g**).

PDA-infiltrating γδT cells do not have direct pro-tumorigenic effects on epithelial cells

We hypothesized that γδT cells may have direct oncogenic effects on transformed epithelial cells. To test this, we co-cultured tumor cells derived from KPC mice with FACS-sorted PDA-infiltrating γδT cells. However, γδT cells failed to enhance proliferation (**Figure S4h**) or deregulate expression of oncogenic or tumor suppressor genes (**Figure S4i)** in transformed epithelial cells. Similarly, γδT cell co-culture did not elicit pro-inflammatory or regulatory cytokine production from tumor cells suggesting that PDA-infiltrating γδT cells do not promote tumorigenesis via direct engagement of cancer cells (**Figure S4j**).

γδT cells support an immune suppressive pancreas tumor microenvironment in invasive and pre-invasive PDA

We postulated that intra-pancreatic γδT cells may promote tumorigenesis by engendering an immune-suppressive pancreatic TME. We found that whereas CD4$^+$ and CD8$^+$ T cells were scarce in invasive PDA tumors, tumor-infiltrating CD4$^+$ and CD8$^+$ T cells increased ~10-fold in absence of γδT cells (**Figure 4a, b**). Moreover, besides expanding in number, PDA-infiltrating αβT cells were markedly activated in Tcrδ$^{-/-}$ hosts. CD8$^+$ T cells infiltrating γδT cell-deficient tumors expressed higher CD44 (**Figure 4c**), ICOS (**Figure 4d**), CTLA4 (**Figure 4e**), and

Granzyme B (**Figure 4f**), each indicative of higher cytotoxic T cell activation. Similarly, $CD4^+$ T cells infiltrating γδT cell-deficient tumors expressed higher CD44 (**Figure 4g**), OX40 (**Figure 4h**), and PD-1 (**Figure 4i**), and lower CD62L (**Figure 4j**). Further, both $CD4^+$ and $CD8^+$ T cells expressed elevated TNF-α and IFN-γ in γδT cell-deleted tumors, indicative of enhanced Th1-differentation and higher $CD8^+$ T cell cytotoxicity (**Figure 5a**). Accordingly, PDA-infiltrating $CD4^+$ and $CD8^+$ T cells each sharply upregulated T-bet expression in the context of γδT cell deletion (**Figure 5b**). GATA-3 and FoxP3 expression in $CD4^+$ T cells were not affected by γδT cell deletion (**Figure 5c, d**). Collectively, these data suggest immunogenic reprogramming of adaptive αβT lymphocytes in PDA in the absence of γδT cells.

To determine whether γδT cells similarly delimit αβT cell expansion and activation in a slowly progressive model of PDA, we compared $CD4^+$ and $CD8^+$ T cell phenotype in KC;$Tcr\delta^{+/+}$ versus KC;$Tcr\delta^{-/-}$ pancreata. We found that while $CD4^+$ and $CD8^+$ T cells were scarce in KC;$Tcr\delta^{+/+}$ controls, both lymphocyte populations were markedly expanded in KC;$Tcr\delta^{-/-}$ pancreata (**Figure S5a, b**). Further, both $CD4^+$ and $CD8^+$ T cells in PDA-draining lymph nodes expressed higher CD44 (**Figure S5c**) and PD-1 (**Figure S5d**) in KC;$Tcr\delta^{-/-}$ animals compared with KC;$Tcr\delta^{+/+}$. Similarly, ICOS and Granzyme B expression were increased in $CD8^+$ T cells in KC;$Tcr\delta^{-/-}$ hosts (**Figure S5e**). Moreover, similar to the orthotopic KPC model, pancreas-draining $CD4^+$ and $CD8^+$ T cells in KC mice upregulated IFN-γ (**Figure S5f**) and T-bet (**Figure S5g**) in the context of γδT cell deletion whereas $CD4^+$ T cell expression of GATA-3 and FoxP3 were unaffected by γδT cell deletion (**Figure S5h, i**).

To definitively test whether enhanced αβT cell immunogenicity accounts for the protection against PDA observed in γδT cell-deficient animals, we depleted $CD4^+$ and $CD8^+$ T cells in $Tcr\delta^{-/-}$ mice and WT controls coincident with KPC-derived orthotopic tumor challenge. Ablation

of αβT cell populations did not accelerate tumor growth in WT hosts but completely reversed the tumor-protective effects of γδT cell deletion. These data suggest that tumor-protection in PDA-bearing Tcrδ$^{-/-}$ mice is mediated by αβT cells (**Figure 5e**). To test whether PDA-infiltrating γδT cell inhibition of CD4^{+} and CD8^{+} T cells requires direct cellular interaction, we activated spleen αβT cells *in vitro* using CD3/CD28 co-ligation alone or in the context of either co-culture with PDA-derived γδT cells or admixture with γδT cell-conditioned media. Direct γδT cell coculture prevented CD4^{+} and CD8^{+} T cells from adopting an activated CD44^{+}CD62L^{-} phenotype (**Figure S6a, b**) and expressing immune-modulatory cytokines (**Figure S6c-e)**; however, γδT cell-conditioned media was non-inhibitory. These data suggest that γδT cells do not inhibit αβT cells via secreted factors but require direct cellular interaction.

Pancreas-infiltrating γδT cells express high T cell exhaustion ligands

We postulated that γδT cells may directly inhibit CD4^{+} and CD8^{+} T cell activation. We discovered that PDA-infiltrating γδT cells in KC mice expressed high PD-L1 (**Figure 6a**) and Galectin-9 (**Figure 6b**) compared with absent expression of these ligands in spleen γδT cells. Similarly, γδT cells in orthotopic KPC tumors also expressed elevated PD-L1 and Galectin-9 (**Figure 6c**). Expression levels of PD-L1 and Galectin-9 in PDA-infiltrating γδT cells were markedly higher than in cancer cells and comparable with that of tumor-infiltrating myeloid cell populations (**Figure 6c**). By contrast, PDA-infiltrating γδT cells expressed elevated B7-1 but low levels of other activating ligands including B7-2, ICOSL, and OX40L in orthotopic KPC (**Figure 6d**) and KC (not shown) tumors. Exhaustion ligand expression in myeloid or tumor cells in PDA was not affected by γδT cell deletion (**Figure 6e**). Notably, besides regulating γδT cell expansion and activation, CCR2, CCR5, and CCR6 signaling were necessary for γδT cell expression of PD-L1 or Galectin-9 (**Figure 6f, g**). To determine whether these findings translated to human

disease, we tested PD-L1 expression in human PDA. Remarkably, PBMC γδT cells in PDA patients expressed elevated PD-L1 compared with absent PD-L1 expression in PBMC γδT cells from healthy subjects (**Figure 6h**). Moreover, PD-L1 was expressed in ~50% of tumor-infiltrating γδT cells in human PDA (**Figure 6i**). Similarly, Galectin-9 was upregulated in human PDA-infiltrating γδT cells (**Figure 6j**).

γδT cells inhibit αβT cell activation via checkpoint receptor ligation

Previous reports have shown that low PD-L1 expression is associated with improved survival in human PDA and that PD-L1 blockade in murine PDA protects mice longitudinally (Nomi et al., 2007). We postulated that γδT cells promote PDA progression by preventing αβT cell activation via checkpoint receptor ligation. To test this, we again activated spleen $CD4^+$ and $CD8^+$ T cells *in vitro* using CD3/CD28 co-ligation alone or in the context of co-culture with PDA-derived γδT cells. Similar to our previous experiments, γδT cells prevented $CD4^+$ (**Figure 7a**) and $CD8^+$ (**Figure 7b**) T cells from adopting an activated $CD44^+CD62L^-$ phenotype; however, γδT cell-mediated suppression was reversed with PD-L1 blockade. Further, whereas PDA-infiltrating γδT cells prevented αβT cell expression of TNF-α *in vitro*, this was again reversed by PD-L1 blockade (**Figure 7c**).

To definitively test whether γδT cells promote PDA progression *in vivo* via checkpoint ligand-dependent immune-suppression, we serially blocked PD-L1 or Galectin-9 using neutralizing mAbs in cohorts of WT and $Tcr\delta^{-/-}$ mice challenged with orthotopic KPC-derived tumor. Consistent with our hypothesis, PD-L1 or Galectin-9 blockade protected WT mice but were ineffective at further inducing tumor-protection in $Tcr\delta^{-/-}$ animals (**Figures 7d**). Moreover, αPD-L1 and αGalectin-9 each substantially increased αβT cell infiltration of PDA in WT mice (**Figure 7e**) but failed to enhance αβT cell infiltration in $Tcr\delta^{-/-}$ hosts (not shown). Similarly,

both PD-L1 and Galectin-9 blockade *in vivo* induced an activated $CD4^+$ and $CD8^+$ T cell phenotype in orthotopic PDA in WT mice but did not enhance αβT cell activation or Th1-polarization in PDA in $Tcr\delta^{-/-}$ hosts (**Figure 7f, g and S6f, g**). To determine whether checkpoint ligand antagonism was also only efficacious in γδT cell-competent hosts in a slowly progressive model of PDA, we serially treated cohorts of 6 week old KC;$Tcr\delta^{+/+}$ and KC;$Tcr\delta^{-/-}$ mice for 8 weeks with an αPD-L1 mAb. Again, PD-L1 inhibition protected KC pancreata from oncogenic progression but offered no benefit in KC;$Tcr\delta^{-/-}$ mice (**Figure S6h**). Moreover, adoptive transfer of PDA-entrained γδT cells to $Tcr\delta^{-/-}$ mice coincident with orthotopic tumor challenge resulted in tumor growth rates comparable to WT mice (**Figure S6i**). However, *ex-vivo* blockade of PD-L1 in γδT cells prior to adoptive transfer failed to accelerate tumor growth (**Figure S6j**). To determine whether PDA-infiltrating γδT cells abrogate antigen-restricted anti-tumor immunity in a PD-L1 dependent manner, we directly inoculated PDA-infiltrating γδT cells into established subcutaneous PDA tumors engineered to express OVA in $Tcr\delta^{-/-}$ hosts. γδT cell administration again accelerated tumor growth and concomitantly diminished OVA-specific $CD8^+$ T cell proliferation and activation. However, *ex-vivo* blockade of PD-L1 blockade in γδT cells abrogated their tumor-promoting and immune-suppressive effects (**Figure S6k-m**). Collectively, these data imply γδT cells are important mediators of checkpoint receptor dependent immune-suppression in PDA.

Notably, whereas γδT cell deletion augmented αβT cell infiltration and activation in PDA, it did not alter the fraction of PDA-infiltrating MDSCs or tumor-associated macrophages (TAMs) (**Figure S7a**). Similarly, γδT cell deletion did not affect the capacity of MDSCs or TAMs to mitigate T cell proliferation in PDA (**Figure S7b, c**). Further, in contrast to the exhaustion ligand-dependent immune-suppressive effects of γδT cells, PDA-infiltrating MDSC

inhibition of αβT cell activation was independent of PD-L1 and macrophage-mediated inhibition was only partially mitigated by PD-L1 blockade based on αCD3/αCD28-mediated αβT cell proliferation (**Figure S7b, c**), expression of TNF-α (**Figure S7d, e**) and adoption of a $CD44^{+}CD62L^{-}$ phenotype (not shown). Moreover, whereas αβT cells were in intimate proximity with γδT cells in the PDA TME, myeloid cells were separated by great distances from αβT cells *in situ* in human PDA (**Figure S7f**), in invasive murine PDA (**Figure S7g**) and in pre-invasive disease (not shown) suggesting enhanced opportunity for direct γδT cell–αβT cell interaction and limited opportunity for direct cross-talk between macrophages and αβT cells. Similarly, whereas αβT cells were in direct contact with PD-$L1^{+}$ γδT cells (**Figure S7h**), αβT cells were not in close proximity of PD-$L1^{+}$ epithelial cells (**Figure S7i**).

Discussion

Immune suppressive inflammation is paramount for PDA progression. Murine modeling of PDA using animals that endogenously express pancreas-specific oncogenic *Kras* revealed that pancreatic dysplasia is preceded by and accompanied by vigorous pancreatitis (Hingorani et al., 2003). Moreover, a driving oncogenic mutation alone is insufficient for disease progression and concomitant pancreatitis is necessary for PDA development (Guerra et al., 2007). The peri-pancreatic immune infiltrate is rife with immune-suppressive elements that support oncogenesis. In particular, innate immune cells within the TME are apt at educating adaptive immune effectors towards a tumor-permissive phenotype. APC populations, including M2-polarized TAMs and myeloid dendritic cells, induce the generation of PDA-promoting Th2 cells over Th1 cells that facilitate cytotoxic T lymphocytes (CTL) (Ochi et al., 2012c; Zhu et al., 2014). Similarly, we and others have shown that GM-CSF-recruited MDSC negate anti-tumor $CD8^+$ CTL responses in PDA and promote metastatic progression (Bayne et al., 2012; Connolly et al., 2010; Pylayeva-Gupta et al., 2012). Effector T cells are also thought to be excluded from the PDA TME by CXCL12 produced by a subset of carcinoma-associated fibroblasts which express fibroblast activation protein (FAP) (Feig et al., 2013). However, a comprehensive understanding of the basis for T cell scarcity and poor immunogenicity in PDA is lacking.

γδT cells have not been well-characterized in PDA and their role in the programming of the TME remains ill-defined. We found that γδT cells are pervasive in human and murine PDA and tumor infiltration with γδT cells promotes oncogenic progression whereas genetic deletion, therapeutic depletion, and blockade of recruitment of γδT cells markedly delays morphologic transformation of the pancreas and increases median animal survival by nearly one year in a slowly progressive model of PDA. In contrast to our findings, γδT cells have long been considered potent anti-tumor entities in diverse tumor subtypes (Cordova et al., 2012; Todaro et

al., 2009). In melanoma, renal cell cancer, and colon cancer the putative protective effects of γδT cells have led to strategies employing exogenous activation of γδT cells to maximize their tumoricidal activity *in vivo* (Gao et al., 2003; Girardi et al., 2001; Lanca and Silva-Santos, 2012). While our findings are ostensibly paradoxical to the described function of γδT cells in these cancer models, we demonstrate that the γδT cells in PDA exhibit a unique phenotype. Most interestingly, PDA-infiltrating γδT cells express substantial FoxP3 which is absent in spleen γδT cells from the same animals. Endogenous FoxP3 expression in γδT cells has not been previously reported. However, FoxP3 can be induced in γδT cells upon *in vitro* stimulation with TGF-β in combination with TCRγδ ligation (Kang et al., 2009). Resultant $FoxP3^+$ γδT cells are potently suppressive to T cell activation and proliferation. We found that chemokine signaling does not influence γδT cell expression of FoxP3 in PDA. However, we and others have shown that the PDA TME is rife with TGF-β which can possibly induce FoxP3 expression (Goggins et al., 1998; Greco et al., 2015). Also consistent with a tumor-permissive phenotype, human PDA-infiltrating γδT cells do not express the Vγ9 TCR whose ligation has been implicated in the direct tumoricidal activity of γδT cells in melanoma and colon cancer (Izumi et al., 2013; Kunzmann et al., 2012).

While most early reports suggested that γδT cells were notable for their anti-cancer properties, emerging data suggest that γδT cells can have pro-tumorigenic effects. Select subsets of tumor infiltrating γδT cells in breast cancer block the maturation of TLR8-sensitive dendritic cells and their capacity to prime αβT cells (Peng et al., 2007). In murine B16 melanoma, $V\gamma4^+$ and $V\gamma1^+$ subsets of γδT cells reportedly have opposing roles in tumorigenesis with $V\gamma4^+$ cells mediating protective anti-tumor immunity via IFNγ and perforin, whereas $V\gamma1^+$ cells produce tumor-permissive Th2-family cytokines (Hao et al., 2011). By contrast, in PDA we found that

tumor-promoting γδT cells are almost exclusively $V\gamma4^{+}V\gamma1^{-}$. Coffelt et al. showed in breast cancer models that IL-17 expression from γδT cells results in G-CSF–dependent expansion of neutrophils which acquire the ability to suppress anti-tumor CTL activity (Coffelt et al., 2015). Similarly, IL-17 production by γδT cells in murine hepatocellular carcinoma and colorectal cancer models mediates MDSC infiltration and their subsequent inhibition of cytotoxic $CD8^{+}$ T cells (Ma et al., 2014; Wu et al., 2014). By contrast, we demonstrate that deletion of γδT cells in PDA does not influence the fraction of myeloid cells in the TME nor does it affect their functional capacity to suppress T cell proliferation. Consistent with recent reports, we show that PDA-infiltrating γδT cells express high IL-17 which can directly promote pancreatic oncogenesis via ligation of IL-17R on transformed epithelial cells (McAllister et al., 2014; Wu et al., 2015). However, our cumulative data suggest that IL-17 may not be critical to the pro-tumorigenic effects of PDA-infiltrating γδT cells since blockade of select chemokine signaling mitigated γδT cell infiltration, activation, and exhaustion ligand expression and was protective against PDA despite IL-17 expression being unaffected. Further, our *in vitro* correlative studies suggested that secreted factors in γδT cell conditioned media were non-inhibitory to $CD4^{+}$ and $CD8^{+}$ T cell activation.

We demonstrate that γδT cells create an immune-suppressive adaptive TME through checkpoint receptor ligation in tumor-infiltrating αβT cells. Deletion of γδT cells in PDA results in a robust influx of $CD4^{+}$ and $CD8^{+}$ T cells. Furthermore, in the absence of γδT cells, $CD4^{+}$ T cells exhibit accentuated Th1-differentiation and $CD8^{+}$ T cells exhibit a heightened cytotoxic phenotype. Moreover, whereas deletion of $CD4^{+}$ and $CD8^{+}$ T cells did not accelerate tumor progression in γδT cell-competent hosts, in $Tcr\delta^{-/-}$ mice αβT cell deletion nearly tripled the rate

of PDA growth. This observation supports the notion that αβT cells are entirely dispensable in PDA, but are reprogramed into powerful anti-tumor entities in the absence of γδT cells.

The volume of T cell exhaustion ligand levels in carcinomas, including in PDA, has been largely attributed to expression from tumor cells and macrophages (Nomi et al., 2007; Sharma and Allison, 2015). However, we show that γδT cells express considerably higher levels of PD-L1 and Galectin-9 in PDA than cancer cells. More importantly, we demonstrate that γδT cells are important contributors to PD-L1 and Galectin-9 induced T cell exhaustion in the TME based on our observation that inhibition of PD-L1 and Galectin-9 in PDA is protective *in vivo* in the presence of γδT cells, whereas in absence of γδT cells PD-L1 or Galectin-9 blockade offers no additional tumor-protective benefit. Even more, PD-L1 or Galectin-9 blockade expand and potently activate PDA-infiltrating $CD4^+$ and $CD8^+$ T cells in γδT cell-competent hosts but do not enhance αβT cell immunogenicity in the absence of γδT cells. It is perhaps surprising that checkpoint receptor blockade would not have potency in $Tcr\delta^{-/-}$ mice considering the substantial myeloid cell infiltrate in PDA (Liou et al., 2015; Pylayeva-Gupta et al., 2012). Indeed we found that myeloid cells from $Tcr\delta^{-/-}$ mice have equivalent T cell inhibitory capacity to their cellular counterparts in WT mice. However, we found that whereas αβT cells are in intimate proximity to γδT cells in the PDA TME, myeloid cells are separated by great distances from αβT cells in situ in human PDA, invasive murine PDA, and pre-invasive disease suggesting enhanced opportunity for γδT cell-αβT cell interaction and limited opportunity for direct cross-talk between macrophages and αβT cells. Further, tumor cell expression of exhaustion ligands is also possibly of lesser significance as T cells are excluded from direct contact with tumor cells via CXCL12 produced from FAP-expressing carcinoma-associated fibroblasts in PDA (Feig et al., 2013; Joyce and Fearon, 2015). Collectively, these data may suggest that αβT cells are prevented from

having immunogenic relevance in PDA via a "double-hit": fibroblast-mediated chemokine signaling excludes αβT cells from the direct tumor environs where γδT cells serve to check their activation via ligation of inhibitory receptors.

In summary, we show that PDA-infiltrating γδT cells are a highly influential lymphocyte subset in human and murine PDA which promote pancreatic oncogenesis and reduce survival via novel cross-talk with the adaptive immune compartment. These data implicate γδT cells as high-yield targets for the development of experimental therapeutics in PDA and has potential implications for the mechanistic progression of oncogenesis in other cancer subtypes. Finally, γδT cells may have prognostic significance in PDA, and may be predictive of response to immunotherapeutic regimens.

Author Contributions

DD (project leadership, data collection and analysis, manuscript preparation), CZ (project conception, data collection), LS (data analysis, manuscript preparation), NA and NM (IHC, flow cytometry, in vivo experiments), GW (manuscript preparation), AA (In vivo experiments, tissue culture), RB (technical assistance), DT (mouse breeding and genotyping), RN (Data analysis), ATH, MH and VRJM (technical assistance), JEJ (intravital imaging), EN (human tissue procurement), VGP (IHC), MLD (intravital imaging), DBS (intravital imaging), CH (human tissue procurement, pathologic analysis), GM (project design, data analysis, manuscript preparation).

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Figure Legends

Figure 1. γδT cells are ubiquitous and activated in human PDA. (a) Frozen sections of human PDA and normal pancreas were stained using a mAb specific for TCRγ/δ or isotype control. Representative images and quantitative data are shown. **(b)** Single cell suspensions from human PDA tumors and PBMC were co-stained for CD45, CD3, and TCRγ/δ. The percentage of γδT cells among $CD3^+$ cells was calculated. Representative contour plots and summary data are shown. Each dot represents a different patient sample. **(c)** The percentage of PDA-infiltrating γδT cells among $CD45^+$ cells was compared with tumor-infiltrating cells expressing select myeloid differentiation markers. **(d)** The percentage of PDA-infiltrating and PBMC γδT cells among $CD3^+$ cells was compared with that of $CD4^+$ and $CD8^+$ αβT cell subsets in each respective compartment. **(e)** PBMC and PDA-infiltrating $CD3^+TCR\gamma/\delta^+$ cells from PDA patients were gated and co-stained using mAbs specific for CD45RA and CD27. The gating paradigms for T_{naive}, T_{CM}, T_{EM}, and $T_{EM\text{-}RA}$ populations are shown. Representative contour plots and quantitative data indicating the fraction of T_{EM} γδT cells in each compartment are indicated. **(f)** PDA-infiltrating and PBMC γδT cells from PDA patients were stained using mAbs specific for CD62L and **(g)** Vγ9. Representative histograms and quantitative data are shown. Human data are based on tumor tissue or PBMC analyzed from 9-13 PDA patients (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

Figure 2. γδT cells are highly prevalent and exhibit a uniquely activated phenotype in murine invasive PDA. (a) C57BL/6-*Trdc*^{tm1Mal} mice whose γδT cells express GFP were orthotopically implanted with KPC-derived tumor and imaged by intra-vital two-photon laser-scanning microscopy at 21 days. **(b)** WT mice were orthotopically implanted with KPC-derived tumor cells. On day 21, single cell suspensions of digested PDA tumors and splenocytes were

co-stained for CD45, CD3, TCRγ/δ, CD4, and CD8 and analyzed by flow cytometry. Representative contour plots and quantitative data are shown. **(c)** WT mice were orthotopically implanted with KPC-derived tumor cells. On day 21 spleen (blue histograms) and PDA-infiltrating (red histograms) γδT cells were gated and tested for co-expression of select surface activation markers and Vγ chains. Representative histogram overlays and summary data from 5 mice are shown. **(d)** Spleen and PDA-infiltrating γδT cells from the same mice were tested for expression of IL-10, **(e)** IL-17, **(f)** NKG2D, **(g)** TLR4, TLR7, TLR9, and **(h)** CCR2, CCR5, and CCR6. Each experiment was repeated at least 3 times using 3-5 mice per data point (*$p<0.05$, **$p<0.01$).

Figure 3. Ablation of γδT cells protects against pancreatic oncogenesis in a slowly progressive model of PDA. (a) KC;Tcrδ$^{+/+}$ and KC;Tcrδ$^{-/-}$ mice were sacrificed at 3, 6, or 9 months of life (n=10-12 mice/cohort). Representative H&E-stained frozen sections are shown. The percentage of pancreatic area occupied by intact acinar structures, and the fractions of ductal structures exhibiting normal morphology, ADM, or graded PanIN I-III lesions were calculated. **(b)** Weights of pancreata were compared in 3 month-old KC;Tcrδ$^{+/+}$ and KC;Tcrδ$^{-/-}$ mice. **(c)** Pancreata from 9 month-old KC;Tcrδ$^{+/+}$ and KC;Tcrδ$^{-/-}$ mice were assayed for peri-tumoral fibrosis using trichrome staining. **(d)** Kaplan-Meier survival analysis was performed for KC;Tcrδ$^{+/+}$ (n=29) and KC;Tcrδ$^{-/-}$ (n=44) mice ($p<0.0001$). **(e, f)** KC;Tcrδ$^{+/+}$ mice were treated with UC3-10A6 or isotype control for 8 weeks beginning at 6 weeks of life. (e) Representative H&E stained pancreatic sections are shown. The percentage of pancreatic area occupied by intact acinar structures, and the fractions of ductal structures exhibiting normal morphology, ADM, or graded PanIN I-III lesions were calculated. (f) Tumor weight was recorded (n=5/group; *$p<0.05$, **$p<0.01$).

Figure 4. γδT cell deletion results in massive CD4$^+$ and CD8$^+$ T cell infiltration and activation in invasive PDA. (a, b) WT and Tcrδ$^{-/-}$ mice were implanted with KPC-derived tumor cells. On day 21 mice were sacrificed. Frozen pancreatic sections were tested for (a) CD8$^+$ and (b) CD4$^+$ T cell infiltration by IHC (n=5/group). **(c)** CD8$^+$ T cells infiltrating orthotopically-implanted KPC-derived tumors in WT and Tcrδ$^{-/-}$ mice were tested for expression of CD44, **(d)** ICOS, **(e**) CTLA-4, and **(f)** Granzyme B. (**g**) Similarly, CD4$^+$ T cells infiltrating orthotopically-implanted KPC tumors in WT and Tcrδ$^{-/-}$ mice were tested for expression of CD44, **(h)** OX40, **(i)** PD-1, and **(j)** CD62L. Experiments were repeated more than 3 times with similar results using 5 mice per group (*p<0.05, **p<0.01, ***p<0.001).

Figure 5. γδT cell deletion results in CD4$^+$ T cell Th1 differentiation, CD8$^+$ T cell activation, and αβT cell-dependent tumor protection in invasive PDA. (a-d) WT and Tcrδ$^{-/-}$ mice were orthotopically implanted with KPC-derived tumor cells. On day 21, tumor-infiltrating CD4$^+$ and CD8$^+$ T cells were interrogated for (a) co-expression of TNF-α and IFN-γ, (b) expression of T-bet, (c) GATA-3, and (d) FoxP3. Representative contour plots and quantitative data are shown. Experiments were repeated twice with similar results (n=5/group; *p<0.05). **(e)** WT and Tcrδ$^{-/-}$ pancreata were orthotopically implanted with KPC-derived tumor cells and serially treated with α-CD4 and α-CD8 neutralizing mAbs or isotype controls. Pancreatic tumors were harvested at 3 weeks. Representative images and tumor weights are shown (n=5/group; *p<0.05, **p<0.01, ***p<0.001).

Figure 6. PDA-associated γδT cells express high levels of T cell exhaustion ligands in multiple murine tumor models and in human disease. (a) Expression of PD-L1 and **(b)** Galectin-9 were compared in pancreas and spleen γδT cells of 3-month-old KC mice by flow cytometry. Representative contour plots and quantitative data are shown (n=5/group). **(c)** WT mice were

orthotopically implanted with KPC-derived tumor cells. Expression of PD-L1 and Galectin-9 were compared in PDA tumor cells, TAMs (Mφ), MDSC, and γδT cells on day 21 (n=5/group). **(d)** WT mice were orthotopically implanted with KPC-derived tumor cells. On day 21, spleen and PDA-infiltrating γδT cells were tested for expression of select activating ligands. Representative histograms and quantitative data are shown (n=5/group). **(e)** Orthotopic PDA-bearing WT and Tcrδ$^{-/-}$ mice were tested for expression of PD-L1 in tumor cells, TAMs, and MDSC (n=5/group). **(f)** WT, CCR2$^{-/-}$, CCR5$^{-/-}$, and CCR6$^{-/-}$ mice were orthotopically implanted with KPC-derived PDA cells (n=5/group). Animals were sacrificed at 3 weeks, and the fraction of tumor-infiltrating γδT cells expressing PD-L1 and **(g)** Galectin-9 were determined by flow cytometry. **(h, i)** PBMC γδT cells from healthy volunteers and PDA patients, and PDA-infiltrating γδT cells and were tested for expression of (h) PD-L1 and (i) Galectin-9. Representative histograms and quantitative data are shown (n=11 patients; *p<0.05, **p<0.01, ***p<0.001).

Figure 7. Exhaustion ligand blockade reverses the direct suppressive effects of γδT cells on αβT cells and on pancreatic tumorigenesis. (a) Splenic CD4^{+} or **(b)** CD8^{+} T cells from untreated WT mice were either unstimulated, or stimulated with αCD3/αCD28 alone or in co-culture with PDA-infiltrating γδT cells (5:1 ratio). αPD-L1 (10μg/ml) was selectively added to each group. The fraction of CD62L^{-}CD44^{+} cells were determined at 72h by flow cytometry. Representative contour plots and quantitative data are shown. **(c)** Similarly, CD4^{+} and CD8^{+} T cell expression of TNF-α was measured. Experiments were performed in quadruplicate and repeated 3 times. **(d)** WT and Tcrδ$^{-/-}$ mice were orthotopically implanted with KPC-derived tumor cells and serially treated with αPD-L1 or αGalectin-9 neutralizing mAbs, or respective isotype controls. Pancreatic tumors were harvested at 3 weeks. Representative gross images are shown (Experiment #1) as are quantitative data on tumor weights from 2 separate experiments using different stocks of KPC-

derived tumor cells (n=5/group for each experiment). **(e-g)** WT and Tcrδ$^{-/-}$ pancreata were again orthotopically implanted with KPC-derived tumor cells and serially treated with αPD-L1 or αGalectin-9 neutralizing mAbs or the respective isotype controls. Pancreatic tumors were harvested at 3 weeks. (e) The fraction of PDA-infiltrating αβT cells among CD45^{+} leukocytes, and (f) CD8^{+} and (g) CD4^{+} T cell adoption of an activated CD62L^{-}CD44^{+} phenotype, were determined by flow cytometry (n=5/group; *p<0.05, **p<0.01, ***p<0.01).

Supplemental Figure Legends

Figure S1. Related to Figure 2. PDA-infiltrating γδT cells express elevated FoxP3. (a) WT mice were orthotopically implanted with KPC-derived tumor cells. On day 21, splenic and PDA-infiltrating $CD3^+$ T lymphocytes were co-stained for CD4, TCRγδ, and FoxP3 or **(b)** CD4, TCRγδ, and T-bet. Representative contour plots and quantitative data from 5 mice per group are shown (*p<0.05). Experiments were repeated twice with similar results.

Figure S2. Related to Figure 2. γδT cells in pancreata of KC mice exhibit a distinct phenotype. (a) Single cell suspensions of pancreata, pancreas-draining lymph nodes, and spleen, from 6 month-old KC mice were co-stained for CD45, CD3, and TCRγ/δ. Representative contour plots and quantitative data are shown. **(b)** Similarly, select CCR expression, **(c)** TLR expression, **(d)** and surface markers were compared in γδT cells harvested from the pancreas and spleen of 6 month-old KC mice. Each result was reproduced at least twice (n=5/group; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

Figure S3. Related to Figure 3. Selective blockade of chemokine signaling mitigates γδT cell expansion and activation in PDA and deletion or depletion of γδT cells or interruption of their recruitment is protective against PDA. (a-e) WT, $CCR2^{-/-}$, $CCL2^{-/-}$, $CCR5^{-/-}$, and $CCR6^{-/-}$mice were orthotopically implanted with KPC-derived PDA cells (n=5/group). Animals were sacrificed at 3 weeks. (a) The fraction of tumor-infiltrating γδT cells determined by flow cytometry. (b) γδT cell expression of TNF-α, (c) IL-13, (d) IL-17, and (e) IFN-γ was determined for each cohort (n=5 /group). **(f)** WT mice were treated with UC3-10A6 and splenocytes from these mice were analyzed for expression of Vγ4 and Vγ1. **(g)** WT and $Tcr\delta^{-/-}$ pancreata were orthotopically implanted with KPC-derived PDA cells. Tumors were harvested and weighed at 3

weeks after implantation. Representative gross images of PDA and quantitative data of tumor weights are shown (n=10/group). **(h)** Pancreata from control WT mice, WT mice treated with a neutralizing α-γδT cell mAb, and Tcrδ$^{-/-}$ mice were orthotopically implanted with KPC-derived PDA cells. Kaplan-Meier survival analysis was performed (n=10/group; WT vs Tcrδ$^{-/-}$: p=0.02; WT vs UC3-10A6: p=0.009; Tcrδ$^{-/-}$ vs UC3-10A6: p=ns). **(i)** WT, CCR5$^{-/-}$, CCR6$^{-/-}$, CCR2$^{-/-}$, and CCL2$^{-/-}$ mice were orthotopically implanted with KPC-derived PDA cells. Animals were sacrificed at 3 weeks and tumor weights were recorded. Data from 2 separate experiments are shown (n=5/group for each experiment; scale bar =2cm; *p<0.05, **p<0.01, ****p<0.0001).

Figure S4. Related to Figure 3. γδT cells do not directly modulate pancreatitis or transformed epithelial cells. (a) Acute pancreatitis was induced using caerulein in C57BL/6-*Trdc*^{tm1Mal} mice, which express GFP exclusively in γδT cells. Pancreata were harvested at 12h and immunohostochemistry for GFP was performed. Arrows indicate GFP^{+} cells. **(b)** Pancreata and spleens of WT mice undergoing caerulein-induced pancreatitis were assessed by flow cytometry for the presence of CD3^{+}TCRγ/δ^{+} cells. The percentage of γδT cells among the intra-pancreatic or spleen T lymphocyte populations, respectively, was calculated at 48h after commencing caerulein treatment (n=5/group; ****p<0.0001). **(c)** WT mice were administered caerulein for up to 48h and then serially observed for a maximum additional 48h. Cohorts were sacrificed at 0 (untreated), 24, 48, 72, or 96 hours from commencing caerulein treatment and the percentage of pancreas-infiltrating CD4^{+} T cells and γδT cells among CD3^{+} T cells was assessed by flow cytometry (n=3 mice/time-point). **(d-g)** WT and Tcrδ$^{-/-}$ mice were induced to develop caerulein pancreatitis for 48h. (d) Severity of pancreatitis was assessed by H&E staining, (e) CD45^{+} pan-leukocyte IHC, and (f) serum amylase and (g) lipase levels (n=5/group). Pancreatitis experiments were repeated more than 5 times with similar results. Representative H&E- and

CD45-stained sections are shown. **(h-j)** KPC-derived PDA cells were co-cultured with PDA-infiltrating or spleen γδT cells in a 1:5 ratio for 24h. (h) Tumor cell proliferation was measured using the XTT assay. (i) Expression of tumor suppressor or oncogenic proteins was assessed by Western blotting. (j) Expression of tumor-modulatory cytokines was determined in a cytometric bead array. Co-culture experiments were repeated 3 times with similar findings.

Figure S5. Related to Figures 4 and 5. γδT cell deletion results in marked αβT cell expansion and activation in a slowly progressive model of PDA. Cohorts of KC;Tcrδ$^{+/+}$ and KC;Tcrδ$^{-/-}$ mice were sacrificed at 3 months of life. Frozen pancreatic sections were tested for **(a)** CD4^{+} and **(b)** CD8^{+} T cell infiltration by immunohistochemistry (n=8/group). **(c)** Pancreas draining lymph nodes from 3 month old KC;Tcrδ$^{+/+}$ and KC;Tcrδ$^{-/-}$ mice were harvested and tested for CD4^{+} and CD8^{+} T cell expression of CD44 and **(d)** PD-1, **(e)** CD8^{+} T cell expression of ICOS and Granzyme B, **(f)** CD4^{+} and CD8^{+} T cell expression of IFN-γ and **(g)** T-bet, and **(h)** CD4^{+} T cell expression of GATA-3 and **(i)** FoxP3. Representative contour plots and quantitative data are shown. Experiments were repeated 2-3 times with similar results (n=5/group; *p<0.05, ****p<0.0001).

Figure S6. Related to Figures 5 and 7. PDA-infiltrating γδT cells inhibit αβT cells but exhaustion ligand blockade is tumor-protective and activates CD8^{+} T cells in γδT cell-competent hosts. (a-e) Splenic αβT cells from untreated WT mice were cultured in 96 well plates either unstimulated, or stimulated with αCD3/αCD28 alone or in co-culture with PDA-infiltrating γδT cells (5:1 ratio) or γδT cell conditioned media. The fraction of CD4^{+} and CD8^{+} T cells (a, b) adopting a CD62L^{-}CD44^{+} phenotype and (c, d) expressing IFN-γ was determined at 72h by flow cytometry. (e) Cytokine expression was also determined by analysis of cell culture supernatant. Experiments were repeated twice (n=4/group). **(f, g)** WT and Tcrδ$^{-/-}$ mice were orthotopically

implanted with KPC-derived tumor cells and serially treated with αPD-L1 or αGalectin-9 neutralizing mAbs or respective isotype controls. Pancreatic tumors were harvested at 3 weeks, and $CD8^+$ T lymphocytes from each cohort were analyzed by flow cytometry for expression of (f) T-bet and (g) TNF-α. **(h)** Cohorts of 6 week old KC;$TCR\delta^{+/+}$ and KC;$TCR\delta^{-/-}$ mice were serially treated with αPD-L1 or isotype control for 8 weeks and pancreata were harvested at 14 weeks. Comparative tumor weights are shown (n=5/group). Controls were previously shown in Figure 3b. **(i, j)** Cohorts of WT mice were orthotopically implanted with KPC-derived tumor cells. In parallel, $Tcr\delta^{-/-}$ mice were similarly treated but tumor cells were co-injected with FACS-sorted PDA-infiltrating γδT cells that were treated ex-vivo with either (i) Rat IgG2b isotype, or (j) αPD-L1. PDA tumors were measured at 21 days (n=4/group). **(k-m)** $Tcr\delta^{-/-}$ mice were subcutaneously implanted with KPC-derived tumor cells engineered to express OVA. On day 10, tumors were directly inoculated with PBS, FACS-sorted PDA-infiltrating γδT cells treated *ex-vivo* with Rat IgG2b, or PDA-infiltrating γδT cells treated with αPD-L1. On day 15 (k) tumor volume (scale bar =1cm), (l) the fraction of $CD8^+$ OVA $Pentamer^+$ T cells among all $CD8^+$ T cells, and (m) OVA $Pentamer^+$ T cell expression of CD107a were recorded (n=5/group;*p<0.05, **p<0.01, ***p<0.001).

Figure S7. Related to Figure 7. γδT cells do not alter myeloid cell infiltration or function in PDA and localize with αβT cells in the TME. (a) WT and $Tcr\delta^{-/-}$ mice were orthotopically implanted with KPC-derived pancreatic tumor cells. Tumors were harvested at 3 weeks and analyzed by flow cytometry. CD11b+ myeloid cells were gated and tested for co-expression of Ly6C and Ly6G. Representative contour plots and quantitative data from 5 mice are shown. **(b, c)** CFSE-labeled splenic $CD3^+$ T cells were either unstimulated, or stimulated with αCD3/αCD28 alone or in co-culture with orthotopic PDA-infiltrating (b) MDSC or (c) TAMs (5:1 ratio) derived

from WT or Tcrδ$^{-/-}$ mice. αPD-L1 was added to select co-culture wells. T cell proliferation was determined by dilution of CFSE on flow cytometry. **(d, e)** CD3^{+} T cells were either unstimulated, or stimulated with αCD3/αCD28 alone or in co-culture with orthotopic PDA-infiltrating γδT cells (d) MDSC or (e) TAMs (5:1 ratio) +/- αPD-L1 (10μg/ml). T cells activation was determined by expression of TNF-α. **(f)** Human PDA and **(g)** orthotopic KPC tumors were co-stained for CD11b/TCRαβ or TCRγδ/TCRαβ. The closest distance between each αβT cell and CD11b^{+} myeloid cell or γδT cell, respectively, were calculated. Representative high and low power images and quantitative data are shown. 10 low power fields were examined per pancreas. **(h)** Orthotopic KPC tumors were co-stained for DAPI, TCRγδ, PD-L1, and TCRαβ or **(i)** DAPI, CK19, PD-L1, and TCRαβ and imaged by confocal microscopy. Two representative images of each combination is shown.

Methods and Resources

Contact for Reagent and Resource Sharing

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Experimental Model and Subject Details

Animals and In Vivo Procedures

C57BL/6 (H-2Kb), C57BL/6-*Trdc*^{tm1Mal}, CCR2$^{-/-}$, CCR5$^{-/-}$, CCR6$^{-/-}$, CCL2$^{-/-}$, and B6.129P2-*Tcrd*^{tm1Mom}/J (Tcrδ$^{-/-}$) mice were purchased from Jackson Labs (Bar Harbor, ME). KC mice (gift of D. Bar-Sagi), which develop pancreatic neoplasia endogenously by expressing mutant *Kras*, were generated by crossing LSL-Kras^{G12D} and p48^{Cre} mice (Hingorani et al., 2003). Tcrδ$^{-/-}$ mice were crossed with KC mice to generate KC;Tcrδ$^{-/-}$ animals. Both male and female mice were used but animals were sex- and age-matched in each experiment. Randomization was not performed. There were no specific inclusion or exclusion criteria. Sample sizes for experiments were determined without formal power calculations. Data for control KC mice have been previously reported (Seifert et al., 2016). For orthotopic tumor challenge, mice were administered intra-pancreatic injections of tumor cells derived from KPC mice ($1x10^5$ cells in Matrigel) and sacrificed at 3 weeks as described (Zambirinis et al., 2015). For subcutaneous tumor challenge, KPC-derived tumor cells ($1x10^6$) engineered to express OVA using pCI-neo-cOVA (gift of Maria Castro; Addgene plasmid # 25097) were administered to the flanks of mice (Yang et al., 2010). In select experiments, FACS-sorted PDA-infiltrating γδT cells were

orthotopicaly transferred ($8x10^5$) together with tumor cells or directly inoculated into subcutaneous tumors ($3x10^5$). In other experiments, animals were treated twice weekly with i.p. injection of neutralizing mAbs directed against TCR Vγ4 (UC3-10A6, 8mg/kg), PD-L1 (10F.9G2, 5mg/kg), or Galectin-9 (RG9-1, 6mg/kg; all BioXCell, West Lebanon, NH). In select experiments CD4 (GK1.5; BioXCell) or CD8 (53-6.72; Monoclonal Antibody Core Facility, Sloan Kettering Institute, New York, NY) T cells were depleted using previously described regimens (Bedrosian et al., 2011). Acute pancreatitis was induced using a regimen of seven hourly i.p. injections of caerulein (50 μg/kg; Sigma, St. Louis, MO) for two consecutive days as we have described (Bedrosian et al., 2011). Serum amylase and lipase levels were measured using commercial kits (Sigma) according to the manufacturer's instructions. Animal procedures were approved by the NYU School of Medicine IACUC.

Human and Murine Cellular Isolation

Pancreatic leukocytes were harvested from mouse PDA as described previously (Ochi et al., 2012a). Briefly, pancreata were resected in total and placed in ice-cold PBS with 1% FBS with Collagenase IV (1 mg/mL; Worthington Biochemical, Lakewood, NJ) and DNAse I (2 U/mL; Promega, Madison, WI). After mincing, tissues were incubated in the same solution at 37°C for 30 minutes with gentle shaking. Specimens were passed through a 70 μm mesh, and centrifuged at 350g for 5 minutes. Human pancreatic tissues and PBMC were collected under an IRB approved protocol and donors of de-identified specimens gave informed consent. Sample sizes for human experiments were not determined based on formal power calculations. Human pancreatic leukocytes were prepared in a similar manner to mice. PBMC were isolated by overlaying whole blood diluted 3:1 in PBS over an equal amount of Ficoll (GE Healthcare,

Princeton, NJ). Cells were then centrifuged at 2100 RPM and the buffy coat harvested as we have described (Rehman et al., 2013).

Method Details

Flow Cytometry and FACS sorting

Cells were suspended in ice-cold PBS with 1% FBS. After blocking FcγRIII/II with an anti-CD16/CD32 mAb (eBioscience, San Diego, CA), cell labeling was performed by incubating 10^6 cells with 1 μg of fluorescently conjugated antibodies directed against murine CD45 (30-F11), CD3 (17A2), CD4 (RM4-5), CD8 (53-6.7), TCR γ/δ (GL3), CD62L (MEL-14), FasL (MFL3), Vγ1 (2.11) , Vγ4 (UC3-10A6), NK1.1 (PK136), CD39 (Duha59), CCR2 (SA203G11), CCR5 (HM-CCR5), CCR6 (29-2L17), CD44 (IM7), JAML (4E10), NKG2D (CX5), CD11b (M1/70), Gr1 (RB6-8C5), PD-1 (29F.1A12), ICOS (15F9), TCR α/β (H57-597), TLR4 (SA15-21), TNF-α (MP6-XT22), IL-17 (TC11-18H10.1), IL-10 (JES5-16E3), INF-γ (XMG1.2), PD-L1 (10F.9G2), Galectin-9 (RG9-35), B7-1 (16-10A1), B7-2 (PO3), ICOSL (HK5.3), OX-40L (RM134L), CD107A (1D4B), CTLA4 (UC10-4B9), Ly6C (HK1.4), Ly6G (1A8), OX-40 (OX-86; all Biolegend, San Diego, CA), TLR7 (IMG-581A), TLR9 (26C593.2; both Imgenex, San Diego, CA), T-bet (eBio4B10), IL-13 (eBio13A), Granzyme B (NGZB), GATA-3 (TWAJ), and FoxP3 (FJK-16s; all eBioscience). OVA-restricted $CD8^+$ T cell proliferation was determined using an H-2kb SIINFEKL OVA Pentamer (ProImmune, Oxford, United Kingdom). Intracellular staining was performed using the FoxP3 Fixation/Permeabilization Solution Kit (eBiosciences). Analysis of human cells was performed using fluorescently conjugated antibodies directed against CD45 (HI30), CD3 (SK7), CD45RA (HI100), CD27 (O323), CD62L (DREG-56), CD14 (HCD14), PD-L1 (29E.2A3), Galectin 9 (9M1-3), CD15 (W6D3), CD11c (3.9), Vγ9 (B3; all Biolegend),

Tcrγ/δ (B1.1; eBioscience). Flow cytometry was performed on the LSR-II (BD Biosciences, Franklin Lakes, NJ). Cytokine levels in cell culture supernatant were measured using a cytometric bead array (BD Biosciences). FACS-sorting was performed on the SY3200 (Sony, Tokyo, Japan). Data were analyzed using FlowJo (Treestar, Ashland, OR).

Western Blotting

Cells or tissues were lysed in ice-cold RIPA buffer. Total protein was quantified using the BioRad DC Protein Assay according to the manufacturer's instructions (BioRad, Hercules, CA). Western blotting was performed as described previously with minor modifications (Ochi et al., 2012a). Briefly, 10% Bis-Tris polyacrylamide gels (NuPage; Invitrogen, Carlsbad, CA) were equiloaded with 10-30μg protein, electrophoresed at 200 V and electrotransferred to PVDF membranes. After blocking with 5% BSA, membranes were probed with primary antibodies to Bcl-XL (54H6), Rb (D20), c-Myc (D84C12), PTEN (26H9), p53 (1C12), and β-actin (8H10D10), all Cell Signaling, Beverly, MA. Blots were developed by ECL (Thermo Scientific, Asheville, NC).

Histology, Immunohistochemistry, and Microscopy

For histological analysis, pancreatic specimens were frozen in OCT medium or fixed with 10% buffered formalin, dehydrated in ethanol, embedded with paraffin, and stained with H&E or Gomori's Trichrome. The fraction of preserved acinar area was calculated as previously described (Ochi et al., 2012a). The fraction and number of ducts containing all grades of PanIN lesions was measured by examining 10 high-power fields (HPFs; 40X) per slide. PanINs were graded according to established criteria (Hruban et al., 2001): In PanIN I ducts, the normal cuboidal pancreatic epithelial cells transition to columnar architecture (PanIN Ia) and gain polyploid morphology (PanIN Ib). PanIN II lesions are associated with loss of polarity. PanIN III

lesions, or in-situ carcinoma, show cribriforming, budding off of cells, and luminal necrosis with marked cytological abnormalities, without invasion beyond the basement membrane. Slides were evaluated by an expert pancreas pathologist (CH). Immunohistochemistry (IHC) was performed using antibodies directed against CD4 (RM4-5; BD Bioscience), CD8 (YTS169.4; Abcam), GFP (D5.1; CellSignalling) and TCRγ/δ (B1; Biolegend). Quantifications were performed by assessing 10 HPF per slide. For immunofluorescent staining, frozen specimens were probed with antibodies directed against TCRγ/δ (GL3; Biolegend), TCRαβ (H57-597; Biolegend), PD-L1 (Polyclonal, Abcam), CK19 (Troma-III; University of Iowa) or CD11b (M1/70; Biolegend). For analysis of human tissues, frozen sections of human pancreatic cancer specimens were probed with antibodies directed against TCRγ/δ (B1.1; eBioscience), TCRαβ (IP26; Biolegend), or CD11b (M1/70; Biolegend). Images were acquired using the Zeiss LSM700 confocal microscope along with ZEN software (Carl Zeiss, Thornwood, New York). The proximity of αβT cells to γδT cells or $CD11b^{+}$ cells, respectively, was determined by measuring the distance between each αβT cell and its spatially closest counterpart. Distances were measured in micrometers on low power fields (20X). The averages distances were calculated for 10 low power fields per pancreas.

Intravital Imaging

Orthotopic pancreas tumor-bearing C57BL/6-*Trdc*^{tm1Mal} mice were anesthetized and a left subcostal laparotomy incision was made. The spleen and pancreatic tumor were externalized. The mouse was then placed prone on a heated (37°C) stage mounted with a coverslip which was in contact with the pancreatic tumor. To visualize the pancreatic vasculature, mice were injected i.v. with 25 µg Evan Blue (Sigma) 10 min before imaging. Images were acquired with a LSM 710 inverted microscope (Zeiss) with a MaiTai Ti:Sapphire laser (Spectra-Physics, Santa Clara, CA) tuned to 910-930 nm. Emitted fluorescence was detected through 420/40, 465/30, 520/30,

575/70, and 660/50 nm band-pass filters and nondescanned detectors to generate second harmonic signals (collagen fibers) and 4-color images. All the images were acquired at least 50 μm below the tumor capsule. ZEN software was used for analysis.

In vitro T cell activation assays

For T cell activation assays, spleen $CD4^+$ or $CD8^+$ T cells ($5x10^4$) were labeled with CFSE (eBioscience) and plated alone or with PDA-infiltrating γδT cells, MDSC, or TAMs (5:1 ratio) in 96 well plates coated with anti-CD3 (145-2C11, 10μg/ml) and anti-CD28 (37.51; 10μg/ml, both Biolegend). After 72 hours, αβT cells were harvested and analyzed by flow cytometry. In selected experiments, cells were treated with a neutralizing mAb directed against PD-L1 (10F.9G2, 10μg/ml; BioXCell).

Quantification and Statistical Analysis

Statistical Analysis

Data is presented as mean +/- standard error. Survival was measured according to the Kaplan-Meier method. The sample size for each experiment, n, is included in the results section and the associated figure legend. Statistical significance was determined by the Student's *t* test and the Wilcoxon test using GraphPad Prism 6 (GraphPad Software, La Jolla, CA). P-values <0.05 were considered significant. P values for each experiment are also included in the associated figure legends.