Unconventional T-lymphocyte populations are emerging as important regulators of tumor immunity. Despite this, the role of TCRαβCD4−CD8−NK1.1− innate αβ T cells (iαβTs) in pancreatic ductal adenocarcinoma (PDA) has not been explored. We found that iαβTs represent ~10% of T lymphocytes infiltrating PDA in mice and humans. Intratumoral iαβTs express a distinct T-cell receptor repertoire and profoundly immunogenic phenotype compared with their peripheral counterparts and conventional lymphocytes. iαβTs comprised ~75% of the total intratumoral IL17+ cells. Moreover, iαβT-cell adoptive transfer is protective in both murine models of PDA and human organotypic systems. We show that iαβT cells induce a CCR5-dependent immunogenic macrophage reprogramming, thereby enabling marked CD4+ and CD8+ T-cell expansion/activation and tumor protection. Collectively, iαβTs govern fundamental intratumoral cross-talk between innate and adaptive immune populations and are attractive therapeutic targets.

SIGNIFICANCE: We found that iαβTs are a profoundly activated T-cell subset in PDA that slow tumor growth in murine and human models of disease. iαβTs induce a CCR5-dependent immunogenic tumor-associated macrophage program, T-cell activation and expansion, and should be considered as novel targets for immunotherapy.

See related commentary by Banerjee et al., p. 1164.
INTRODUCTION

The phenotypic composition of inflammatory cells infiltrating pancreatic ductal adenocarcinoma (PDA) influences disease outcome (1). Depending on the signals released, innate immune cells entrain the adaptive immune compartment toward an immunogenic or tolerogenic response, affecting PDA growth and metastasis. Classically activated M1-like macrophages secrete proinflammatory mediators, such as TNFα, IFNγ, and IL12, which in turn polarize CD4+ T cells toward a Th1 phenotype and activate cytotoxic CD8+ T cells. More commonly, alternatively activated M2-like macrophages promote tolerogenic Th2 and T regulatory cell (Treg) differentiation (2). However, this relationship is bidirectional, as adaptive immune cells can in turn influence innate immune programming. For example, we previously showed that CD4+ T cells can augment tolerogenic macrophage polarization via IL4 (3). Similarly, FOXP3+ Tregs can influence dendritic cell capacity for cross-presentation of tumor antigen (4). These data suggest bidirectional cross-talk between innate and adaptive immune subsets in the PDA tumor microenvironment (TME).

There is growing evidence illustrating the contribution of unconventional T lymphocytes in shaping the immune milieu in PDA. γδT cells, characterized by expression of TCRγδ, expand early in pancreatic oncogenesis. We showed that γδT cells exert direct immune-suppressive influences on conventional T cells in PDA via the PD-L1–PD-1 axis (5). By contrast, natural killer T cells (NKT cells) have recently been shown to protect against PDA by modulating the phenotype of tumor-associated macrophages (TAM) through mPGES-1 and 5-LOX (6).

We observed that CD3+ TCRδδ+CD4–CD8–NK1.1– cells represented ~10% of T cells in murine and human PDA. Given the emerging role for unconventional T cells in PDA, we were spurred to investigate the function of these iαβTs in this disease. iαβTs exhibited a unique cellular program that was influenced by diverse microbial and sterile stimuli within the TME. We further hypothesized that iαβTs may have important immune-modulatory functions in PDA. We found that iαβTs exhibited marked tumor-protective properties in both murine and human PDA by driving a CCR5-dependent immunogenic macrophage polarization, resulting in conventional T-cell activation in situ. Collectively, our work uncovers fundamental intratumoral cross-talk between iαβTs and the innate and adaptive immune compartments, and suggests that strategies targeting iαβTs may be effective for reprogramming of the TME in human cancer.
RESULTS

**αβTs Are a Prominent T-cell Population in Pancreatic and Human PDA**

To assess the significance of αβTs in PDA, we determined their prevalence in an invasive orthotopic model of PDA using tumor cells derived from Pdx1Cre;KrasG12D;Tpr53−/−/+ (KPC) mice that express mutant Kras and Tpr53 in their pancreatic progenitor cells, a slowly progressive preneoplastic autochthonous model of PDA using p48Cre;KrasG12D (KCC) mice whose pancreatic exocrine progenic Kras alone, and human disease. We discovered that CD4+CD8−NK1.1+ αβTs constitute ~10% of TCRαβ+ T cells in orthotopic KPC tumors (Fig. 1A; Supplementary Fig. S1a) and KC pancreata (Fig. 1B). Human PDA similarly possessed an increased CD4+CD8− T-cell infiltrate compared with normal adjacent pancreas or peripheral blood mononuclear cells (PBMC; Fig. 1C; Supplementary Fig. S1b). By contrast, αβTs were scarce in murine spleen and human PBMCs. PDA-infiltrating αβTs did not express characteristic functional markers of NK T cells and ~10% bound an MR1-specific tetramer, compared with ~40% in the gut (Supplementary Fig. S1c-f). Single-cell RNA sequencing (RNA-seq) of PDA-infiltrating CD3+ cells further confirmed that this population is transcriptomically distinct from CD4+ CD8− γδ T, and NK T cell populations (Fig. 1D). αβTs upregulated Ccr7 expression and exhibited reduced Il2rb in comparison with other lymphocyte populations (Supplementary Fig. S1g and h). These observations were confirmed by flow cytometry (Supplementary Fig. S1i and j). In-depth analysis comparing αβT with NK T cells revealed that αβTs downregulated the NK T markers Klrk1, Klrk7, Klrk11, Nkg7, Klrk2, and Ly6c2 but upregulated the transcription factors Socs3 and Junb (Supplementary Fig. S1k). Interestingly, αβTs increased as a fraction of TCRβ+CD4+CD8− T cells as tumors progressed (Fig. 1E). γδ T cells remained stable over the course of oncogenesis, as previously reported (1). Multiplex IHC suggested that αβTs were interspersed among CD4+ CD8− T cells within the TME (Fig. 1F). Of note, PDA-infiltrating αβTs constituted ~30% of TCRβ+ cells in Fas+/− mice (Fig. 1G), known to accumulate αβTs in secondary lymphoid organs (7). To determine whether the thymic production of αβTs is accelerated during pancreatic oncogenesis, we interrogated T-cell populations in the thymus of 6-month-old KC mice. Thymic αβTs were not increased in prevalence in KC mice compared with wild-type (WT; Fig. 1H). Adoptive transfer tracking experiments suggested that neither CD4+ nor CD8+ T cells converted to the αβT phenotype in PDA. Likewise, αβTs did not gain CD4 or CD8 expression (Fig. 1I). Interestingly, cellular proliferation was higher in PDA-infiltrating αβTs than in either CD4+ or CD8+ T cells (Fig. 1J). We recently reported that unconventional T cells, particularly γδ T cells, are recruited to the PDA TME via diverse chemokine signaling networks (5). We observed that PDA-infiltrating αβTs express CCR2, CCR5, and CCR6 (Fig. 1K), and CCR2 deletion trended to mitigate αβT recruitment in PDA (Fig. 1L).

**αβTs Exhibit a Distinctive Phenotype in PDA and Are Targets of Immunotherapy**

PDA-infiltrating αβTs acquire a distinctive phenotype relative to the periphery. Tumor-penetrating αβTs expressed the adhesion ligand JAML and the cytotoxic marker CD107a (Fig. 2A and B). Additionally, αβTs in the TME upregulated the checkpoint receptors CTLA4, TIM3, PD-1, and LAG3, ectonucleotidases CD39 and CD73, costimulatory receptors CD40L and ICOS, and the C-type lectin receptor Dectin-1 (Fig. 2C–G). PDA-associated αβTs were also highly activated compared with their splenocyte counterparts, downregulating CD62L (Fig. 2H). Moreover, the T-cell receptor (TCR) nucleotide sequences of tumor-infiltrating αβTs exhibited minimal overlap with those of splenic αβTs or conventional T cells infiltrating PDA (Fig. 2I; Supplementary Table S1). αβTs in the TME were further distinct from both intratumoral CD4+ and CD8+ T cells and splenic αβTs in their high expression of the Th1-family transcription factor T-bet (Fig. 2J). STAT1 signaling, associated with Th1 differentiation, was similarly upregulated in αβTs in PDA (Supplementary Fig. S2a). Expression of the Th17-family transcription factor RORγt was also increased (Supplementary Fig. S2b). Accordingly, PDA-infiltrating αβTs expressed high IFNγ and TNFα compared to their splenic counterparts and to both intratumoral CD4+ and CD8+ T cells (Supplementary Fig. S2c and d). IFNγ and IL17 were distinctively coexpressed in the αβT-cell
Innate T Cells Promote Antitumor Immunity

**A**
- Spleen: CD4+ 28%, CD8+ 42%
- Pancreas: CD4+ 10%, CD8+ 31%
- SSA: CD4+ 31%, CD8+ 11%

**B**
- Spleen: CD4+ 87%, CD8+ 5%
- Pancreas: CD4+ 44%, CD8+ 4%

**C**
- % CD4+CD8+ (of CD3+): Nml 27%, PDA 72%

**D**
- tSNE plot showing CD4+ and CD8+ T-cell distribution

**E**
- % of iκBα+ T cells per NK1.1+ TCRαβ: Day 7 SSA: 27%, Day 21 SSA: 87%

**F**
- CD3+ CD4+ CD8+ expression

**G**
- Fas+ CD4+ CD8+ expression: WT 25%, Fas+ 0%

**H**
- % iκBα+ T cells: WT 5%, KC 4%

**I**
- Recipients (CD45.2+): CD4+ 13%, CD8+ 15%, iκBα+ 41%

**J**
- % Ki67+ T cells: CD4+ 28%, CD8+ 87%

**K**
- % expression of CCR2, CCR5, CCR6: Spleen vs. PDA

**L**
- % iκBα+ T cells: WT vs. PDA, WT vs. PDA+−
Innate T Cells Promote Antitumor Immunity

Innate T Cells Promote Antitumor Immunity or MHC II restricted, we administered orthotopic PDA tumor αT cells, and CD8βi option in select subsets of patients with PDA (8). Because S2e–g). Analysis of iαβαβ combination of receptor based therapy in PDA whose impact must be con-

immunotherapy has emerged as an efficacious treatment option in select subsets of patients with PDA (8). Because iαβTs express high levels of costimulatory and checkpoint receptors in PDA, we postulated that immune-based therapies targeting these entities would activate iαβTs in situ. Accordingly, we found that ICOS ligation upregulated IFNγ and IL17 expression in splenic iαβTs in vitro (Supple-

mentary Fig. S3a and b). Further, treatment of PDA-bearing mice with an ICOS agonist was tumor-protective and was associated with marked iαβT cellular expansion and activation (Supplementary Fig. S3c–e). Given our observation that PDA-infiltrating iαβTs upregulate PD-1, we also tested the effect of PD-L1 blockade on the iαβT phenotype (Fig. 2E). αPD-L1 mAb treatment promoted iαβT activation in the TME (Supplementary Fig. S3f). These data suggest that iαβTs are distinctive targets of costimulatory and checkpoint receptor based therapy in PDA whose impact must be con-

considered in immune-based therapeutics. Moreover, the combination of αPD-L1 treatment and iαβT cell transfer offered additional protection compared with either monotherapy (Supplementary Fig. S3g).

To investigate whether PDA-infiltrating iαβTs are MHC I or MHC II restricted, we administered orthotopic PDA tumor to β2-microglobulin−/− and MHC II−/− mice, respectively. PDA tumors in MHC II−/− hosts did not alter iαβT-cell recruitment (Supplementary Fig. S3h), whereas β2-microglobulin–deficient mice exhibited reduced iαβT-cell recruitment to the PDA TME and lower cellular expression of T-bet and TNFα (Supplementary Fig. S3i–k). In contrast to CD8β T cells, how-

ever, iαβT-infiltrating Ova-expressing PDA tumors lacked Ova-pentamer staining and were thus not MHC I antigen restricted, lending support to their innate immune properties (Supplementary Fig. S3l and m). Accordingly, intratumoral iαβTs were less clonal than both CD4αβ and CD8βαβT cells (Supplementary Fig. S3n).

iαβT Cell Phenotype in PDA Is Influenced by Diverse Inflammatory and Microbial Signals

Because CCR2 recruits innate T cells to the PDA TME (Fig. 1K; ref. 5), we postulated that CCR2 signaling also modulates iαβT cellular phenotype in situ. Accordingly, intra-
tumoral iαβT-cell expression of T-bet and CD4αβ was reduced in CCR2−/− hosts, expression of ICOS, CTLA4, and TIM3 was upregulated, and cytokine expression was not altered (Sup-

plemenitary Fig. S4a). Because we previously reported that Dectin-1 can regulate cytokine secretion in unconventional T cells (9), we postulated that, unlike CCR2, Dectin-1 signaling may govern iαβT-cell cytokine profile. PDA-infiltrating iαβTs in Dectin-1−/− mice expressed reduced IL17 and IFNγ, whereas TNFα expression was unchanged (Supplementary Fig. S4b). Accordingly, ligating Dectin-1 upregulated IFNγ and IL17 in naïve splenic iαβTs (Supplementary Fig. S4c). Collectively, these data suggest that CCR2 and Dectin-1 signals influence distinct aspects of iαβT-cell programming in PDA.

We recently reported that the host microbiome in PDA exerts suppressive influences on intratumoral CD4αβ and CD8αβ T-cell differentiation (10). We therefore postulated that the PDA microbiome has similar tolerogenic effects on iαβTs. To test this, we ablated the microbiome in PDA-bearing mice using an established oral antibiotic regimen (Supplementary Fig. S4d; ref. 11). Consistent with our hypothesis, microbial ablation activated iαβTs, increasing their expression of T-bet, CD44, LFA-1, ICOS, IL17, IFNγ, and TNFα (Supplementary Fig. S4e). Mechanistically, we reported that the microbiome directs immune suppression in PDA via TLR4 signaling (10, 12). Interestingly, we found that iαβTs upregulate TLR4 expression in PDA (Supplementary Fig. S4f). Consistent with our analysis of PDA-infiltrating CD4αβ and CD8αβ T cells (10), TLR4 ligation mitigated iαβT-cell activation (Supplementary Fig. S4g).

iαβTs Protect against PDA and Induce Immunogenic CD4αβ and CD8αβ T-cell Activation in Mouse and Human Models

To determine the influence of iαβTs on pancreatic onco-
genesis, we adoptively transferred iαβTs to mice coincident with subcutaneous tumor challenge. iαβT-cell administration mitigated pancreatic tumor growth (Fig. 3A). iαβT-cell transfer resulted in intratumoral CD8αβ T-cell activation (Fig. 3B). Similarly, intra-

pancreatic iαβT cellular transfer with orthotopically implanted KPC tumor cells protected against tumor growth (Fig. 3C) and again activated CD4αβ and CD8αβ T cells in the TME, inducing higher expression of CD44, ICOS, and TNFα (Fig. 3D–F). To determine whether iαβTs are suitable agents for cellular therapy, we serially administered iαβTs intravenously to mice bear-

ing established orthotopic PDA tumors. iαβT administration protected against tumor growth and immunogenically repro-
grammed the adaptive TME (Fig. 3G–J). Furthermore, analysis of tumors treated with iαβTs by single-cell RNA-seq suggested a marked CD4αβ and CD8αβ T-cell expansion compared with controls. Specifically, conventional T-cell populations increased from 3% to 20% of leukocytes with iαβT-cell administration (Fig. 3K and L). Other lymphocyte subsets were also expanded. Consistent with our flow cytometry data, conventional T cells...
Innate T Cells Promote Antitumor Immunity

To determine whether iαβTs have the capacity to immunogenically reprogram adaptive immunity in human disease, we generated patient-derived organotypic tumor spheroids (PDOTS) from freshly harvested human tumors using a microfluidic-based system that we recently described (13). We selectively added FACS-sorted autologous iαβTs or vehicle to the PDOTS and analyzed the system at 3 days. Consistent with our murine data, iαβTs induced marked conventional T-cell activation in the human PDOTS system (Fig. 3N; Supplementary Fig. SSb).

PDA-Infiltrating iαβTs Induce Effector T Cell-Dependent Tumor Protection, but Paradoxically Suppress Conventional T Cells In Vitro

In addition to expressing high levels of the cytotoxicity marker CD107a (Fig. 2B), PDA-associated iαβTs also upregulate Fasl, Perforin, and Granzyme B (Fig. 4A). We therefore postulated that iαβTs may be directly cytotoxic to PDA tumor cells. However, iαβTs did not mitagate tumor cell proliferation, induce tumor cell lysis, or promote apoptosis (Fig. 4B–D). Because iαβT cellular transfer enhanced T-cell immunity in situ, we speculated that iαβTs mediate tumor protection by directly activating CD4+ and CD8+ T cells in the PDA microenvironment. Accordingly, whereas CD4+/CD8+ T-cell depletion did not accelerate tumor progression in control mice as we previously reported (5, 10), CD4+ T-cell depletion abrogated the tumor protection associated with iαβT transfer (Fig. 4E). We therefore used in vitro modeling to determine whether iαβTs directly enhance iαβT cell immunogenicity. Belying our in vivo data, we found that PDA-infiltrating iαβTs were highly inhibitory to CD4+ and CD8+ T-cell activation in vitro and prevented their upregulation of IFNγ, TNFα, T-bet, CD69, and CD44 in response to CD3/CD28 oligation (Figs. 4F–I; Supplementary Fig. S5C), iαβTs similarly mitigated polyclonal T-cell secretion of IFNγ, TNFα, and IL2 in cell culture supernatant (Fig. 4J). Further, iαβTs promoted upregulation of FOXP3 expression in CD4+ T cells (Fig. 4K). The inhibitory effects of iαβTs on conventional T cells were independent of TGFβ secretion (Supplementary Fig. SSd–f). We previously reported that γδT cells can inhibit CD4+ and CD8+ T-cell activation in cancer via the PD-L1–PD-1 axis (5). Therefore, we postulated that iαβTs, which upregulate PD-L1 in PDA (Fig. 4L), suppress effector T cells through PD-L1–PD-1 interactions. PD-L1 blockade partially reversed the inhibitory effects of PDA-infiltrating iαβTs on effector T-cell activation (Fig. 4M and N).

PDA-Associated iαβTs Promote Immunogenic Macrophage Polarization via CCR5 Activation

We previously showed that macrophage programming governs the balance between T-cell tolerance and immunogenicity in PDA (5, 14). Therefore, we postulated that iαβTs may indirectly augment intratumoral adaptive immunity by inducing immunogenic macrophage polarization, which in turn reprograms effector T-cell populations. Accordingly, iαβTs directly upregulated macrophage expression of MHCII, CD38, IL6, and TNFα in coculture experiments (Fig. 5A). iαβTs also promoted increased CD86 and IFNγ expression in macrophages and downregulated CD206 and IL10 (Fig. 5B and C). In vivo iαβT-cell transfer in PDA also induced higher MHCII, iNOS, TNFα, IFNγ, and IL12 expression in TAMs, whereas CD206 and IL10 expression was reduced (Fig. 5D). iαβT-cell transfer also significantly increased STAT1 signaling in TAMs, which is linked to M1-like macrophage polarization (Fig. 5E). Further, our single-cell RNA-seq data indicated a marked contraction of F480+ macrophages in tumors after serial iαβT-cell transfer. Specifically, macrophages represented 84% of leukocytes in control tumors compared with only 24% in iαβT-cell–treated mice (Fig. 3L and M). Moreover, consistent with our flow cytometry data, iαβT-cell transfer resulted in upregulation of genes related to antigen presentation (e.g., H2-family genes, Cd74, B2m), T-cell chemotraction (Cd5, Cxcl9, Cxcl10), IFN signaling (Stat1, Ifnar1, Ifng1, Ifrg1, Ifrr5, Ifr7, Ifrb8), and M1 polarization (Tnf, Ccl5, Hba, Ccr5) on macrophages, whereas M2-associated transcription factors were downregulated (Stat6, Socs3, Il1b; Supplementary Fig. S6a–e). Ingenuity pathway analysis of upstream regulators indicated that Stat1, Ifng, and Tnfα signaling was significantly activated in TAM-infiltrating tumors of iαβT-cell–treated mice relative to controls (Supplementary Fig. S6f–h). Further, gene set enrichment analysis (GSEA) indicated significant enrichment of “KEGG_Antigen processing and presentation” and “KEGG_Chemokine signaling pathway” in macrophages of iαβT cell–treated tumors (Supplementary Fig. S6i and j).

**Figure 3.** iαβTs protect against PDA and enhance intratumoral T-cell immunity. A, WT mice were administered KPC tumor cells subcutaneously, either alone or admixed with iαβTs. Tumor growth was serially measured (n = 5/group). B, WT mice were administered KPC tumor cells subcutaneously, either alone or admixed with PDA-infiltrating iαβTs. On day 21, tumor-infiltrating CD8+ T cells were analyzed for expression of TNFα (n = 5/group). C-F, WT mice were orthotopically administered KPC tumor cells, either alone or admixed with iαβTs. Tumors were harvested on day 21. C, Representative pictures of tumors and quantitative analysis of tumor weight are shown. Scale bar, 5 mm. D-F, PDA-infiltrating CD4+ and CD8+ T cells were analyzed for expression of CD44 (D), ICOS (E), and TNFα (F, n = 5/group). Each mouse experiment was repeated more than 3 times. G-J, Mice with established orthotopic KPC tumor were serially transferred intravenously twice weekly with iαβTs or vehicle beginning on day 5 (n = 5). Mice were sacrificed at day 21 after tumor implantation. G, Representative pictures of tumors and quantitative analysis of tumor weight are shown. Scale bar, 5 mm. H, PDA-infiltrating CD4+ and CD8+ T cells were analyzed for expression of CD44 (H), ICOS (I), and IFNγ (J). Subcutaneous and orthotopic tumor experiments were each repeated at least 4 times. K-M, Mice with established orthotopic KPC tumors were serially transferred intravenously twice weekly with iαβTs or vehicle beginning on day 5. Mice were sacrificed at day 21 and single-cell RNA-seq performed on FACS-purified CD4+ tumor-infiltrating leukocytes. K, The distribution of cellular clusters was determined using the t-SNE algorithm. Each cluster is identified by a distinct color. L, A t-SNE plot overlay of tumor-infiltrating leukocytes in tumors of mice treated with iαβTs (red) vs. vehicle (blue) is shown. Percent cellular abundance in each cluster is depicted in pie charts and specified in the accompanying legend. M, Violin plots comparing normalized log expression of select genes in the T-cell cluster for both treatment groups are shown. N, iαβTs derived from rescued human tumor cells were treated with autologous iαβTs or vehicle at 72 hours. Overlay of CD4+ T-cell activation for expression of CD44, ICOS, IFNγ, and TNFα. Data are indicated as fold change in the iαβT cell–treated group compared with vehicle treated. n = 5 patients. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.
**Figure 4.** iaβTs induce T cell–dependent tumor immunity but are directly suppressive to conventional T cells. A, WT mice bearing orthotopic KPC tumors were sacrificed on day 21. Spleenic and PDA-infiltrating iaβTs were tested for expression of FasL, Perforin, and Granzyme B (n = 5 mice). B and C, iaβTs were harvested by FACS from orthotopic PDA tumors and cultured in various ratios with KPC tumor cells. B, Proliferation of KPC tumors cells was tested using the XTT assay. C, Cytotoxicity against KPC tumor cells was determined in an LDH release assay. D, iaβTs were harvested by FACS from orthotopic PDA tumors and cultured in 1:1 ratio with KPC tumor cells. KPC tumor cell apoptosis was determined by costaining for Annexin V and PI. E, WT mice were orthotopically administered KPC tumor cells admixed with iaβTs. Cohorts were either serially depleted of both CD4+ and CD8+ T cells or administered isotype control before sacrifice on day 21. Representative images and quantitative analysis of tumor weights are shown (n = 5/group). This experiment was repeated twice. F-I, Polyclonal splenic CD4+ or CD8+ T cells were cultured without stimulation, stimulated by CD3/CD28 coligation, or stimulated by CD3/CD28 coligation in coculture with iaβTs. CD4+ and CD8+ T-cell activation was determined at 72 hours by their expression of IFNγ (F), TNFα (G), T-bet (H), and CD69 (I). J, Representative contour plots and quantitative data are shown. J, Polyclonal splenic CD4+ T cells were stimulated by CD3/CD28 coligation, either alone or in coculture with iaβTs. Cell culture supernatant was tested for expression of IFNγ, TNFα, and IL2 at 72 hours. K, Polyclonal splenic CD4+ T cells were cultured without stimulation, stimulated by CD3/CD28 coligation, or stimulated by CD3/CD28 coligation in coculture with iaβTs. CD4+ T cells were tested for expression of FOXP3 at 72 hours. L, Spleen and PDA-infiltrating iaβTs were tested for expression of PD-L1 by flow cytometry. M and N, Polyclonal splenic CD4+ T cells from PD-L1−/− mice were cultured without stimulation, stimulated by CD3/CD28 coligation, or stimulated by CD3/CD28 coligation in coculture with WT iaβTs, either alone or with an antiPD-L1–neutralizing mAb. CD4+ T cells were tested for expression of CD44 (M) and IFNγ (N) at 72 hours. Experiments were performed in replicates of 5 and repeated at least 4 times. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.
Figure 5. iαβTs induce immunogenic reprogramming of macrophages. A–C, Splenic macrophages were cultured alone or cocultured with iαβTs for 24 hours. Macrophages were then harvested and tested for expression of MHC II, CD38, IL6, and TNFα (A), CD86 and CD206 (B), IFNγ and IL10 (C). Select contour plots and quantitative data are shown. This experiment was repeated 5 times. D and E, KPC tumor-bearing mice were adoptively transferred with iαβTs. Tumors were harvested on day 21 and TAMs were analyzed for expression of MHCII, iNOS, TNFα, IFNγ, IL12, CD206, and IL10. I. This experiment was repeated twice (n = 5/group). F and G, iαβT cell–entrained splenic macrophages and control splenic macrophages were pulsed with Ova223-239 peptide and used to stimulated Ova-restricted CD4+ T cells. T-cell activation was determined at 96 hours by expression of (F) CD44 and LFA-1. G, Cell culture supernatant was harvested and tested for expression of TNFα, IL4, IL6, and IL10. In vitro experiments were performed in replicates of 5 and repeated 3 times. H–K, Cohorts of WT mice were administered orthotopic KPC tumor either alone, admixed with control macrophages or macrophages that had been cocultured with iαβTs. Pancreatic tumors were harvested on day 21 (n = 5/group). H, Tumor weights were recorded. Scale bar, 5 mm. I, Tumor-infiltrating CD8+ T cells were analyzed for expression of CD44. Tumor-infiltrating CD4+ T cells were analyzed for expression of FOXP3 (J) and IL10 (K). This experiment was repeated twice. *, P < 0.05; **, P < 0.01; ***, P < 0.001; *****, P < 0.0001.
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* ns: not significant
** ns: significant at P < 0.01
*** ns: significant at P < 0.001
**** ns: significant at P < 0.0001

**P = 0.08**

**P = 0.06**

**P = 0.1**

**P = 0.09**
Finally, we noted the expansion of a distinct Cx3cr1<sup>hi</sup>F4/80<sup>hi</sup> macrophage population in the TME of i<sub>αβ</sub>T cell–treated mice (Fig. 3L and M). This population upregulated C11S, but did not exhibit upregulation of the other genes and pathways related to antigen presentation and proinflammatory signaling observed in the broader macrophage population (Supplementary Fig. S6K). Collectively, these data show that i<sub>αβ</sub>Ts induce profound immunogenic macrophage programming in PDA.

We postulated that i<sub>αβ</sub>Ts secondarily augment adaptive T-cell immunity in situ via their immunogenic influence on macrophage differentiation. To test this, antigen-pulsed macrophages entrained by coculture with i<sub>αβ</sub>Ts were used to stimulate antigen-restricted CD4<sup>+</sup> T cells. Consistent with our hypothesis, i<sub>αβ</sub>T-entrained macrophages were more efficient than controls at antigen presentation based on T-cell expression of activation markers and secreted cytokines (Fig. 5F and G). Moreover, in vitro adoptive transfer of macrophages entrained by i<sub>αβ</sub>T coculture was markedly protective against PDA, whereas transfer of control macrophages was not (Fig. 5H). Adoptive transfer of i<sub>αβ</sub>T-entrained macrophages also activated intratumoral conventional T cells and reduced Treg differentiation in situ (Fig. S1–K). Collectively, these data suggest that i<sub>αβ</sub>Ts can induce macrophage-mediated adaptive antitumor immunity in PDA. To determine whether i<sub>αβ</sub>Ts also activate macrophages in human systems, we cocultured FACS-sorted i<sub>αβ</sub>Ts with human PBMC-derived macrophages. Consistent with our murine data, i<sub>αβ</sub>Ts activated human macrophages (Fig. 6A). Furthermore, autologous treatment of organotypic 3-D models of human PDA with i<sub>αβ</sub>Ts similarly resulted in marked activation of TAMs (Fig. 6B).

Mechanistically, we postulated that i<sub>αβ</sub>Ts secrete specific inflammatory mediators that may drive immunogenic macrophage programming. To test this, we cultured naïve splenic macrophages with i<sub>αβ</sub>T cell–conditioned media or control media. i<sub>αβ</sub>T-conditioned media upregulated MHC II, CD38, and TNFα in macrophages and downregulated CD206, suggesting that i<sub>αβ</sub>T-derived secreted factors are sufficient to induce an activated macrophage phenotype (Fig. 6C). To determine the mechanism through which i<sub>αβ</sub>Ts regulate macrophage polarization, we performed unbiased analysis of inflammatory mediators secreted by i<sub>αβ</sub>Ts. We found that i<sub>αβ</sub>Ts produced markedly high levels of CCL3, CCL4, and CCL5, each of which ligate CCR5 (Fig. 6D; ref. 15). Consistently, i<sub>αβ</sub>T-conditioned media upregulated MHC II, CD86, and TNFα (Fig. 6E). Thus, although the cytokine profiles remain similar, we are unable to classify i<sub>αβ</sub>Ts as a single cytokine family given their unique expression of cytokines, including IL17 and IFNγ, as well as production of cytokytic molecules such as Perforin and Granzyme B, we probed the antigen specificity of PDA-infiltrating i<sub>αβ</sub>T and gut-derived MAIT cells (17). The MHC class I-like protein MR1 is responsible for presenting bacterially produced vitamin B metabolites to MAIT cells. In turn, recognition of antigen results in MAIT cell stimulation and secretion of proinflammatory cytokines (18). However, we found that only ~10% of i<sub>αβ</sub>Ts in PDA recognize a murine MR1-specific tetramer, as compared with ~40% of CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>+</sup> cells in the gut. CD4<sup>+</sup> and CD8<sup>+</sup> T cells in PDA expressed similar levels of the MR1-specific tetramer compared with i<sub>αβ</sub>Ts. Thus, although the cytokine profiles remain similar, we are unable to define our population as MAIT cells and prefer their more general description as i<sub>αβ</sub>Ts. We maintain the possibility that i<sub>αβ</sub>Ts in PDA encompass a diversity of phenotypes, as do most broadly described T-cell populations, but the precise cellular ontogeny is beyond the scope of our work. Rather, our investigations focus on the impact of these cells in interfacing with other innate and adaptive immune subsets, as novel targets of immunotherapy, and their role in combating oncogenic progression.

**DISCUSSION**

We show that TCR<sup>αβ</sup>-CD4<sup>+</sup>CD8<sup>+</sup>-NK1.1<sup>+</sup> represent ~10% of T cells in the mouse and human PDA TME. These cells differ from innate lymphoid cells by expression of the CD3–TCR complex and are distinguished from NKT cells by the absence of obligate marker expression on transcriptomic and flow-cytometric analyses. Interestingly, our i<sub>αβ</sub>Ts share some phenotypic features with mucosal associated invariant T (MAIT) cells, a subset of CD3<sup>+</sup> T cells that can express CD4 and CD8, but are classically CD4<sup>+</sup>CD8<sup>+</sup>. MAIT cells predominate in the gut, where they defend against microbial infection, but have been described in the blood, liver, and lungs (16). Given that PDA-infiltrating i<sub>αβ</sub>Ts and MAIT cells both exhibit high expression of cytokines, including IL17 and IFNγ, as well as production of cytokytic molecules such as Perforin and Granzyme B, we probed the antigen specificity of PDA-infiltrating i<sub>αβ</sub>T and gut-derived MAIT cells (17). The MHC class I-like protein MR1 is responsible for presenting bacterially produced vitamin B metabolites to MAIT cells. In turn, recognition of antigen results in MAIT cell stimulation and secretion of proinflammatory cytokines (18). However, we found that only ~10% of i<sub>αβ</sub>Ts in PDA recognize a murine MR1-specific tetramer, as compared with ~40% of CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>+</sup> cells in the gut. CD4<sup>+</sup> and CD8<sup>+</sup> T cells in PDA expressed similar levels of the MR1-specific tetramer compared with i<sub>αβ</sub>Ts. Thus, although the cytokine profiles remain similar, we are unable to define our population as MAIT cells and prefer their more general description as i<sub>αβ</sub>Ts. We maintain the possibility that i<sub>αβ</sub>Ts in PDA encompass a diversity of phenotypes, as do most broadly described T-cell populations, but the precise cellular ontogeny is beyond the scope of our work. Rather, our investigations focus on the impact of these cells in interfacing with other innate and adaptive immune subsets, as novel targets of immunotherapy, and their role in combating oncogenic progression.
The PDA microenvironment is regulated by diverse cellular and biochemical inflammatory signals that modulate tumor growth. However, the cross-talk between cellular subsets in the inflammatory TME remains incompletely understood. The importance of unconventional T-cell subsets in this immune interplay is becoming increasingly recognized in both hematologic and solid organ malignancies. For example, in PDA we showed that γδT cells cripple conventional CD4+ and CD8+ T-cell immunogenicity via upregulation of PD-L1 and Galactin-9 (5). By contrast, NKT cells exhibit protective effects in preclinical models of PDA via monocytic cross-talk (6). Consistent with the role of NKT cells as first responders in inflammation (19, 20), we found that NKT cell levels steadily declined in PDA as tumors progressed, whereas iοβTs sharply increased in the TME over the course of disease progression. This latter observation is consistent with high levels of CCL2 in mature PDA tumors (21), as we discovered that iοβTs are recruited to the TME via CCR2 signaling.

We found that iοβTs are distinctly proinflammatory in PDA, promiscuously expressing high costimulatory (ICOS and CD40L) and checkpoint (CTLA4, PD-1, TIM3, and LAG3) receptors and coexpressing both Th1 and Th17 families of fate-determining transcription factors (T-bet and RORγt) and cytokines (IFNγ and IL17). However, in contrast to the tolerogenic role of iοβTs in the context of organ transplantation (19), PDA-infiltrating iοβTs minimally express FOXP3 and IL10, consistent with their more immunogenic function. Interestingly, the tumor-protective effects of iοβTs belie the fact that these cells are the highest producers of IL17 in PDA, a cytokine that has been shown to have directly proliferative effects on tumor cells (20). Though we observed that iοβTs possess a cytotoxic phenotype in PDA (expression of perforins, granzymes, and FasL), they were incapable of directly inhibiting tumor proliferation or inducing tumor cell death in vitro. This contrasts with findings in melanoma and lymphoma where iοβTs exhibit potent tumor-specific cytotoxicity (22, 23). This observation could in theory be due to the paucity of tumor-associated antigens (TAA) and neoantigens in PDA relative to other cancers and the consequent limitation of MHCI-dependent killing. However, we observed that iοβTs in PDA do not recognize tumor antigen; therefore, their lack of directly tumoricidal properties is unlikely solely due to the scarcity of TAA.

We found that iοβTs delimit PDA progression by shaping the innate and adaptive immunologic landscape. Specifically, iοβTs promote macrophage reprogramming to a distinctly immunogenic phenotype, which in turn enhances effector CD4+ and CD8+ T-cell function. Several studies have shown that TAMs drive tumor progression in PDA by possessing potent immunosuppressive properties (24–26). Interestingly, we show that treatment with iοβTs not only results in the global contraction of TAMs in the PDA TME, but also confers significant proinflammatory phenotypic changes to the remaining macrophages, leading to the expansion of effector T-cell populations. We also found that deletion of TAMs abrogated the tumor protection and enhancement in adaptive immunity associated with iοβT-cell transfer. Programs upregulated in TAMs by treatment with iοβTs included antigen presentation, T-cell chemoattraction, and IFN, TNF, and STAT1 signaling. In this way, iοβTs may shift the balance toward antitumor immunity by dually abrogating immune suppression and promoting increased antigen presentation capacity to effector T cells and their activation (Supplementary Fig. S7). We found that the iοβT-cell-macrophage cross-talk was linked to the high levels of CCR5 ligands secreted by iοβTs. Moreover, considering the central role of TAMs in regulating the adaptive immune program and the protective tumor immunity associated with iοβT cellular transfer, our data suggest that iοβTs may be attractive vehicles for cell therapy in PDA.

This work also highlights paradoxical effects regarding the influence of iοβTs on CD4+ and CD8+ T cells. We show that iοβTs directly inhibit CD4+ and CD8+ T cells in situ in coculture experiments via the PD-L1–PD-1 axis. This parallels the suppressive effects of γδT cells in PDA (5). Conversely, the broader in vivo effect of iοβTs is a downstream immunogenic activation of effector T cells via macrophage activation. These findings are consistent with previous work suggesting that TAM programming is the dominant driver of the adaptive immune landscape in PDA (14, 24). This functional hierarchy is even more plausible considering that the iοβT-mediated suppressive effect on effector T cells requires direct cell contact via PD-L1–PD1 interaction, whereas macrophage activation can occur remotely via secretion of CCL3, CCL4, and CCL5 (CCR5 ligands). The contact-dependent inhibitory effects of iοβTs on effector T cells, activating effects (including PD-1 upregulation) observed after iοβT-cell transfer on effector T cells, and the observed activation of iοβTs in response to in vitro PD-L1 therapy in vivo suggest that immunotherapy targeting the PD-L1–PD-1 axis could synergistically accentuate the tumor-protective and immunologic benefits of iοβT cell–directed therapies.

The signals that drive the distinctive phenotype of iοβTs in PDA are diverse and modifiable and collectively account for their complex features. Signaling via Dectin-1, a pattern recognition receptor capable of binding Galactin-9 and fungal cell β-glucans, promoted IFNγ and IL17 production in iοβTs, but did not appreciably modulate iοβT surface phenotype. By contrast, CCR2 signaling activated iοβT cell–surface phenotype and upregulated expression of costimulatory and checkpoint receptors, but did not alter cytokine production. The PDA-associated microbiome and LPS have broadly suppressive effects on iοβTs, reducing both cytokine and surface activation marker expression. Therefore, tailored approaches can be used to modulate iοβT cell phenotype in situ or ex vivo to optimize phenotypic outcomes. Finally, the use of iοβTs as an adoptive cell therapy into human organotypic PDOT systems of pancreatic cancer is the first application of its kind. The ability for iοβTs to induce significant immunogenic activation in these 3-D human systems illustrates their potential as a novel cell therapy. In aggregate, our work suggests that iοβT-cell cross-talk serves to connect innate and adaptive immunity in PDA by reprogramming of macrophages that leads to conventional CD4+ and CD8+ T-cell activation. This work thus describes a critical new cellular entity in the landscape of the TME and suggests that iοβTs can be utilized for adoptive cell therapy or as targets of activation for immune-based therapies in situ in PDA.
METHODOLOGIES

Animals and In Vivo Procedures

C57BL/6 (H-2bKb), B6.MRL-Fas(−/−)/J, Dectin-1−/−, CCR2−/−, CCR5−/−, CCR6−/−, β2m−/−, MHCII−/−, OT-II, and CD45.1 mice were purchased from The Jackson Labs and bred in-house. KC mice, which express KrasG12D in the progenitor cells of the pancreas, were a gift of Dafna Bar-Sagi of New York University (1). Both male and female mice were used, but animals were gender-matched within each experiment. For orthotropic pancreatic tumor challenge, 8- to 10-week-old mice were administered intrapancreatic injections of FC124 mouse tumor cells derived from KC mice, as we previously described (5). In select experiments, we utilized KC tumor cells, which we engineered to express Ovalbumin using the pCIneo-OVA vector (Addgene). PDA cells (1 × 10^6) were injected subcutaneously in PBS with 50% Matrigel (BD Biosciences) and were injected into the body of the pancreas via laparotomy. Mice were sacrificed 3 weeks later for analysis. Alternatively, PDA cells and were injected into the body of the pancreas via laparotomy. Mice (3 × 10^5) were implanted subcutaneously and tumor growth was serially measured. In some experiments, iNKTs or macrophages were mixed with tumor cells in a 1:3 ratio before orthotopic or subcutaneous injection. In other experiments, iNKTs were harvested by FACS and administered intravenously (5 × 10^6) twice weekly to orthotopic PDA-bearing mice beginning on day 5 after tumor implantation. For tracking experiments, iNKTs from CD45.1 hosts were cojected with PDA cells into pancreata of CD45.2 mice and harvested 96 hours later. In select experiments, animals were treated with neutralizing mAbs directed against CD4 (GK1.5), CD8 (Lyt 2.1), F4/80 (C1:A3-1), TCRγδ (UC3-10A4), NK1.1 (PK136), and PD-L1 (10F.9G2; all Bio X Cell) using regimens we have previously described (5, 10). Alternatively, mice were treated with an agonizing ICOS mAb (7E.17G9, 100 μg, days 4, 7, and 10 after tumor challenge; Bio X Cell) or TLR4 ligand (LPS, 5 μg, intraperitoneally, 3x/week; Invivogen). Antimicrobial ablation was performed as previously described (10). Briefly, mice were administered an antibiotic cocktail by oral gavage daily for 5 consecutive days. Controls were gavaged with PBS. The oral gavage cocktail contained vancomycin (50 mg/mL; Sigma), neomycin (10 mg/mL; Sigma), metronidazole (100 mg/mL; Santa Cruz Biotech), and amphotericin (0.5 mg/mL; Sigma). Additionally, for the duration of the experiments, mouse drinking water was mixed with ampicillin (1 mg/mL; Santa Cruz Biotech), vancomycin (0.5 mg/mL; Sigma), neomycin (0.5 mg/mL; Sigma), metronidazole (1 mg/mL; Santa Cruz Biotech), and amphotericin (0.5 μg/mL; MP Biomedicals). All studies were approved by the New York University School of Medicine Institutional Animal Care and Use Committee.

Sequential IHC, Image Acquisition, and Processing for Human Tissues

Human tissues used for IHC analysis were obtained with written informed consent in accordance with the Declaration of Helsinki and were acquired through the Oregon Pancreas Tissue Registry. All studies were approved by an Institutional Review Board (IRB) under Oregon Health and Science University IRB protocol #3609. Human PDA and murine orthotopic PDA tissues were fixed with 10% buffered formalin, dehydrated in ethanol, and embedded with paraffin. Sequential IHC was performed on 5-μm formalin-fixed, paraffin-embedded (FFPE) sections using an adapted protocol based on methodology we previously described (27). Briefly, slides were deparaffinized and stained with hematoxylin (S3001, Dako), followed by whole-slide scanning at 20× magnification on an Aperio AT2 (Leica Biosystems). Tissues then underwent 15 minutes of heat-mediated antigen retrieval in pH 6.0 Citra solution (BioGenes), followed by 20 minutes of endogenous peroxidase blocking in 0.6% H2O2 (mouse) or 10 minutes of blocking in Dako Dual Endogenous Enzyme Block (S2003, Dako; human), then 10 minutes of protein blocking with 5% normal goat serum and 2.5% BSA in TBST. For staining of mouse tissue, primary antibody incubations were carried out overnight at 4°C with CD4 (D7D2Z, 1:50, Cell Signaling Technology), CD3 (SP7, 1:300, Thermo Fisher), and CD8 (48S15, 1:100, eBioscience). For staining of human tissues, primary antibody incubations were carried out for 30 minutes at room temperature using CD4 (SP35, 1:4, Ventana), CD8 (PG-M1, 1:50, Abcam), CD3 (SP7, 1:150, Thermo Fisher), and Pan Cytokeratin (AE1/AE3, 1:2000, Abcam), and 60 minutes at room temperature using CD45 (H130, 1:100, Thermo Fisher). After washing off primary antibody in TBST, either anti-rat, anti-mouse, or anti-rabbit Histone Simple Stain MAX PO horseradish peroxidase (HRP)-conjugated polymeric (Nichirei Biosciences) was applied for 30 minutes at room temperature, followed by AEC chromogen (Vector Laboratories). Slides were digitally scanned following each chromogen development, and the staining process was repeated starting at the Citra step for all subsequent staining cycles. Scanned images were registered in MATLAB version R2018b using the SURF algorithm in the Computer Vision Toolbox (The MathWorks, Inc.). Image processing and cell quantification were performed using Fiji (Fiji Is Just ImageJ; ref. 28). CellProfiler Version 3.5.1 (29), and FCS Express 6 Image Cytometry RUO (De Novo Software). AEC signal was extracted for quantification and visualization in Fiji using a custom macro for color deconvolution. Briefly, the Fiji plugin Color_Deconvolution (H AEC) was used to separate hematoxylin, followed by post-processing steps for signal cleaning and background elimination. AEC signal was extracted in Fiji using the NIH plugin RGB_to_CMKY. For visualization, signal-extracted images were overlaid in pseudocolor in Fiji. Color deconvoluted images were processed in CellProfiler to quantify single-cell mean intensity signal measurements for every stained marker, and human T cells were identified and quantified by image cytometry in FCS Express based on the expression of known markers as follows: pan cytokeratin (CD45^+ CD68^- CD3^+ CD4^+), CD8^-.

Murine and Human Cellular Isolation, Flow Cytometry, and FACS

Single-cell suspensions of mouse PDA tumors were prepared for flow cytometry as described previously, with slight modifications (5). Briefly, pancreata were placed in cold 2% FACS (PBS with 2% FBS) with collagenase IV (1 mg/mL; Worthington Biochemical), Trypsin inhibitor (1 mg/mL; EMID Milipore), and DNase I (2 U/mL; Promega), and minced with scissors to submillimeter pieces. Tissues were then incubated at 37°C for 20 minutes with gentle shaking every 5 minutes. Specimens were passed through a 70-μm mesh and centrifuged at 350 × g for 5 minutes. Splenocytes and thymocytes were prepared by manual disruption, as we described (5). Cell pellets were resuspended and cell labeling was performed after blocking FcRRII/II with an anti-CD16/CD32 mAb (eBioscience) by incubating 1 × 10^6 cells with 1 μg of fluorescently conjugated mAbs directed against mouse CD45 (30-F11), CD3 (17A4), CD4 (RM4-5), CD8 (33-6-7), CD62L (MEL-14), FasL (M33), NK1.1 (PK136), CD39 (Duha59), CCR2 (K036C2), CCR5 (HM-C5R5), CD86 (292L17), CD44 (IM7), CD106 (C06BC2), CD107α (D48), JAM1 (E101), CD46 (GL1), Gr1 (RB6-8C5), MHCII (M5/114.15.2), PD-1 (29F.1A12), ICOS (15F9), TGFβ (MP-6XT22), IL17A (TC11-18H10.1), CD4 (RB7-15H10.1), and CD8 (21F.812). Immunofluorescence (IF) and tetramer staining were performed on 2 × 10^6 cells in 1 mL of PBS with 2% FBS with monoclonal antibodies against the following surface markers: CD3 (SP7, 1:150), CD4 (D7D2Z, 1:50, Cell Signaling Technology), CD8 (45D10, 1:100, eBioscience). Flow cytometric analysis was performed on a FACSCalibur (BD Biosciences) and images were overlaid in pseudocolor in FIJI. Flow cytometric analysis was performed using FlowJo version 10.4.7 (Treestar).
For histologic analysis, murine orthotopic PDA tissues were fixed with 10% buffered formalin, dehydrated in ethanol, and embedded with paraffin. Sequential IHC was performed on 5-μm FFPE murine PDA using a staining methodology we previously described (27). Briefly, slides were deparaffinized and stained with hematoxylin (S3301, Dako), followed by whole-tissue scanning at 20× magnification on an Aperio AT2 (Leica Biosystems). Slides then underwent 20 minutes of endogenous peroxidase blocking in 0.6% H2O2 followed by 15 minutes of heat-mediated antigen retrieval in pH 6.0 Citra solution (BioGenex), followed by 10 minutes of protein blocking with 5% normal goat serum and 2.5% BSA in TBST. Primary antibody incubations were carried out overnight at 4°C using rabbit anti-CD4 (D7D2Z, 1:50, Cell Signaling Technology), rabbit anti-CD3 (SP7, 1:300, Thermo Fisher), and rat anti-CD8 (45-1151, 1:100, ebioscience). After washing off primary antibody in TBST, either anti-rat or anti-rabbit Histofine Simple Stain MAX PO HRP-conjugated polymer (Nichirei Biosciences) was applied for 30 minutes at room temperature, followed by AEC chromagen (Vector Laboratories). Slides were digitally scanned following chromagen development, and the staining process was repeated starting at Citra step for all subsequent staining cycles. Images were coregistered using MATLAB software, followed by AEC color deconvolution and pseudocoloring in Fiji software.
log-transformed the result. The final data set included 807 cells with a median of 1,330 detected genes. For analysis of the total leukocyte population in tumors treated with iosts, we normalized the data by the total expression, multiplied this by a scale factor of 10,000, and log-transformed the result. The final data set included 3,839 cells with a median of 1,418 detected genes.

**Visualization and Clustering of Single-Cell RNA-seq Data**

To visualize the data, we further reduced the dimensionality of the data set to project the cells in two-dimensional space using principal component analysis followed by t-distributed stochastic neighbor embedding (t-SNE) based on the aligned CCA. Aligned CCA was also used as a basis for partitioning the data set into clusters using a smart local moving community detection algorithm (https://arxiv.org/ftp/arxiv/papers/1308/1308.6604.pdf). To find markers that define individual clusters, we performed differential expression analysis using Wilcoxon rank sum test for each cluster compared with all other individual clusters, we performed differential expression analysis using Wilcoxon rank sum test for each cluster compared with all other

**Statistical Analysis**

Data are presented as mean ± standard error. Statistical significance was determined by the Student t test and the log-rank test using GraphPad Prism 7 (GraphPad Software). P values ≤0.05 were considered statistically significant. Significance for GSEA and differential gene expression based on single-cell RNA-seq was determined using the Wilcoxon rank sum test with Bonferroni multiple-comparison correction.

**Disclosure of Potential Conflicts of Interest**

D. Saxena has ownership interest (including stock, patents, etc.) in Periomicscare LLC. L.M. Coussens reports receiving commercial research grants from Acerta Pharma, LLC, Deciphera Pharmaceuticals, Roche Glycated Ag, and Syndax Pharmaceuticals, Inc.; reports receiving other commercial research support from PlexusInc, PharmaciesInc., Inc., Acerta Pharma, LLC, Deciphera Pharmaceuticals, Genentech Inc., Roche Glycated Ag, and NanoString Technologies, Inc.; is a member of the Pharmacy, Inc. steering committee for PCYC-1137-CA (NCT02436668); and is a consultant/advisory board member for Syndax Pharmaceuticals, Inc., Cancer Research Institute (CRI), The V Foundation for Cancer Research, Cancer Research United Kingdom (CRUK) Early Detection (EDx) Research Committee, Starr Cancer Consortium, NIH/NCI-Frederick National Laboratory Advisory Committee (FNLAC), Cell Signaling Technologies, Carisma Therapeutics Inc., Verseau Therapeutics, Inc., Zymeworks, Inc., (P30) Melvin and Ben Simon Cancer Center, Indiana University, (P30) Koch Institute for Integrated Cancer Research, Massachusetts Institute of Technology, (P30) Salk Institute Cancer Center, Bloomberg-Kimmel Institute for Cancer Immunotherapy, Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, and Dana-Farber Cancer Center Breast SPORE. No potential conflicts of interest were disclosed by the other authors.

**Authors’ Contributions**

Conception and design: M. Hundein, E. Kurz, J. Leinwand, B. Wadowski, G. Miller


Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Hundein, E. Kurz, A. Mishra, S.M. Liudahl, M. Kim, L.E. Torres, S. Sivagnanam,
REFERENCES


Immunogenic Macrophage Programming T Cells Mediate Antitumor Immunity by Orchestrating

Material

Supplementary

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