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ORIGINAL RESEARCH

Regulatory T cells that co-express RORγt and FOXP3 are pro-inflammatory and immunosuppressive and expand in human pancreatic cancer

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ABSTRACT

Pancreatic ductal adenocarcinoma (PDAC) is highly infiltrated by $CD4^+$T cells that express RORγt and IL-17 (T_H17). Compelling evidence from the tumor microenvironment suggest that regulatory T cells (T_{reg}) contribute to T_H17 mediated inflammation. Concurrently, PDAC patients have elevated levels of pro-inflammatory cytokines that may lead to T_H17 associated functional plasticity in T_{reg}. In this study, we investigated the phenotype and functional properties of T_{reg} in patients with PDAC. We report that PDAC patients have elevated frequency of $FOXP3^+T_{reg}$, which exclusively occurred within the $FOXP3^+ROR\gamma t^+T_{reg}$ compartment. The $FOXP3^+ROR\gamma t^+T_{reg}$ retained $FOXP3^+T_{reg}$ markers and represented an activated subset. The expression of RORγt in T_{reg} may indicate a phenotypic switch toward T_H17 cells. However, the $FOXP3^+ROR\gamma t^+T_{reg}$ produced both T_H17 and T_H2 associated pro-inflammatory cytokines, which corresponded with elevated T_H17 and T_H2 immune responses in PDAC patients. Both the $FOXP3^+T_{reg}$ and $FOXP3^+ROR\gamma t^+T_{reg}$ from PDAC patients strongly suppressed T cell immune responses, but they had impaired anti-inflammatory properties. We conclude that $FOXP3^+ROR\gamma t^+T_{reg}$ have a dual phenotype with combined pro-inflammatory and immunosuppressive activity, which may be involved in the pathogenesis of PDAC.

Abbreviations: CFSE, Carboxyfluorescein succinimidyl ester; FOXP3, Forkhead box P3; HD, Healthy donor; IL, Interleukin; INF, Interferon; MFI, Median fluorescence intensity; PBMC, Peripheral blood mononuclear cells; PDAC, Pancreatic ductal adenocarcinoma; Treg, Regulatory T cell; RORγt, RAR related orphan receptor gammat; TH cells, T helper cells; Tresp cells, T responder cells.

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Introduction

$CD4^+FOXP3^+T_{reg}$ cells (T_{reg}) constitute a separate thymus-derived $CD4^+$T cell lineage that is pivotal in maintaining immune tolerance.[1] The dominant role of T_{reg} in maintaining immune tolerance and homeostasis in humans is demonstrated in the fatal autoimmune disorder IPEX (immune dysregulation, polyendochrinopathy, enteropathy, X-linked syndrome), which is caused by mutations in the *FOXP3* gene.[1] Although T_{reg} ensure a protective and balanced immunity to the host, they may also contribute to the suppression of antitumor immunity initiated by tumor-infiltrating and tumor-associated T cells (TILs and TALs).[2] Therefore, analysis of T_{reg} may be an interesting prognostication tool in many cancer types.[3,4] However, high levels of T_{reg} have also been reported to correlate with both poor and favorable prognosis in various cancer types, which suggests that T_{reg} may have multiple effects on antitumor immunity.[5]

Pancreatic cancer is the fourth leading cause of cancer-related deaths,[6] and is characterized by aggressive growth and poor prognosis even in early stage disease. Adenocarcinoma in the region of the pancreatic head can have different histological types, where the pancreaticobiliary type is most common and most aggressive.[7] The putative origin of these tumors, either the pancreatic tissue or distal bile duct is difficult to establish with certainty and does not have prognostic significance given stage parity.[8,9]

PDAC is associated with chronic inflammation,[10] and inflammation combined with expansion of T_{reg} in peripheral blood and in the tumor tissue correlates with poor prognosis.[11-13] In addition, infiltration of IL-17 producing T_H17 and γδT cells into pancreatic stroma facilitates the initiation and progression of pancreatic intraepithelial neoplasia (PanIN) into PDAC.[14] In colon cancer, the infiltration of T_H17 cells and the expansion and conversion of T_{reg} into pro-

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inflammatory IL17^{+}T$_{reg}$ with reduced IL-10 secretion is associated with disease progression.[15-18] This suggests that T$_{reg}$ not only suppress antitumor immunity, but they may also contribute to the inflammation.

Here, we show that the frequency of T$_{reg}$ is increased in the peripheral blood of PDAC patients compared to healthy blood donors. However, the expansion occurs exclusively within a subset of T$_{reg}$ that co-express FOXP3 and RORγt. Detailed phenotypic analyses revealed that the FOXP3^{+}RORγt^{+}T$_{reg}$ retained the FOXP3^{+}T$_{reg}$ related markers and were a highly activated T$_{reg}$ subset. T$_{reg}$ from PDAC patients suppressed T cells, but they did not suppress inflammatory immune responses, and our results demonstrate that the expression of RORγt in FOXP3^{+}T$_{reg}$ is associated with pro-inflammatory properties. Due to their suppressive activity of adaptive immune responses combined with pro-inflammatory activity, these cells may represent an attractive therapeutic target in PDAC patients. However, due to the small cohort presented in this study, this must be further investigated in a larger cohort of PDAC patients.

Results

FOXP3^{+}RORγt^{+}T$_{reg}$ expand in peripheral blood of PDAC patients

The frequency of CD4^{+}CD25^{+}T$_{reg}$ is elevated in both peripheral blood and in pancreatic tumors ranging from low-grade pancreatic intraepithelial neoplasia (PanIN) to highly invasive adenocarcinoma.[11-13] To assess whether this expansion occurs within the T$_{reg}$ compartment and not in the FOXP3^{+} non-T$_{reg}$ population, we used the mutually exclusive marker CD127,[19] to distinguish CD4^{+}FOXP3^{+}CD127^{-}T$_{reg}$ (total T$_{reg}$) from CD4^{+}FOXP3$^{+/-}$ non-T$_{reg}$ cells (total T$_{H}$ cells) (Fig. S1). Total T$_{reg}$ frequency was significantly increased in peripheral blood mononuclear cells (PBMCs) of PDAC patients compared to that of HDs (Fig. 1A). A small fraction of IL17^{+}FOXP3^{+}T$_{reg}$ that co-express the FOXP3 and RORγt transcription factors has been shown to be present in peripheral blood from healthy donors (Fig. 1B).[20-22] Recent reports suggest that inflammation associated with T$_{H}$17 immune response in gastro-intestinal cancers can lead to accumulation of IL17^{+}FOXP3^{+}T$_{reg}$ and FOXP3^{+}RORγt^{+}T$_{reg}$.[15-17] T$_{H}$17 associated inflammation has also been reported to fuel the progression of PDAC.[14,23] However, appearance of FOXP3^{+}RORγt^{+}T$_{reg}$ has not been reported in PDAC patients. Here, we analyzed the expression of the T$_{H}$17 lineage specific master transcription factor RORγt in the total T$_{reg}$ population and found that the frequency of T$_{reg}$ that co-express the FOXP3 and RORγt transcription factors was substantially elevated, whereas T$_{reg}$ that expressed FOXP3 alone were significantly decreased in PBMCs of PDAC patients compared to HDs (Fig. 1B and Fig. S2). More than 80% of the total T$_{reg}$ from PDAC patients and HDs were of the CD45RA^{-} memory phenotype (data not shown). However, the presence of FOXP3^{+}RORγt^{+}T$_{reg}$ was elevated in both the CD45RA^{+} naïve and the CD45RA^{-} memory compartments of the total T$_{reg}$ pool in PDAC patients compared to HDs (Fig. 1C). FOXP3 has been shown to inhibit the transcriptional activity of RORγt,[24] and the increase in RORγt expression in FOXP3^{+}RORγt^{+}T$_{reg}$ from PDAC (Fig. 1D) could potentially indicate T$_{reg}$ lineage instability. However, the expression of FOXP3 was similar in the FOXP3^{+}T$_{reg}$ and FOXP3^{+}RORγt^{+}T$_{reg}$ from PDAC patients and

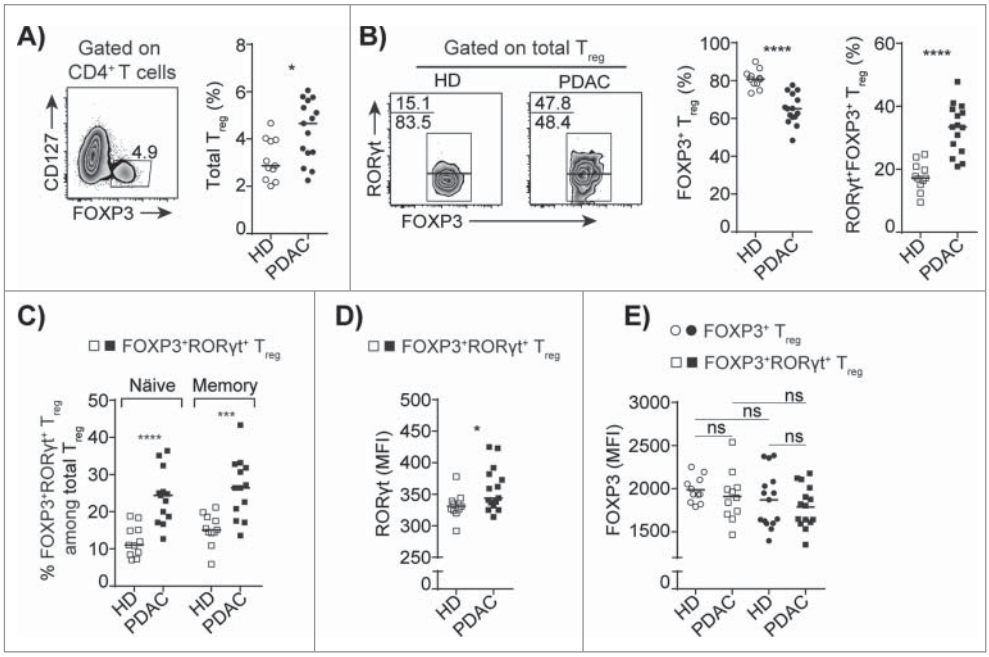

Figure 1. Increased frequency of FOXP3^{+}RORγt^{+}T$_{reg}$ in peripheral blood of PDAC patients. (A) A representative flow cytometry dot plot and compiled frequencies of CD4^{+}FOXP3^{+}CD127^{-}T$_{reg}$ (total T$_{reg}$) from PBMCs of HD and PDAC. (B) A representative flow cytometry dot plots and compiled frequencies of FOXP3^{+}T$_{reg}$ and FOXP3^{+}RORγt^{+}T$_{reg}$ from PBMCs of HD and PDAC. (C) Percentages of FOXP3^{+}RORγt^{+}T$_{reg}$ in naïve (CD45RA^{+}) and memory (CD45RA^{-}) fraction of total T$_{reg}$ population from HD and PDAC PBMCs. (D) Expression level of RORγt in FOXP3^{+}RORγt^{+}T$_{reg}$ from PBMCs of HD and PDAC. (E) Expression level of FOXP3 in FOXP3^{+}T$_{reg}$ and FOXP3^{+}RORγt^{+}T$_{reg}$ from PBMCs of HD and PDAC. HD (n=11) and PDAC (n=15). Horizontal bar represents median, each dot represents one patient. ns=non-significant, $^{*}p \le 0.05$, $^{***}p \le 0.001$, $^{****}p \le 0.0001$.

HDs (Fig. 1E). Taken together, these results suggest that FOXP3$^+$RORγt$^+$T$_{reg}$ constitute the expanding fraction of total FOXP3$^+$T$_{reg}$ in PDAC patient peripheral blood.

FOXP3$^+$RORγt$^+$T$_{reg}$ represent a highly activated T$_{reg}$ subset with transition capabilities toward T$_H$17 cells

T$_H$17 and T$_{reg}$ cells share a similar ontogeny, and the expression of RORγt could mark a transitional intermediate stage between T$_{reg}$ and T$_H$17 cells.[25] Therefore, we analyzed the expression of a panel of established T$_{reg}$ associated markers on circulating FOXP3$^+$RORγt$^+$T$_{reg}$ and compared their phenotype with that of FOXP3$^+$T$_{reg}$ from PDAC patients (Fig. 2A to C). The phenotypic characterization demonstrated that FOXP3$^+$RORγt$^+$T$_{reg}$ is a unique T$_{reg}$ subset that has retained and upregulated a majority of the markers associated with FOXP3$^+$T$_{reg}$ (Fig. 2B and C), which includes the inhibitory related markers CTLA-4, PD-1, LAP (TGFβ1), and CD39 and markers associated with activation that include HLA-DR, ICOS, TNFR2, OX40, LAG3, and CD147.[26] Next, we used the thymus derived T$_{reg}$ marker Helios to study the origin of FOXP3$^+$RORγt$^+$T$_{reg}$. Although the majority of FOXP3$^+$RORγt$^+$T$_{reg}$ were positive for Helios, we observed a significant decrease in the Helios positive subset compared to the FOXP3$^+$T$_{reg}$ in PDAC patients (Fig. 2D), which indicates that the expansion takes place in the periphery. In contrast, T$_H$ cells as expected did not express Helios. Human T$_H$17 cells have previously been shown to originate from CD161$^+$CD4$^+$T cell precursors,[27] and we found that FOXP3$^+$RORγt$^+$T$_{reg}$ did not express CD161, which strongly indicates that FOXP3$^+$RORγt$^+$T$_{reg}$ from PDAC patients originate from FOXP3$^+$T$_{reg}$ rather than from T$_H$17 cells (Fig. 2E). However, we noticed that FOXP3$^+$RORγt$^+$T$_{reg}$ were positive for CD25, but the level of expression was downregulated (Fig. 2B and C). Expression of CD25 is required for the maintenance of the FOXP3$^+$T$_{reg}$ pool in periphery,[28] whereas IL-2 signaling

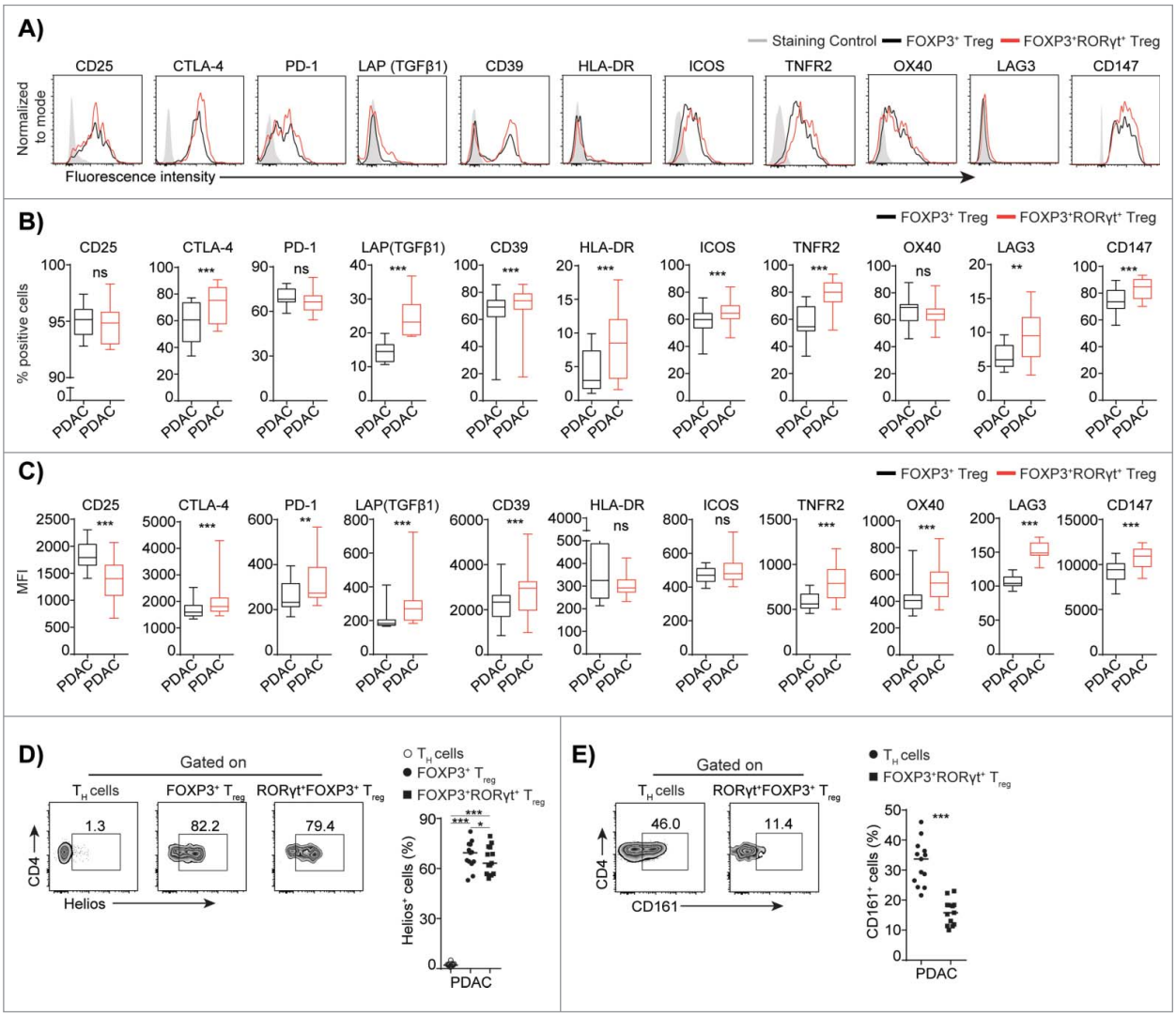

Figure 2. Phenotypic characteristics of FOXP3$^+$RORγt$^+$T$_{reg}$ from PDAC patients. Phenotypic analysis of PBMCs from PDAC shows the expression level of indicated markers on FOXP3$^+$RORγt$^+$ T$_{reg}$ compared to FOXP3$^+$T$_{reg}$. (A) A representative flow cytometry histogram from one donor is shown. The Box plots shows, (B) compiled frequencies and (C) expression level of indicated markers. (D) A representative flow cytometry dot plots and compiled frequencies of Helios$^+$T$_H$, FOXP3$^+$T$_{reg}$ and FOXP3$^+$RORγt$^+$T$_{reg}$ from PDAC PBMCs. (E) A representative flow cytometry dot plots and compiled frequencies of CD161$^+$T$_H$, FOXP3$^+$T$_{reg}$, and FOXP3$^+$RORγt$^+$T$_{reg}$ from PDAC PBMCs. HD (n = 11) and PDAC (n = 13). Horizontal bar represents median, each dot represents one patient. ns = non-significant, $^*p \le 0.05$, $^{**}p \le 0.01$, $^{***}p \le 0.001$.

through CD25 interferes with T_H17 cells differentiation,[29] and downregulation of CD25 indicates that FOXP3$^+$RORγt$^+$T$_{reg}$ may convert into T_H17 cells. Together these results suggest that FOXP3$^+$RORγt$^+$T$_{reg}$ represent a distinct subset that is in a transition stage from T$_{reg}$ to T_H17 cells.

Expression of RORγt in FOXP3$^+$T$_{reg}$ imprints pro-inflammatory features of T_H2 and T_H17 lineages

The RORγt transcription factor is essential for the generation of classical IL-17A producing CD4$^+$T$_H$ cells.[24] To investigate whether FOXP3$^+$RORγt$^+$T$_{reg}$ also display features of T_H17 cells, we analyzed the production of cytokines by intracellular cytokine staining after 4 h of stimulation of PBMC. We found that the total T$_{reg}$ population from PDAC patients produced significantly increased levels of IL-17A when compared to that of HDs (Fig. 3A), whereas the production of the inhibitory cytokine IL-10, which is required for T$_{reg}$ to maintain immune homeostasis in the gastrointestinal tract (Fig. 3A),[30-32] was significantly reduced from total T$_{reg}$ population from PDAC when compared to HDs. However, the production of other pro-inflammatory cytokines associated with T_H2 and T_H1 cells was not significantly altered comparing the total T$_{reg}$ from PDAC to that of HDs. Further analysis of intracellular cytokine profiles in FOXP3$^+$T$_{reg}$ compared to FOXP3$^+$RORγt$^+$T$_{reg}$ from PDAC patients showed that only FOXP3$^+$RORγt$^+$T$_{reg}$ produced the T_H17 associated cytokine IL-17A (Fig. 3B and C). However, the secretion of TNF-α, which is another T_H17 associated cytokine, was significantly upregulated in FOXP3$^+$RORγt$^+$T$_{reg}$, but was also produced by FOXP3$^+$T$_{reg}$. Unexpectedly, we found that FOXP3$^+$RORγt$^+$T$_{reg}$, but not FOXP3$^+$T$_{reg}$ were able to produce the T_H2 associated cytokines IL-6 and IL-13 (Fig. 3B and C). The production of T_H1 associated cytokine INFγ and the inhibitory cytokine IL-10 were produced at low levels by the FOXP3$^+$T$_{reg}$ and FOXP3$^+$RORγt$^+$T$_{reg}$ (Fig. 3B and C). We also found that a

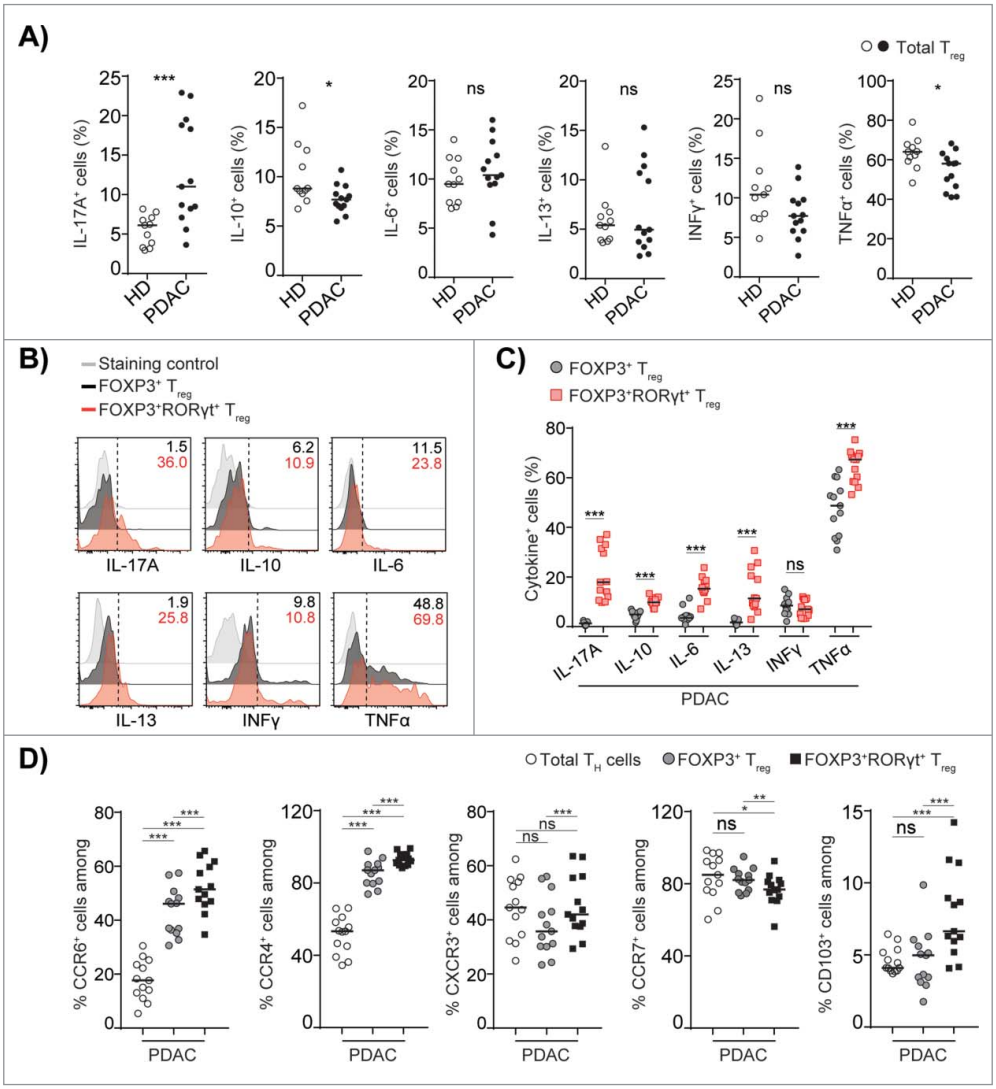

Figure 3. FOXP3$^+$RORγt$^+$T$_{reg}$ share pro-inflammatory cytokines and chemokine receptors of T_H2 and T_H17 lineages. (A) Compiled frequencies of IL-17A$^+$, IL-10$^+$, IL-6$^+$, IL-13$^+$, TNFα^+, and INFγ^+ total T$_{reg}$ from HD and PDAC PBMCs. (B) A representative overlaid flow histograms shows the expression levels of indicated cytokines in FOXP3$^+$ T$_{reg}$ and FOXP3$^+$RORγt$^+$T$_{reg}$ from PDAC PBMCs. (C) Compiled frequencies of indicated cytokines expressed by FOXP3$^+$T$_{reg}$ and FOXP3$^+$RORγt$^+$T$_{reg}$ from PDAC PBMCs. (D) Compiled frequencies of expression of indicated non-lymphoid and lymphoid homing receptors on FOXP3$^+$T$_{reg}$ and FOXP3$^+$RORγt$^+$T$_{reg}$ from PDAC PBMCs. HD (n = 11) and PDAC (n = 13). Horizontal bar represents median, each dot represents one patient. ns=non-significant, $^*p \leq 0.05$, $^{***}p \leq 0.001$.

significant fraction of FOXP3$^+$RORγt$^+$T$_{reg}$ were able to produce IL-17A, IL-6, IL-13, and TNF-α concurrently, whereas FOXP3$^+$T$_{reg}$ mostly produced TNF-α, INFγ but little IL-10 and IL-6 (Fig. S3A). We found that in correspondence with their cytokine secretion pattern, the T$_H$17 and T$_H$2 associated chemokine receptors CCR4, CCR6, and intra-epithelial homing marker CD103 were significantly upregulated in FOXP3$^+$RORγt$^+$T$_{reg}$ compared to the FOXP3$^+$T$_{reg}$ and the T$_H$ cells (Fig. 3D). Whereas, the expression of the T$_H$1 associated chemokine receptor CXCR3 was not altered. Although the majority of the FOXP3$^+$RORγt$^+$T$_{reg}$ retained the lymphoid homing chemokine receptor CCR7, the expression was significantly reduced (Fig. 3D). These results demonstrate that FOXP3$^+$RORγt$^+$T$_{reg}$ are capable of producing both T$_H$2 and T$_H$17 associated cytokines and expresses chemokine receptors for homing to both non-lymphoid and lymphoid tissues.

Higher frequency of RORγt$^+$T$_H$ cells subset with features of both T$_H$2 and T$_H$17 lineages

Next, we investigated the whether expansion of FOXP3$^+$RORγt$^+$T$_{reg}$ concurrently lead to an increase in T$_H$2 and T$_H$17 cells in PDAC patients. We found that the frequency of circulating RORγt$^+$ and IL-17A producing T$_H$17 cells in the total T$_H$ cell population was strongly increased in PDAC patients compared to HDs (Fig. 4A, right and left panels). Similarly, the circulating IL-13 producing T$_H$2 cells were elevated in the total T$_H$ cell population in PDAC patients compared to HDs (Fig. 4B). However, there was no change in T$_H$1 response as the INFγ producing total T$_H$ cells in PDAC patients were similar to the level in HDs (Fig. 4C). This indicates that there is a skewing from an antitumor T$_H$1 immunity toward a pro-carcinogenic T$_H$17 and T$_H$2 responses in PDAC patients.[33] We noticed that expression of RORγt was not only restricted to T$_H$17 cells in PDAC and HDs, but was also expressed in T$_H$1, T$_H$2, and T$_H$17/1 subsets (Fig. 4D). However, the expression level of RORγt was increased in all T$_H$ subsets identified in PDAC patients (Fig. 4D), which further supports the notion that there is a T$_H$17 associated plasticity in PDAC patients. In humans, T$_H$17 cells are highly plastic and contribute to inflammatory conditions,[34] and owing to the elevated T$_H$17 and T$_H$2 responses in PDAC patients, we next analyzed the cytokine expression in RORγt$^-$ and RORγt$^+$ T$_H$ cells. Notably, we found that the expression of the T$_H$17 associated cytokine IL-17A and the T$_H$2 associated cytokine IL-13 were strictly confined to the RORγt$^+$T$_H$ cells (Fig. 4E). However, RORγt$^+$T$_H$ cells and RORγt$^-$T$_H$ cells expressed equal levels of INFγ (Fig. 4E). Furthermore, we confirmed that the PDAC patients had elevated levels of circulating T$_H$17 and T$_H$2 associated cytokines, which included IL-6, IL-4, and IL-33 with no change in the level of INFγ (Fig. 4F). Together, these results indicate that RORγt$^+$T$_H$ cells appear to have a T$_H$17/T$_H$2 phenotype and are capable of producing the corresponding T$_H$17 and T$_H$2 cytokines. These findings mirrored the phenotypical features of FOXP3$^+$RORγt$^+$T$_{reg}$ and indicate that FOXP3$^+$RORγt$^+$T$_{reg}$ and RORγt$^+$T$_H$ contribute to elevated T$_H$17 and T$_H$2 immune responses in PDAC patients.

T$_{reg}$ from PDAC suppress T cell immune responses, but enhance inflammation

To examine the suppressive function of circulating T$_{reg}$ isolated from PDAC patients, we used CD4$^+$FOXP3$^+$CD127$^{dim/-}$ T$_{reg}$ (total T$_{reg}$) and T$_{reg}$ depleted PBMCs from the same donor as T$_{resp}$

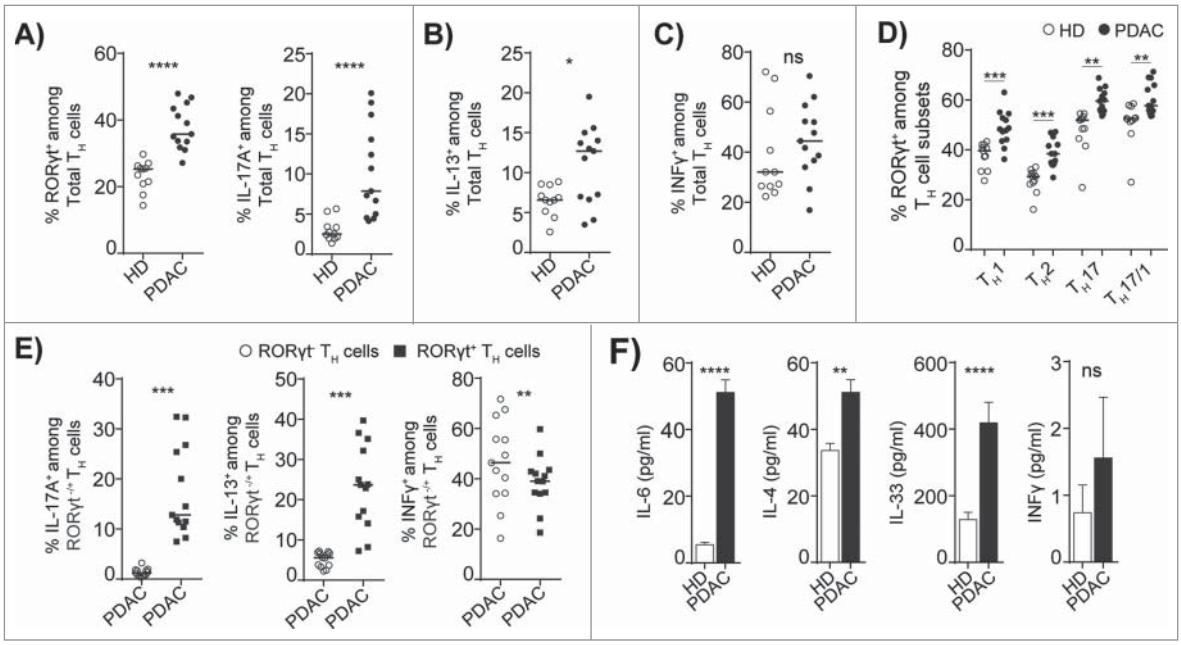

Figure 4. A subset of RORγt$^+$T$_H$ cells displays features of both T$_H$2 and T$_H$17 lineages. (A) Compiled frequencies of RORγt$^+$ (left panel) IL-17A$^+$ (right panel), (B) IL13$^+$, and (C) INFγ$^+$T$_H$ cells from HD and PDAC PBMCs. (D) Compiled frequency of RORγt expression in T$_H$ cell subsets from HD and PDAC PBMCs. (E) Compiled frequencies shows the IL-17A$^+$, IL-13$^+$, and INFγ$^+$ RORγt$^-$ and RORγt$^+$T$_H$ cells from PDAC PBMCs. HD (n = 11) and PDAC (n = 13). (F) Cytokines in HD and PDAC serum by ELISA HD (n = 10 to 11) and PDAC (n = 12 to 15). Error bars represents mean ± SEM, Horizontal bar represents median, each dot represents one patient. ns=non-significant, $^*p \le 0.05$, $^{**}p \le 0.01$, $^{***}p \le 0.001$, $^{****}p \le 0.0001$.

cells in co-culture experiments. T_{reg} from the PDAC patients strongly suppressed the proliferation of $CD4^+T_{resp}$ and $CD8^+T_{resp}$ cells at a similar potency as T_{reg} from HDs (Fig. 5A). The suppression of T_{reg} was dependent on the T_{reg}: T_{resp} ratio and T_{reg} from PDAC patients retained suppression at all the ratios tested (Fig. 5B). The T_{reg} also suppressed significantly the secretion of IL-2, TNF-α, and IL-10, but increased the production of IL-17A (Fig. 5C). T_{reg} in presence of TGFβ and IL-6 have previously been shown to promote IL-17A induction in T_H cells.[35-37] As already shown (Fig. 2B and C), TGFβ is expressed significantly higher in $FOXP3^+ROR\gamma t^+T_{reg}$ compared to $FOXP3^+T_{reg}$ and are also able to secrete IL-6 (Fig. 3B and C), thus the $FOXP3^+ROR\gamma t^+T_{reg}$ may induce IL-17A secretion from T_H cells by this mechanism. However, the expanded $FOXP3^+ROR\gamma t^+T_{reg}$ also expressed IL-17A at the same level as T_H cells from PDAC patients, and both the $FOXP3^+ROR\gamma t^+T_{reg}$ and the T_H cells may therefore contribute to the elevated level of IL-17A in the co-culture supernatants. Since $FOXP3^+ROR\gamma t^+T_{reg}$, constitute more than 35% of total T_{reg} population, the data suggest that PDAC T_{reg}, which include both the $FOXP3^+T_{reg}$ and the $FOXP3^+ROR\gamma t^+T_{reg}$ subsets are T cell immunosuppressive. T_{reg} and mast cells share substantial co-localization in tissues and lymph nodes, and both cell types infiltrate the tumor microenvironment in PDAC.[38] The interaction between T_{reg} and mast cells enhances mast cell degranulation and promotes skewing of T_{reg} from anti-inflammatory properties toward pro-inflammatory functions without losing its suppressive function.[39] To test whether T_{reg} enhance mast cell degranulation, we co-cultured T_{reg} from patients and HDs with mast cell in a 1:1 ratio. T_{reg} from PDAC patients significantly enhanced mast cell degranulation compared to T_{reg} from HDs (Fig. 5D). Furthermore, we confirmed that PDAC patients have increased plasma tryptase activity compared to HDs (Fig. 5E), which indicates that there is enhanced mast cell degranulation in PDAC patients. Finally, we investigated the possibility to target the $FOXP3^+ROR\gamma t^+T_{reg}$ using the RORγt specific inhibitor digoxin, which inhibits the development of T_H17 cells by antagonizing RORγt function and IL-17A production.[40] We analyzed the production of cytokines by the $FOXP3^+ROR\gamma t^+T_{reg}$ compared to that of the $FOXP3^+T_{reg}$ from digoxin pre-treated PBMCs from PDAC patients. We found that the production of IL-17A was strongly inhibited in the $FOXP3^+ROR\gamma t^+T_{reg}$ (Fig. 5F), whereas the production of IL-10 was largely unaltered in both the $FOXP3^+ROR\gamma t^+T_{reg}$ and the $FOXP3^+T_{reg}$ (Fig. 5F). Furthermore, we found that the digoxin treated PBMCs demonstrated a significant decrease in the total T_{reg} population and that this effect modestly influenced the composition of $FOXP3^+ROR\gamma t^+T_{reg}$ versus $FOXP3^+T_{reg}$ (Fig. 5G). Similar results were observed for the $ROR\gamma t^+T_H$ cells (data not shown). Thus, T_{reg} from PDAC patients, which includes both $FOXP3^+T_{reg}$ and $FOXP3^+ROR\gamma t^+T_{reg}$, retain their T cell suppressive activity. However, the T_{reg} population undergoes a shift from anti-inflammatory to pro-inflammatory activity, which can be inhibited using RORγt specific inhibitors.

Discussion

The $FOXP3^+T_{reg}$ have diverse roles in the pathogenesis of human malignancies and have been associated with both favorable prognosis and progressive disease.[2,5] The T_{reg} population is heterogeneous and the composition could explain why T_{reg} may have opposing effects on the course of the disease in cancer patients. T_{reg} is one of the major T cell populations that infiltrate cancer tissues. Here, we demonstrate that $FOXP3^+T_{reg}$ isolated from peripheral blood of patients with PDAC expand, but the expansion occurs only within the $FOXP3^+ROR\gamma t^+T_{reg}$ subset, which have combined inflammatory and immunosuppressive properties. This population may be particularly detrimental for the prognosis by suppressing adaptive antitumor immunity, whereas the pro-inflammatory nature may support malignant transformation and tumor growth by contributing to a microenvironment enriched in growth factors, pro-inflammatory cytokines, pro-angiogenic factors, and reactive oxygen species. Furthermore, recent evidence suggests that IL-17A signaling promotes the initiation of pancreatic intraepithelial neoplasia (PanIN) and disease progression.[14] It is also interesting to note that immunosuppressive treatment in solid organ recipients and patients with chronic inflammatory diseases have been associated with an increased risk of developing pancreatic cancer.[41, 42]

Pro-carcinogenic processes that involve elevated T_H17 immune response are involved in the pathogenesis of pancreatic, gastric, lung, and colon cancer.[14,15,18,43,44] T_H17 cells that express RORγt infiltrate tumor tissue in patients with PDAC, but T_{reg} comprise a larger fraction of the total tumor infiltrating $CD4^+T$ cells compared to the T_H17 cells. Even though $FOXP3^+T_{reg}$ are a stable T cell lineage, phenotypic plasticity has been demonstrated in inflammatory conditions where the $FOXP3^+T_{reg}$ may acquire pro-inflammatory properties. This suggests that the T_H17 cells and the $FOXP3^+T_{reg}$ may act in concert to propagate the disease. This is further supported by the identification of a small fraction of T_{reg} that co-express FOXP3 and RORγt,[20,21] which expands particularly in patients with chronic inflammation,[42,43] and colon cancer.[15-17]

In our study, we found a significantly increased number of $FOXP3^+ROR\gamma t^+T_{reg}$ in peripheral blood of PDAC patients. The $FOXP3^+ROR\gamma t^+T_{reg}$ were present in both the naive and memory compartment, which indicates that these cells persist beyond the initial immune response and acquire the ability to persist and engage in a secondary immune response. At the molecular level, FOXP3 interacts with RORγt and antagonize its transcriptional function and subsequently hampers the induction of IL-17 production.[24] This is thought to be the basis for the reciprocal balance between suppression of adaptive immunity versus inflammation. However, our results demonstrated that the level of expression of RORγt in $FOXP3^+ROR\gamma t^+T_{reg}$ was similar to the level in T_H17 cells. This indicates that FOXP3 does not impair the RORγt function in the $FOXP3^+ROR\gamma t^+T_{reg}$ in these patients. T_{reg} from healthy donors express equal level of two isoforms of FOXP3 by alternate splicing.[45] The isoform lacking exon 2 does not interact with RORγt, and it is therefore likely that the expanding $FOXP3^+ROR\gamma t^+T_{reg}$ that we identified in PDAC patients may overexpress this FOXP3 isoform to facilitate FOXP3 and RORγt co-expression in T_{reg}.

Phenotypic plasticity enables the T_{reg} to downregulate FOXP3 expression and acquire T_H cell properties.[34] The enhanced expression of RORγt in T_{reg} could also mark their potential plasticity toward T_H17 cells. However, we found that

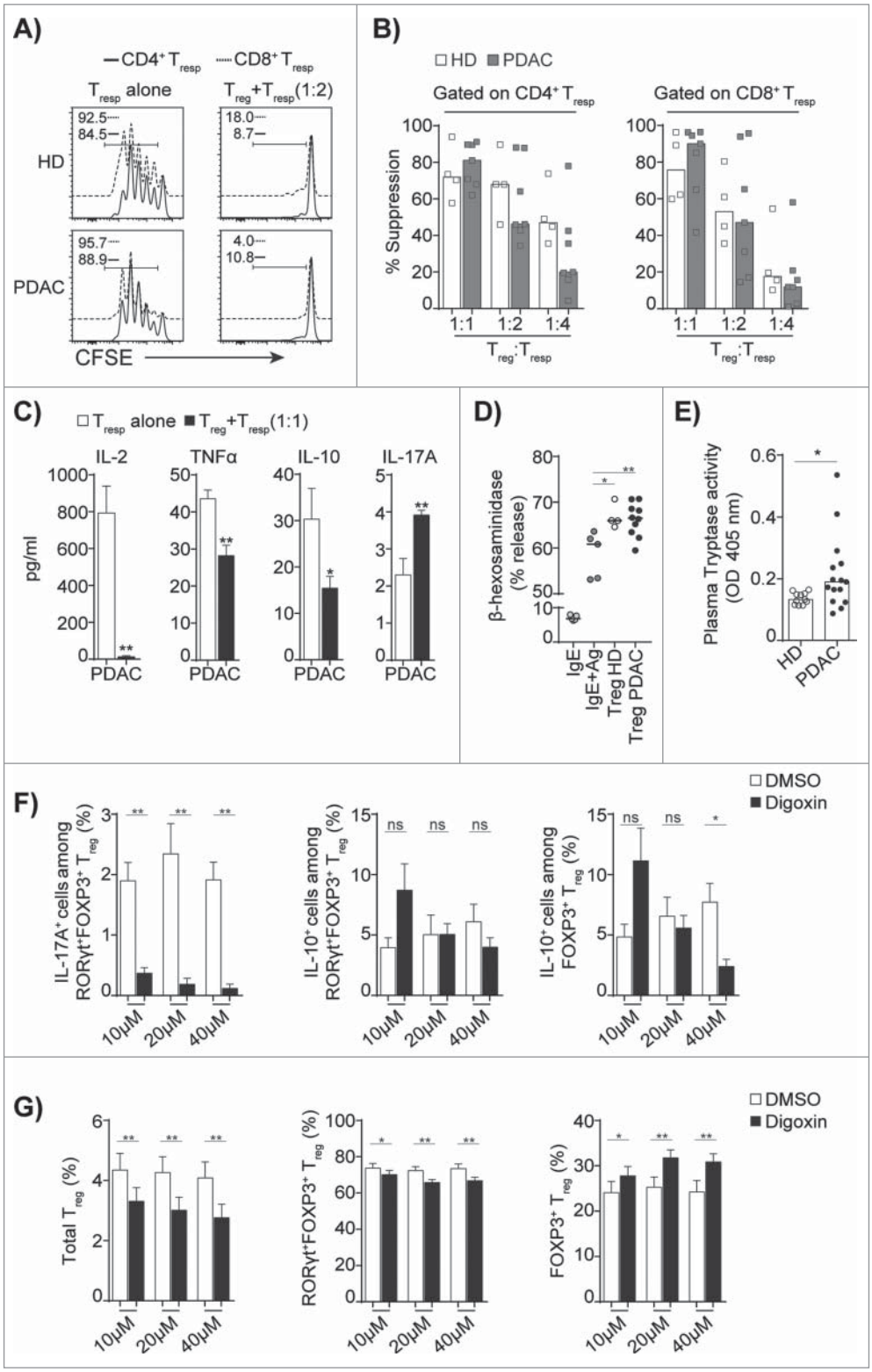

Figure 5. T_{reg} that include $FOXP3^+T_{reg}$ and $FOXP3^+ROR\gamma t^+T_{reg}$ from PDAC are immunosuppressive but enhance mast cell degranulation. (A) A representative overlaid flow histograms shows the suppressive capacity of T_{reg} against $CD4^+T_{resp}$ (black), $CD8^+T_{resp}$ (blue) from HD and PDAC PBMCs. Left panel shows T_{resp} stimulated in the absence of T_{reg} and right panel shows T_{resp} stimulated in presence of T_{reg} (T_{reg}:T_{resp} = 1:2 ratio). (B) Compiled percentage of suppression by T_{reg} from HD and PDAC PBMCs with increasing T_{resp} to T_{reg} ratio in the co-culture. (C) Cytokines from co-cultures of T_{reg} and T_{resp} from PDAC PBMCs by ELISA. HD (n=4) and PDAC (n = 7). (D) Compiled percentage degranulation of LAD2 human mast cells sensitized with IgE alone, IgE + antigen cross-linked, or co-cultured with purified T_{reg} (T_{reg}:MC = 1:1 ratio) from HD and PDAC PBMCs. HD (n = 4) and PDAC (n = 10). (E) Tryptase activity of HD and PDAC serum was measured by spectrophotometric assay HD (n = 11) and PDAC (n = 15). (F) Compiled bar graph shows the IL-17A and IL-10 expressing $FOXP3^+ROR\gamma t^+T_{reg}$ and $FOXP3^+T_{reg}$, and (G) The frequency of total T_{reg}, $FOXP3^+ROR\gamma t^+T_{reg}$ and $FOXP3^+T_{reg}$ from PDAC PBMCs that were pretreated with indicated concentrations of the RORγt inhibitor digoxin or with dimethyl sulfoxide (DMSO) as control. PDAC (n = 10). Error bars represents mean ± SEM, horizontal bar represents median and each dot represents one patient. ns = non-significant, $^*p \leq 0.05$, $^{**}p \leq 0.01$.

the FOXP3$^+$RORγt$^+$T$_{reg}$ in PDAC patients retained the expression of FOXP3. The phenotypical features of FOXP3$^+$RORγt$^+$T$_{reg}$ have not been extensively reported, and our analyses herein demonstrate that both surface and intracellular markers associated with FOXP3$^+$T$_{reg}$ activation and function were expressed either equally or at elevated levels in FOXP3$^+$RORγt$^+$T$_{reg}$. Constitutive expression of LAG3, CTLA-4, and PD-1 has been shown to be essential for T$_{reg}$ suppressive function in the periphery.[1] The enhanced expression of these receptors on T$_{reg}$ from PDAC patients could be associated with their suppressive function and blockade may lead to impaired T$_{reg}$ functions in both the FOXP3$^+$RORγt$^+$T$_{reg}$ and the FOXP3$^+$T$_{reg}$ compartment and thereby improve T cell dependent antitumor immunity. Further, the expression of Helios, a marker for thymus derived FOXP3$^+$T$_{reg}$ was expressed at a similar level in the FOXP3$^+$RORγt$^+$T$_{reg}$ as in the FOXP3$^+$T$_{reg}$, whereas the absence of the human T$_H$17 marker CD161 excluded the possibility that these cells represent a subset that is derived from T$_H$17 cells. Although FOXP3$^+$RORγt$^+$T$_{reg}$ retained FOXP3$^+$T$_{reg}$ associated markers, the surface expression level of CD25 was significantly reduced in FOXP3$^+$RORγt$^+$T$_{reg}$. The maintenance of FOXP3$^+$T$_{reg}$ frequency in the periphery requires stable CD25 expression,[28] and indicates that FOXP3$^+$RORγt$^+$T$_{reg}$ may undergo plasticity toward T$_H$ cells.

T$_{reg}$ inhibit inflammation in the gastrointestinal tract through secretion of IL-10.[30-32] Our observations in PDAC patients suggest that FOXP3$^+$T$_{reg}$ and FOXP3$^+$RORγt$^+$T$_{reg}$ produce less IL-10, whereas production of IL-17A was restricted to FOXP3$^+$RORγt$^+$T$_{reg}$. Our observations also suggest that the FOXP3$^+$RORγt$^+$T$_{reg}$ were capable of expressing the poly-functional cytokines IL-6, IL-13, TNF-α which are associated with both T$_H$17 and T$_H$2 responses. It has previously been reported that IL-17A-secreting FOXP3$^+$RORγt$^+$ T$_{reg}$ and IL-10-secreting FOXP3$^+$T$_{reg}$ are in equilibrium during infections and inflammation,[46] however our findings in PDAC patients demonstrated that this balance is altered in the direction of pro-inflammatory function with production of T$_H$17 and T$_H$2 cytokines. The FOXP3$^+$RORγt$^+$T$_{reg}$ were also CD45RA- and upregulated the T$_H$17 and T$_H$2 associated non-lymphoid tissue homing receptors CCR6, CCR4, the intra-epithelial homing receptor CD103 and had significantly reduced expression of the lymphoid homing receptor CCR7 compared to the FOXP3$^+$T$_{reg}$ and the T$_H$ cells. This suggests that the FOXP3$^+$RORγt$^+$T$_{reg}$ in PDAC are posed to migrate to inflamed tissue.[47]

Our results also show that the number of circulating RORγt and IL-17A expressing T$_H$17 cells increase in parallel with expansion of FOXP3$^+$RORγt$^+$T$_{reg}$ in the PDAC patients. In addition, PDAC patients also had increased levels of circulating IL-13 expressing T$_H$2 cells. Furthermore, we confirmed that not only the intracellular expression, but also the level of cytokines that are associated with T$_H$17 and T$_H$2 cells were elevated in peripheral blood of the PDAC patients. Unexpectedly, the expression of IL-13 was restricted to RORγt$^+$T$_H$ cells. This further adds complexity to CD4$^+$T cell mediated inflammatory responses in PDAC patients. T$_H$17/T$_H$2 hybrid T$_H$ cells were demonstrated to exacerbate inflammation in chronic asthma.[48-50] Here, we show for the first time that the increased numbers of T$_H$17 cells and T$_H$2 cells in PDAC patients are confined to RORγt$^+$T$_H$ cells that produce both IL-13 and IL-17A and represent T$_H$17/T$_H$2 hybrid cells. Furthermore, RORγt$^+$T$_H$ cells also share features with FOXP3$^+$RORγt$^+$T$_{reg}$ both in terms of cytokine production and tissue trafficking receptors, which indicates that these cells co-localize at the same sites.

We further assessed the suppressive activity of the T$_{reg}$ from the PDAC patients on CD4$^+$ and CD8$^+$T cells. In malignancies, this implies suppression of anticancer immunity. However, recent studies suggest that T$_{reg}$ promote T$_H$17 differentiation and enhance their function.[51] We found that T$_{reg}$ from PDAC patients were able to suppress the proliferation and IL-2 production of the T$_H$ cells completely, but the inhibitory effect on IL-17A, IL-10, and TNF-α secretion was compromised. This clearly indicates that T$_{reg}$ from PDAC patients enhance T$_H$17 and T$_H$2 responses, but suppress T$_H$ cells. Mast cells also infiltrate PDAC tissue,[10] and the level of tryptase in peripheral blood was elevated. T$_{reg}$ interact with mast cells through the OX40 receptor,[52] and mast cells have been shown to reduce the suppressive function of T$_{reg}$ and contribute to an additive effect to T$_H$17 responses in cancer microenvironments.[39,53] FOXP3$^+$ T$_{reg}$ and FOXP3$^+$RORγt$^+$T$_{reg}$ from PDAC patients expressed OX40 receptor and significantly enhanced degranulation from mast cells. Together, this shows that T$_{reg}$ retain their suppressive function toward T$_H$ cells while enhancing inflammation by inducing T$_H$17 and T$_H$2 responses in addition to stimulate mast cell degranulation. Finally, we show that specific inhibition of RORγt using digoxin,[40] inhibited IL-17A production by FOXP3$^+$RORγt$^+$T$_{reg}$. These findings indicate a potential future therapeutic strategy to target the inflammatory activity of the FOXP3$^+$RORγt$^+$T$_{reg}$ in the PDAC patients.

In conclusion, our identification, phenotypic, and functional characterization of FOXP3$^+$RORγt$^+$T$_{reg}$ provides an insight into their pro-inflammatory and suppressive functions in PDAC patients. We were able to confirm that FOXP3$^+$RORγt$^+$T$_{reg}$ and RORγt$^+$T$_H$ cells exhibit overlapping functions and lead to T$_H$17/T$_H$2 associated immune responses and inflammation that likely contribute to the progression of the disease. Together, these results identify a cellular target that may prove to be therapeutically important in order to resolve inflammation and reverse the suppression of antitumor immunity in PDAC patients.

Materials and methods

Patient samples

Patient studies were approved by the Regional Ethics Committee of the South-Eastern Norway Regional Health Authority. Informed consent was obtained from 53 patients and blood samples were collected preoperatively. Fifteen of the patients treated with Whipple operation with curative intent had localized adenocarcinoma with pancreaticobiliary histology. No patients had received pre-surgical chemo- or radiotherapy. Patients with other tumor origin, histology, and advanced tumors were excluded from this analysis. Blood from age-matched healthy controls (HDs) were obtained from Oslo University Hospital Blood Center (Oslo, Norway). PBMCs were isolated by Ficoll-paque (Axis-Shield Poc AS) buoyant density

Table 1. The clinicopathological characteristics and immunological parameters in pancreatic cancer patients and healthy donors

Parameters	PDAC	HD
Number (n)	15	11
Age (years)[a]	69 (51–78)	68 (65–69)
Male-female ratio	8:7	7:4
Tumor Stage (n)[b]		
Ia	1	
Ib	1	
IIa	4	
IIb	8	
III	1	
Tumor Grade (n)		
II	11	
III	4	
Residual Tumor (n)		
0	8	
I	7	
Histology (n) Pancreaticobiliary	15	
Neoadjuvant/adjuvant therapy	0/8	
One-year overall surival, %	80	
CA19–9, U/mL	649 (13–36831)	
PBMC, $\times 10^6$ cells/mL	2.12 (0.92–3.6)	2.4 (1.84–4.24)
$CD4^+$ cells, %	51.3 (27.1–67.8)	48.9 (33.1–56.9)
$CD8^+$ cells, %	34.7 (18.2–49.7)	22.7 (13.1–45.3)
Among $CD4^+CD127^{+/-}FOXP3^+T_{reg}$		
$FOXP3{+}T_{reg}$, %	65.1 (56–77.6)	80.8 (73.4–90)
$RORyt{+}FoxP3{+}T_{reg}$, %	33.4 (21.7–47.8)	17.2 (9.54–24.7)

Abbreviations: PDAC, pancreatic ductal adenocarcinoma; HD, Healthy donor. The data are represented as median and range. [a]Age at diagnosis. [b]According to The American Joint Committee on Cancer.

medium centrifugation. PBMCs were cryopreserved in FBS with 10% DMSO.

Cell culture and purification

PBMCs were thawed and rested overnight at 37°C with 5% CO_2 in RPMI 1640 with GlutaMax supplemented with 10% FCS, 1% penicillin-streptomycin (GIBCO), 1% sodium pyruvate, and 1% minimum non-essential amino acids before using it for immunophenotyping and functional assays. The T_{reg} from rested PBMCs were purified using human $CD4^+CD25^+CD127^{dim/-}$ T_{reg} isolation kit II (Miltenyi Biotech) according to the manufacturer's instructions. The T_{reg} depleted $CD3^+$T cells fraction from the same donor was used as T_{resp} cells. The LAD2 mast cell line was cultured in serum free StemPro→-34 SFM media (Life Technologies) supplemented with 2 mM L-Glutamine, 100 IU/mL penicillin, 50 μg/mL streptomycin, 100 ng/mL rhSCF (Peprotech) at 37°C with 5% CO_2.

T_{reg} suppression assays

T_{reg} were pre-activated for 48 h using α-CD2/CD3/CD28 coated beads (T cell activation/Expansion Kit, Miltenyi Biotech) in a 1:2 ratio (beads to cells). T_{reg} and CFSE (5μM/mL) labeled T_{resp} cells were mixed in a 1:1 to 1:4 (T_{reg}:T_{resp}) ratio and stimulated with α-CD2/CD3/CD28 coated beads in a 1:5 ratio (beads:cells). CFSE dilution in T_{resp} was analyzed after 4 d of co-culture by flow cytometry. For mast cell degranulation inhibition by T_{reg}, LAD2 cells were pre-sensitized over night with 100 ng/mL of biotin-conjugated human immunoglobulin E (IgE). T_{reg} and LAD2 cells were mixed in 1:1 ratio in Tyrode's buffer and incubated for 10 min at 37°C, stimulated with Streptavidin (100 ng/mL, 30 min) and centrifuged. The supernatants and cell lysates (0.1% Triton X-100) were incubated with p-nitrophenyl n-acetyl-β-D-glucosaminide in 0.1 M citrate buffer (pH 4.6) for 60 min at 37°C and neutralized with 400 mM glycine (pH 10.7). Subsequently, β-hexosaminidase release was measured as described previously.[54]

Cytokine analysis

For intracellular cytokine staining, PBMCs were stimulated with phorbol 12-myristate 13-acetate (50ng/mL) and ionomycin (1 μg/mL) in presence of Brefeldin A (10μg/mL) for 4 h, in some experiments PBMCs were pretreated with 10–40 μM digoxin or equivalent dimethyl sulfoxide (DMSO) for 24 h prior to stimulation and analyzed by flow cytometry. The cytokines from plasma were quantified with ELISA kits (eBiosciences) or Bio-Plex Pro™ Human cytokine kit (Bio-Rad Laboratories) according to manufacturer's instructions.

Flow cytometry reagents

Antibodies used for human Treg phenotyping, α-CD3 PerCP 5.5 (UCHT1), α-$CD4^+$ APCH7 (RPA-T4), α-CD25 V421 (M-A251), α-CD45RA V510 (HI100), α-CD127 BV786 (HIL-7R-M21), α-Foxp3 Ax488 (259D/C7), α-CD3 V421 (UCHT1), α-ICOS PE (DX29), α-HLA-DR PE (TU36), α-CD147 PE (HIM6), α-CXCR3 PE-Cy (1C6/CXCR3), α-CCR6 APC (11A9), α-CD38 APC (HIT2), α-IL-17A BV421 (N49–653), α-IL-13 BV421 (JES10–5A2), α-IFNγ PE-Cy7 (B27), α-TNFα APC (6401.1111), and Human FOXP3 buffer were from BD Biosciences. α-CD161 PerCP 5.5 (HP-3G10), α-RORγt PE (AFKJS−9), and α-IL-6 APC (MQ2–13A5) were from eBiosciences. α-RORγt PerCP (600380), and α-LAG3 APC (FAB2319A) was from R&D Systems. α-LAP PE (TW4–6H10), α-TNFRII PE (3G7A02), α-PD-1 PE-Cy7 (EH12.2H7), α-CD39 PE-Cy7 (A1), α-CD103 PE-Cy7 (Ber-ACT8), α-OX40 PE-Cy7 (Ber-ACT35), α-CCR7 PE-Cy7 (G043H7), α-Helios APC (22F6), α-CTLA-4 APC (L3D10), α-CCR4 APC (L291H4), and α-IL10 PE-Cy7 (JES3–9D7) were from BioLegend. All flow cytometry data was acquired with BD LSR Fortessa™ and analyzed using FlowJo™ version 10 (TreeStar Inc.).

Statistics

p values were calculated using a two-tailed, non-parametric, Wilcoxon signed rank test or Mann–Whitney test (Wilcoxon rank-sum test) for respective paired and unpaired groups. Error bars represents mean ± SEM, horizontal line represent median, and each dot represents one donor. $p \leq 0.05$ was considered statistically significant. All statistical values were generated using GraphPad Prism version 6.

Contributors

S.C. and E.M.A. designed the experiments; S.C. conducted and analyzed the experiments. S.C., K.T., E.M.A. interpreted the

data. S.C. wrote the manuscript together with E.M.A. K.T. and E.M.A. obtained funding, supervised the research and edited the manuscript; H.H., K.J.L., and G.W. provided the patient materials and together with P.D.L contributed to data analysis and interpretation. M.H. did some of the cytokine quantification experiments. All authors reviewed the manuscript and approved the final version.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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