Immunotherapy Converts Nonimmunogenic Pancreatic Tumors into Immune Foci of Immune Regulation

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) is considered a "nonimmunogenic" neoplasm. Single-agent immunotherapies have failed to demonstrate significant clinical activity in PDAC and other "nonimmunogenic" tumors, in part due to a complex tumor microenvironment (TME) that provides a formidable barrier to immune infiltration and function. We designed a neoadjuvant and adjuvant clinical trial comparing an irradiated, granulocyte-macrophage colony-stimulating factor (GM-CSF)–secreting, allogeneic PDAC vaccine (GVAX) given as a single agent or in combination with low-dose cyclophosphamide to deplete regulatory T cells (Treg) as a means to study how the TME is altered by immunotherapy. Examination of resected PDACs revealed the formation of vaccine-induced intratumoral tertiary lymphoid aggregates in 33 of 39 patients 2 weeks after vaccine treatment. Immunohistochemical analysis showed these aggregates to be regulatory structures of adaptive immunity. Microarray analysis of microdissected aggregates identified gene-expression signatures in five signaling pathways involved in regulating immune-cell activation and trafficking that were associated with improved postvaccination responses. A suppressed Treg pathway and an enhanced Th17 pathway within these aggregates were associated with improved survival, enhanced postvaccination mesothelin-specific T-cell responses, and increased intratumoral Teff:Treg ratios. This study provides the first example of immune-based therapy converting a "nonimmunogenic" neoplasm into an "immunogenic" neoplasm by inducing infiltration of T cells and development of tertiary lymphoid structures in the TME. Post-GVAX T-cell infiltration and aggregate formation resulted in the upregulation of immunosuppressive regulatory mechanisms, including the PD-1–PD-L1 pathway, suggesting that patients with vaccine-primed PDAC may be better candidates than vaccine-naïve patients for immune checkpoint and other immunomodulatory therapies. Cancer Immunol Res; 2(7); 1–16. ©2014 AACR.
with resected and metastatic PDAC (12, 14–18). Mesothelin is an antigen expressed by virtually all PDACs, and posttreatment detection of enhanced mesothelin-specific T-cell responses in peripheral blood lymphocytes (PBL) is associated with improved disease-free survival (DFS) and overall survival (OS) in GVAX-treated patients (12, 16–18). Despite evidence of peripheral immune activation and antitumor activity in some patients, immune tolerance mechanisms within the tumor microenvironment (TME) likely inhibit the full potential of vaccines alone (13). Thus, measures of peripheral immune activation following treatment with immunotherapy may not represent the immune activation status within the TME.

Tumors evolve numerous mechanisms to escape immune recognition (19). For PDAC, suppressive monocyes, including dendritic cells (DC), neutrophils, and myeloid-derived suppressor cells (MDSC), immune checkpoints (CTLA-4 and PD-1), and CD4+CD25+FoxP3+ Tregs have been reported in preclinical and clinical studies (13). Tregs have been found infiltrating the TME of many human tumors, including PDAC, and elevated Treg numbers are generally associated with shorter patient survival (6, 20–23). Previous studies have suggested that Tregs can be depleted with immune-modulating doses of cyclophosphamide to enhance immunotherapies (24–28). We previously reported the induction of higher avidity mesothelin-specific T-cell responses in the periphery of patients with metastatic PDAC when low-dose cyclophosphamide is given 1 day before vaccination (16). Furthermore, our preclinical studies suggest that cyclophosphamide primarily affects subsets of Tregs found infiltrating tumors, and that studying peripheral Tregs does not provide insight into the mechanisms by which Tregs regulate immune responses within the TME (29). However, the effect of cyclophosphamide on intratumoral Tregs and other immune-cell populations within human cancers has not been well studied.

In this study, we tested the hypothesis that vaccine-based immunotherapy can convert PDACs from "nonimmunogenic" into "immunogenic" tumors with infiltrating effector lymphocytes. We evaluated the effects of GVAX, given alone or in combination with Treg-modulating doses of cyclophosphamide, on lymphocytes infiltrating PDAC tumors. Neoadjuvant therapy was initiated 2 weeks before surgical resection to enable the direct assessment of the TME following treatment. Here, we show for the first time that an immune-based therapy induces the development of tertiary lymphoid aggregates within this "nonimmunogenic" neoplasm that resemble ectopic lymph node-like structures observed in subsets of immunotherapy-naïve patients with more "immunogenic" cancers such as melanoma and NSCLC (30–33). The development of intratumoral tertiary lymphoid aggregates in PDAC tumors was dependent on vaccination, supporting GVAX as a trigger of antigen-specific immune responses at the tumor site. Microdissection and gene array analyses demonstrated that decreased Treg and increased Th17 immune effector signatures within the vaccine-induced intratumoral lymphoid aggregates were associated with enhanced systemic postvaccination mesothelin-specific T-cell responses, higher intratumoral Teff:Treg ratios, and longer patient survival. However, as has been observed for melanomas (34, 35) that are naturally infiltrated with T cells, the T-cell infiltration following treatment with GVAX was also associated with the upregulation of immunosuppressive regulatory mechanisms within the PDAC TME that can be targeted by immune modulation. This process has been termed adaptive resistance. Thus, these data support a new model for developing immunotherapy approaches in tumors lacking natural T-cell infiltration.

Materials and Methods

Study subjects and tissue specimens

Between July 2008 and September 2012, 59 patients were enrolled into an ongoing study (NCT00727441) of an irradiated, allogeneic granulocyte-macrophage colony-stimulating factor (GM-CSF)–secreting pancreatic tumor vaccine (GVAX) administered intradermally either alone or in combination with immunomodulatory doses of cyclophosphamide as neoadjuvant and adjuvant treatment for patients with resectable PDAC. Patients were randomized 1:1:1 into three treatment arms (Supplementary Fig. S1, study schema). In arm A, patients received GVAX alone; in arm B, patients received GVAX plus a single intravenous dose of cyclophosphamide at 200 mg/m² 1 day before each vaccination; in arm C, patients received GVAX plus oral cyclophosphamide at 100 mg once daily for 1 week on and 1 week off (36). Up to six GVAX treatments were administered and all the patients remained in their initial treatment arms throughout the duration of the study. All 59 patients received the first GVAX treatment 2 weeks ±4 days before surgery. Fifty-four patients successfully underwent pancreatec- toduodenectomy (the Whipple surgery) and received the second GVAX treatment. Five patients were found to have liver metastases during surgery, which were not radiographically identified before the surgery, and instead these patients underwent bypass surgery. Among the 54 patients who had pancreatecduodenectomy, 1 patient was found to have ampullary cancer, 1 to have neuroendocrine tumor, 2 to have undifferentiated carcinoma, and 1 to have autoimmune pancreatitis. These patients’ preoperative CT scans did not distinguish their disease process from PDAC. In addition, 1 patient had grossly residual tumors and another 9 patients had recurrence immediately following surgery. All of these patients were taken off the study postoperatively. The 39 patients remaining on the study received standard adjuvant chemo- and radiotherapy. Patients remaining disease free following chemoradiotherapy received up to four additional PDAC GVAX treatments every 4 weeks. A full clinical report, including survival analyses, will be submitted at the completion of the study.

Tumor specimens from the 39 patients who remained in the study and continued with adjuvant chemotherapy were analyzed in this study. Formalin-fixed paraffin-embedded (FFPE) tissue blocks were obtained from our pathology archive. For most tumors, a piece was also stored frozen at –80 °C in optimal-cutting-temperature (OCT) freezing medium. PDAC tumor specimens collected before vaccine exposure from the 54 subjects treated in a previous adjuvant...
vaccine study (17) and from 4 unvaccinated patients who underwent surgery concurrently at our institution were obtained from an Institutional Review Board (IRB)–approved retrospective database and used as controls in this study.

Isolation of PDAC tumor-infiltrating lymphocytes

After keeping adequate tissue for surgical pathology diagnosis, the majority of the remaining tissue was processed for isolating tumor-infiltrating lymphocytes (TIL). The spared portion of each tumor was digested with enzymes into single-cell suspensions as described previously (15). Lymphocytes were isolated from these tumor-digested single-cell suspensions by Percoll density gradient centrifugation and stored frozen at −140° C. To isolate TIL subpopulations, TIL were thawed and labeled with anti-human CD3-PE, anti-human CD4-PECy5, anti-human CD8-APC, and anti-human CD19-FITC (all monoclonal antibodies were from BD Pharmingen). CD3+CD4+ cells (CD4+ T cells), CD3+CD8+ cells (CD8+ T cells), CD3+CD19+ cells (B cells), and CD3+CD4+CD8+CD19+ cells (non-T or -B cells) were isolated by fluorescence-activated cell sorting (FACS) using a Beckman Coulter MoFlo FACS sorter. Dead cells were excluded using LIVE/DEAD Fixable Blue Dead Cell Stain (Invitrogen).

Immunohistochemistry

Immunohistochemistry (IHC) was performed on FFPE 5-μm sections of resected PDAC. Immunostaining for CD1a, CD3, CD4, CD8, CD20, CD56, CD68, and CD163 was performed on Ventana autostainer using Ultra-view detection (Ventana Medical Systems, Inc.). The protocols and sources of antibodies are summarized in Supplementary Table S1. Staining for IL17A and RORγt was performed on Leica auto stainers using Bond Refine detection (Leica Microsystems). For double staining of IL17A and RORγt, Chromplex detection was used as per the manufacturer’s guidelines (Bond Leica, Leica Microsystems). For some protocols, antigen retrieval was performed manually as indicated. PD-L1 staining was performed as previously described (37). Following standard protocol, slides were deparaffinized, hydrated, and heat-induced antigen retrieval was performed. Incubation with the primary antibody using optimal conditions was followed by development of immunostaining. Counterstaining with hematoxylin and eosin (H&E) was applied, and slides were dehydrated and coverslipped.

Microarray analysis

Approximately 20 3-μm sections of FFPE tissue from each subject were anonymized and stained with H&E immediately before the lymphoid aggregates were microdissected by a pathologist (J. Wang) using a dissection microscope. The microdissected FFPE tissue was stored in RNA later solution (Ambion) until RNA was purified using the RecoverAll Total Nucleic Acid Isolation Kit for FFPE (Ambion) and amplified by the WT-Ovation FFPE System (NuGEN Technologies) as per the manufacturers’ protocols. Gene expression microarray analysis was performed using the Affymetrix Human U133 Plus 2.0 array chips. The microarray data have been submitted to the Gene Expression Omnibus (Accession number: GSE52171).

IHC image analysis

All slides were anonymized and scanned, and whole-slide images were analyzed using the Image Analysis Software (Aperio Technologies). The area of tumor to be analyzed was circled by a pathologist (R.A. Anders) on the anonymized H&E-stained slides, with attention to the inclusion of the largest area possible of continuous neoplastic tissue while excluding, to the extent possible, normal pancreatic, intestinal, and smooth muscle tissue. The Positive Pixel Count algorithm was used to quantify IL17 expression. The Cell Surface Count algorithm and the Nuclear Count algorithm were used to quantify the number of cells positive for other immune-cell surface or nuclear markers, respectively. The automatic quantification results were anonymized and validated first by manual quantification by a pathologist (R.A. Anders).

Detection of mesothelin-specific T-cell responses by IFNγ ELISPOT

The mesothelin peptide sequences and enzyme-linked ImmunoSpot (ELISPOT) assay used in this study have been described previously (17, 18). PBLs were isolated by density gradient centrifugation using Ficoll-Hypaque from peripheral blood collected at baseline, before, and following each Panc GVAX treatment. The postvaccination blood sample after the first vaccination was obtained either the day before surgery or on the day of surgery before anesthesia. Postvaccination blood samples for all subsequent vaccinations were obtained 1 month after each vaccination (Supplementary Fig. S1). Only PBLs from 27 of 39 patients expressed the HLA-A*0101 and/or HLA-A*0201 alleles and were analyzed by ELISPOT. T-cell responses to mesothelin peptides were adjusted for background measured against irrelevant melanoma or RCC control peptides. Responses were measured to eight HLA-A*0101 and six HLA-A*0201 mesothelin peptides. Postvaccination responses were considered enhanced when the frequency of mesothelin-specific T cells increased by at least 2-fold when compared with pretreatment baseline frequencies. Mesothelin-specific T-cell levels measured in 16 patients (8 responders and 8 nonresponders) whose microdissected lymphoid aggregates and/or TIL were used for gene-expression or FACS analysis are shown in Supplementary Fig. S2A and S2B.

Quantitative real-time PCR analysis

Quantitative real-time PCR was performed with the SYBR Green system (SABiosciences) as per the manufacturer’s protocol. Prevalidated PCR primers for each gene listed in Supplementary Table S2 were obtained from Real Time Primer, LLC, or SABiosciences. The expression levels of each gene were normalized to the same scale (1–4) as previously described (Supplementary Table S3; ref. 38). The average score for the set of genes representing each pathway was used to compare Th pathway gene expression between grouped patients’ TIL specimens.

Intracellular cytokine and FoxP3 staining analysis of TIL

Cryopreserved TIL isolated from 10 vaccinated patients treated in this study and 4 unvaccinated control subjects were analyzed. TIL were thawed and incubated at 37°C overnight.
either alone (“unstimulated”) or at a 1:1 ratio with human T-cell Activator CD3/CD28 Dynabeads (Invitrogen; “stimulated”) in Iscove’s Modified Dulbecco’s Medium (IMDM) supplemented with 5% FBS. After 16 hours, Protein Transport Inhibitor Cocktail (eBioscience) was added and the TIL were cultured for an additional 4 hours. Unstimulated and stimulated TIL were then harvested and surface stained with anti-CD3-PECy7 (BD Pharmingen), anti-CD4-PECF594 (BD Horizon), and anti-CD8-APC/780 (eBioscience), and intracellular staining was performed following permeabilization and fixation using eBioscience FoxP3/Transcription Factor Staining Buffers with anti-IFNγ-FITC (BD Pharmingen), anti-IL17A-PE (eBioscience), and anti-FoxP3-V450 (BD Horizon). Dead cells were excluded using LIVE/DEAD Fixable Aqua Dead Cell Stain (Invitrogen). Stained samples were analyzed using a Beckman Coulter Galios flow cytometer and FlowJo software (TreeStar, Inc.).

Statistical analysis
OS is defined as the time from surgery to death from any cause. Comparisons between groups were made using two-tailed unpaired Student t tests or Wilcoxon signed-rank tests. Graphing and statistical analyses were performed using either GraphPad Prism software (GraphPad Software) or SAS version 9.3 statistical software (SAS Inc.). Gene set enrichment analysis (GSEA) was performed according to the algorithm described by Subramanian and colleagues (39), using the analysis software provided by the Broad Institute (Cambridge, MA).

Results

Vaccine-based immunotherapy induces the neogenesis of intratumoral tertiary lymphoid aggregates that resemble ectopic lymph node–like structures

Thirty-nine patients with resectable PDAC were randomized to receive either GVAX alone, GVAX with one dose of intravenous cyclophosphamide 1 day before vaccine, or GVAX with daily oral cyclophosphamide for 1 week on and 1 week off (Supplementary Fig. S1). Patients underwent pancreaticoduodenectomy 2 weeks following GVAX treatment and the resected PDAC tumor specimens were analyzed histologically. The majority (33/39 or 85%) of tumors contained intratumoral lymphoid aggregates (Fig. 1A and B). In contrast, even though lymphocytes were sometimes present, organized intratumoral lymphoid aggregates like the ones observed in tumors from immunotherapy-naive patients with other cancers that are associated with longer survival (30–33). Consistent with these prior studies, there was an association between the formation of postvaccination lymphoid aggregates and longer survival (Supplementary Fig. S4). Because lymphoid aggregates formed in most (85%) vaccinated patients/tumors (Fig. 1B), not all PDAC tumors from patients who survived <1.5 years lacked intratumoral lymphoid aggregates (Supplementary Fig. S4). Thus, the induction of lymphoid aggregates alone did not accurately predict the postvaccination induction of a clinically effective antitumor response.

Postvaccination effector T-cell trafficking into the TME to form intratumoral lymphoid aggregates is associated with evidence of early T-cell activation, increased recruitment of Tregs, and upregulation of IFNγ and the PD-1–PD-L1 T-cell suppressive pathway

The postvaccination formation of intratumoral lymphoid aggregates suggests that treatment with GVAX triggers an adaptive T-cell response at the tumor site. To assess the activation status and functional polarity of T cells within these aggregates, we evaluated the expression of a number of T-cell markers by IHC. The lymphoid aggregates contained both CD45RA+naïve and CD45RO+ antigen-experienced T cells (Fig. 2A). CD45RA+ T cells were clustered in B-cell zones, whereas CD45RO+ T cells were more enriched in T-cell zones located in the periphery of the aggregates. These aggregates did not contain many granzyme B–expressing T cells (Fig. 2B), which were found frequently in lymphocytes outside of the aggregates (Supplementary Fig. S5). In contrast, the early T-cell activation marker CD69 and the activated T-cell trafficking chemokine receptor CXCR3 were abundantly expressed in the lymphoid aggregates (Fig. 2B). The aggregates also contained T-bet+ cells consistent with the induction of IFNγ-expressing effector T-cell responses (Fig. 2B). These data suggest that vaccine-induced aggregates are not major sites for cytotoxic T-cell lysis of target cells, but more likely are sites of initial T-cell activation within the TME.
Along with signals of effector T-cell trafficking and early activation, negative regulatory signals were also present in these aggregates. FoxP3-expressing Tregs were present in most aggregates (Fig. 2B). In addition, PD-L1, an inhibitory ligand that signals through the negative regulatory receptor PD-1 on activated T cells (42), was commonly expressed by cells in...
aggregates with morphologies consistent with monocytes/macrophages (Fig. 2C). These PD-L1⁺ cells were surrounded by lymphocytes that expressed PD-1. Clusters of PD-L1⁺ cells were also seen at the edges of intratumoral lymphoid aggregates where T cells tended to be located (Fig. 2D). In contrast, PD-L1⁻ expressing cells were rarely detected in PDAC tumors from vaccine-naive patients (Fig. 2E), supporting our hypothesis that PD-L1 expression is induced by vaccine treatment.

Some functional markers are difficult to assess by immunohistochemical analysis. To explore the functional activity of these aggregates, we next studied postvaccination TIL isolated from portions of the resected tumors. We compared IFNγ production by unstimulated TIL from vaccinated and unvaccinated patients by FACS. Analysis was limited to specimens from 10 vaccinated patients and 4 unvaccinated patients who had enough TIL recovered for the assays. Elevated numbers of IFNγ-producing T cells were detected in TIL from vaccinated patients (Fig. 3A). To evaluate whether Treg infiltration into tumors was affected by GVAX, we compared the levels of PDAC tumor-infiltrating Tregs between the same 10 vaccinated and 4 unvaccinated patients. As shown in Fig. 3B, the levels of CD3⁺CD4⁺FoxP3⁺ Tregs in TIL, quantified by FACS, were higher in vaccinated patients compared with that in unvaccinated patients. We observed a similar GVAX-induced increase in tumor-residing Tregs in a murine PDAC model (Soares and colleagues; submitted for publication). Thus, the activities of GVAX included both the recruitment of effector T cells into the TME and the upregulation of immunosuppressive regulatory mechanisms, specifically the expression of PD-L1 and Treg infiltration.

To estimate the net impact of the simultaneous infiltration of both effector T cells and Tregs in the TME, we compared the ratios between the number of IFNγ-producing effector T cells to the number of CD4⁺FoxP3⁺ Tregs, both measured by FACS directly in TIL without stimulation. Higher Teff:Treg ratios measured by CD8⁺/Foxp3⁺ T-cell ratio in TIL in untreated patients have been reported to correlate with better prognosis in multiple human cancers, including PDAC (3, 43–45). We found that the ratios of IFNγ-producing Teff:Tregs were...
Figure 3. Postvaccination effector T-cell (Teff) activation in intratumoral lymphoid aggregates is associated with Treg infiltration into the TME. Percentages of IFNγ-producing CD4+CD8+ effector T cells (A) and CD3+CD4+FoxP3+ Tregs (B) quantified by FACS in unstimulated TIL isolated from 10 vaccinated patients compared with those isolated from 4 unvaccinated patients. C, IFNγ-producing Teff:Treg ratios measured in unstimulated TIL from the same sets of vaccinated and unvaccinated patients. Comparisons between vaccinated and unvaccinated TIL were made using Wilcoxon signed-rank tests, and the calculated $P$ values are shown.

higher in 7 of the 10 vaccinated patients with over a log-fold increase in 5 of these 7 vaccinated patients when compared with unvaccinated patients (Fig. 3C). These data suggest that GVAX can alter the balance of Teff to Tregs in favor of an antitumor response.

**Specific gene expression signatures in intratumoral lymphoid aggregates define regulatory structures that correlate with immune responses and OS**

To evaluate possible mechanisms regulating vaccine-induced lymphoid aggregates, and to assess whether particular functional immune signatures within these aggregates were associated with response to treatment, we first performed gene microarray analysis on RNA isolated from microdissected lymphoid aggregates (Supplementary Fig. S6). Gene expression was compared among samples grouped according to three different correlates of vaccine response: patient OS, postvaccination mesothelin-specific T-cell responses in PBL (17), and the intratumoral CD8+ Teff to FoxP3+ Treg ratio. Mesothelin-specific CD8+ T cells specific for six HLA-A2–binding and eight HLA-A1–binding mesothelin epitopes were measured using IFNγ ELISPOT assays as described previously (17). Mesothelin-specific T-cell levels measured before and 12 to 14 days following the first vaccination just before surgery are shown for 16 patients who were either HLA-A1+ or -A2+: 8 responders who developed enhanced (at least 2-fold higher than baseline) postvaccination mesothelin-specific T-cell responses and 8 nonresponders who did not (see Supplementary Fig. S2A and S2B, respectively). The postvaccination levels of mesothelin-specific T cells were 2.6- to 54-fold higher than baseline in responders compared with 0.97- to 57-fold lower than baseline in nonresponders.

Gene ontology analysis of the microarray data revealed that genes involved in regulating immune-cell trafficking and function were differentially expressed, including genes encoding multiple integrins, chemokines and chemokine receptors, members of the NF-κB pathway, and the ubiquitin–proteasome system (UPS; Supplementary Fig. S7A–S7D, respectively). These pathways are involved in regulating both adaptive and innate immune cells. A few themes among the signatures were present. Specifically, chemokine and integrin signals involved in recruiting immunosuppressive populations were downstreamed in patients who had longer survival. These include the chemokines CXCL9, CCL18, CCL19, and CCL21, and the chemokine receptor CCR7 involved in recruiting Tregs (34, 46–48), chemokine CCL2 involved in recruiting immunosuppressive populations of monocytes/macrophages (49, 50), and the integrins ITGA4 and ITGB1 that are expressed by the suppressive MDSCs (Fig. 4A; ref. 51). A specific NF-κB signature defined by the downregulation of canonical NF-κB genes (NFκB1, RELA, and REL) combined with the upregulation of noncanonical NF-κB genes (NFκB2 and RELB) was associated with improved postvaccination responses (Fig. 4B). Although NF-κB is a known regulator of all immune subsets, this signature is consistent with polarization toward Th17 and away from Tregs (52, 53). The UPS signature may also represent both adaptive and innate immune signals but the underlying mechanisms of this signature are less clear. However, it is interesting to note that the downregulation of the UBE2N gene that encodes an E2 ubiquitin-conjugating enzyme important for maintaining Treg function and preventing Tregs from acquiring effector T cell-like function (54) was specifically associated with longer survival and higher intratumoral Teff:Treg ratios (Fig. 4B). In addition, multiple genes involved in the Treg pathway were downregulated, whereas multiple genes in the Th17 pathway were upregulated in lymphoid aggregates from patients who survived longer and developed enhanced immune responses (Fig. 4C and Supplementary Fig. S8). In contrast, the expression of Th1 and Th2 effector pathway genes was not uniformly upregulated or downregulated (Fig. 4C). Gene set enrichment analysis (GSEA; ref. 39) of the microarray data further supported the hypothesis that the differences in gene expression in the Treg and Th17
pathways, but not in the Th1 or Th2 pathways, in these aggregates may predict clinical and immune responses (Fig. 5).

Because PD-L1 protein expression was shown by IHC to be induced by vaccination, we next compared PD-L1 (CD274) and PD-1 (PDCD1) gene expression with respect to OS, enhanced T-cell responses, and Teff:Treg ratios (Fig. 4D). CD274/PD-L1 expression was downregulated in patients who survived >3 years, whereas there was no correlation with enhanced T-cell responses and Teff:Treg ratios. In contrast, PDCD1/PD-1 expression was increased in lymphoid aggregates from patients who survived >3 years and who demonstrated enhanced mesothelin-specific T-cell responses. Because PD-1 upregulation was associated with improved survival and immune responses, it may be a marker of postvaccination

Figure 4. Gene expression signatures in lymphoid aggregates associated with immune responses and OS. Gene expression measured by microarray was compared between microdissected lymphoid aggregates from 14 tumors grouped according to overall patient survival (OS > 3 years vs. <1.5 years), postvaccination induction of enhanced mesothelin-specific T-cell responses in PBL (Enh vs. Unenh), and the intratumoral CD8+ T effector (Teff)/FoxP3+ Treg ratio (high vs. low CD8:Foxp3 ratio). Gene ontology analysis revealed the enrichment of differentially expressed genes encoding numerous chemokine and chemokine receptor, integrin and adhesion molecule, NF-κB pathway, and UPS components (Supplementary Fig. S6). Differences in the Treg and Th17 pathway genes, but not the Th1 or Th2 pathway genes, were also associated with improved postvaccination responses and survival (Supplementary Fig. S7). The linear fold-change in expression for each comparison is shown for representative genes in chemokine, chemokine receptor, and integrin genes (A), NF-κB pathway genes (B), Th pathway genes (C), and PD-1 and PD-L1 (D).
T-cell activation in these aggregates, independent of its role in downregulating antitumor T-cell responses when engaged by its ligand, PD-L1.

Decreased Tregs are associated with enhanced Th1/Th17 signals in lymphoid aggregates and prolonged survival following vaccine therapy.

Microarray analysis of microdissected intratumoral lymphoid aggregates was performed in a subset of vaccinated patients and IHC was used to further evaluate the in situ expression of Th pathway genes in a larger cohort of patients. We examined the expression of representative transcription factors known to drive Th1 (T-bet), Th2 (GATA-3), Treg (Foxp3), and Th17 (RORγt) differentiation. As shown in Fig. 6A–D, T-bet and RORγt protein expression was increased, whereas Foxp3 expression was decreased in the immune aggregates from patients who survived longer. No difference in GATA3 expression was observed. Co-staining of RORγt and
IL17A (Fig. 6E) confirmed that RORγt+ cells also expressed IL17A, although many IL17A+ T cells did not express RORγt, likely because Th17 cytokine expression is also regulated by other transcription factors (55). Depending on the cytokine milieu, Th17 cells can also express T-bet and possess Th1-like effector functions (56), but it is not yet clear whether T-bet+ cells in these lymphoid aggregates were producing Th1- or Th17-like cytokines, or both. Regardless of the specific transcription factor(s) driving IL17A expression, we found that IL17A protein expression increased significantly in lymphoid aggregates from patients who survived >3 years compared with those who survived <1.5 years (Fig. 6F).

**Signatures within TIL reveal reduced Tregs but no differences in Th1/Th2/Th17 gene expression in association with survival**

We next evaluated the gene expression in TIL isolated from PDAC tumor specimens to determine whether the same T-cell signatures in the aggregates were also present in TIL. TIL were sorted from 20 PDAC TIL specimens for the analysis of gene expression in individual CD4+, CD8+, and non-T cell subpopulations. Consistent with signatures in the aggregates, Th1 and Th2 pathway gene expression was not associated with any parameters of response (Supplementary Table S3), and Treg pathway gene expression was decreased in CD4+ TIL from patients with longer survival (Supplementary Table S3 and Supplementary Fig. S9A). However, unlike signatures in the aggregates, Th17 pathway gene expression in CD4+ TIL and CD8+ TIL was not different between patients who survived >3 years and those who survived <1.5 years (Supplementary Fig. S9B and S9C, respectively, and Supplementary Table S3). This result may not be surprising as some key Th17 pathway cytokines, such as IL23A, are not expressed by T cells but by other cell types (Supplementary Table S4) that are abundant in the aggregates. However, the majority of TIL specimens also did not have detectable levels of IL17 expression (Supplementary Table S4). These data suggest that gene expression signatures in the total population of T cells that infiltrate tumors are similar, but not identical to gene signatures in T cells within the lymphoid aggregates. Furthermore, these data suggest that IL17A-producing T cells represent a relatively small population of T cells within the TME that are enriched in lymphoid aggregates of vaccine responders. In support of this notion, only 0.124% to 2.27% of CD4+ TIL and 0% to 0.410% of CD8+ TIL from vaccinated patients produced IL17A following overnight stimulation with anti-CD3- and anti-CD28-coated beads (Supplementary Fig. S10). Taken together, these data suggest that vaccine-induced Th17 signatures reside mainly within the lymphoid aggregates.
Figure 7. Low-dose cyclophosphamide (Cy) reduces intratumoral Treg numbers and promotes enhanced T-cell trafficking and activation within the tertiary lymphoid aggregates. A, comparison of the densities of Foxp3⁺ T cells in lymphoid aggregates between patients receiving GVAX alone (GVAX) and patients receiving GVAX plus Treg-modulating doses of cyclophosphamide (Cy+GVAX). B, IHC staining of Foxp3, T-bet, CD45RA, and CD45RO in representative lymphoid aggregates in PDACs from patients with high Teff:Treg ratios in the TME compared with those with low Teff:Treg ratios in the TME. C, IHC staining of CD69 and CXCR3 in representative PDAC tumor areas from patients with high versus low Teff:Treg ratios in the TME. All positive IHC signals are in brown. D, proposed model of vaccine-induced T-cell infiltration into the PDAC TME. At baseline, the "nonimmunogenic" PDAC TME is primarily immunosuppressed and infiltrated with Tregs and other immunosuppressive populations of innate immune cells, and low numbers of effector T cells. Vaccination induces antigen-specific T cells systematically that traffic to the TME and initiate an inflammatory reaction. (Continued on the following page.)
LOW TREG NUMBERS IN LYMPHOID AGGREGATES ENABLE ENHANCED T-CELL ACTIVATION AND TRAFFICKING WITHIN PDAC TUMORS

Because decreased Treg signatures in the aggregates and TIL are associated with improved postvaccination responses, and the intended purpose of low-dose cyclophosphamide is Treg depletion, we next evaluated by IHC the effects of cyclophosphamide on the number of Tregs in vaccine-induced aggregates. A trend toward increased numbers of aggregates infiltrating PDACs was observed in patients treated with GVAX plus cyclophosphamide (Supplementary Fig. S11A). Although the overall numbers of CD4⁺ T cells were not different (Supplementary Fig. S12), fewer Foxp3⁺ Tregs were detected in the aggregates from patients treated with GVAX plus cyclophosphamide versus with GVAX alone (Fig. 7A). Because both forms of cyclophosphamide used to deplete Tregs showed similar results, the two cyclophosphamide groups were combined for the analyses. Even though we only evaluated TIL from 10 treated patients (Fig. 3C), consistent with the IHC data, CD3⁺ CD4⁺ Foxp3⁺ Treg levels measured in TIL by FACS were also lower in patients who received GVAX plus cyclophosphamide versus GVAX alone (Supplementary Fig. S11B). These data suggest that cyclophosphamide can lower Treg levels in the TME, and Tregs may inhibit lymphoid aggregate formation.

To assess the impact of Teff:Treg ratios on T-cell activation within the TME, we evaluated the expression of T-cell activation markers in tumors with low and high Teff:Treg ratios defined by IHC. Teff:Treg ratios were considered high if they were above 10, and low if they were below 5. Increased numbers of CD45RO⁺ antigen-experienced and T-bet⁺ T cells and decreased numbers of CD45RA⁻ naive T cells were detected in lymphoid aggregates from vaccinated patients who had higher Teff:Treg ratios in their TME (Fig. 7B). In patients with higher Teff:Treg ratios, greater numbers of activated lymphocytes expressing CD69 and CXCR3 were also found trafficking outside of lymphoid aggregates into the stroma surrounding neoplastic cells (Fig. 7C). Thus, these data suggest that treatment with cyclophosphamide skews the postvaccination intratumoral Teff:Treg ratio and promotes effector T-cell activation and trafficking within the TME.

Discussion

Here, we report the first description of tertiary lymphoid aggregates infiltrating human PDAC. These aggregates develop 2 weeks after a single treatment with a PDAC vaccine. These novel structures express TME signatures that describe previously unrecognized foci of immune regulation within PDAC, uncover PDAC signaling pathways associated with long-term survival, and identify potential targets for collaborative immune modulation in the setting of a PDAC vaccination. Taken together, these lymphoid aggregates provide the first demonstration that an immune-based therapy can convert a "nonimmunogenic" neoplasm into one that is similarly "immunogenic" to melanomas and RCCs.

Tertiary lymphoid structures play a critical role in numerous autoimmune and inflammatory diseases (57). Different from primary and secondary lymphoid structures, their formation depends on antigen stimulation and represents an ongoing adaptive immune response. However, their role in cancer development and cancer therapy is not clear. In immunotherapy-naïve patients with colon cancer, NSCLC, and melanoma, increased numbers of lymphoid structures have correlated with better prognoses (31–33) and may be useful for selecting patients who are better candidates for immunotherapy (31). However, whether these structures are formed in response to tumor-antigen stimulation or to the pathogens and inflammatory conditions to which they are often exposed is unknown. Furthermore, the anticancer role of these structures is not established. In fact, under chronic inflammatory conditions, similar lymphoid structures are also associated with cancer development (58).

We have established for the first time that GVAX can induce similar intratumoral tertiary lymphoid structures not naturally formed in PDAC. Our data support the notion that these structures are newly formed on the basis of the expression of neogenesis markers within these structures together with antigen-experienced and activated T cells not commonly found in untreated PDACs. Whether these tertiary lymphoid structures are unique to GVAX or can be elicited by other forms of immune-based therapy is not yet known. Similar to reports in the literature (32, 33), these structures were associated with prolonged survival in some but not all GVAX-treated patients. This is not surprising as our analyses support their role as organized structures of immune regulation. Evidence supporting their role as regulators includes the expression of early markers of T-cell activation, IFNγ, as well as the recruitment of suppressive cell populations and the upregulation of T-cell–suppressing regulatory pathways. It is possible that the (Continued)
formation of lymphoid aggregate results from the activation and re-organization of immune cells present in the TME before vaccination. Because GVAX is delivered intradermally and not at the tumor site, presumably additional immune cells may traffic into the tumors following vaccination. In this study, we did not obtain prevaccination tumor biopsies because it is not the standard of care at our institution. For future studies, collection of pretreatment tumor biopsies will be necessary to evaluate changes in the TME following vaccination and to elucidate the mechanisms driving the development of post-vaccination lymphoid aggregates. Additional studies will be required to determine whether T cells that are activated within the lymphoid aggregates migrate directly into the tumor tissue or reenter the circulation first. We have assumed that mesothelin-specific T cells measured in the PBL are activated in the vaccine-draining lymph nodes. However, it is possible that some mesothelin-specific T cells are actually activated within the aggregates. In fact, it is possible that different routes can be used to enter the tumor depending on whether a T cell is activated in the periphery or within a lymphoid aggregate.

In some PDACs, the lymphoid aggregates were dominated by immunosuppressive signals, such as IL6, STAT3, and TGFβ, while others had proinflammatory signals, such as IL12, IFNγ, and IL17, which are associated with activated T-cell responses. The immunosuppressive signaling proteins PD-1 and PD-L1 were present in all intratumoral lymphoid aggregates. Because IFNγ can induce PD-L1 expression (34, 35), it is likely that the PD-L1 expressed in the intratumoral lymphoid aggregates was induced by the IFNγ produced by the lymphoid aggregate-residing CD4+ and CD8+ T cells. This could explain partially why IFNγ gene expression was lower in lymphoid aggregates in patients who demonstrated improved postvaccination responses and longer survival. In contrast, PD-1 was upregulated in the lymphoid aggregates from patients who demonstrated improved survival and enhanced postvaccination peripheral T-cell responses. PD-1 is a marker of T-cell activation and likely requires the engagement with PD-L1 to confer a downregulatory signal to the T cell. However, PD-L1 was not naturally upregulated in treatment-naive patients, or in untreated mice bearing implanted PDAC tumors (Soares and colleagues; submitted for publication). Our data support prior reports that PD-L1 is upregulated by IFNγ-secreting T cells recruited into the TME (34, 35). However, patients with lower levels of PD-L1 within their lymphoid aggregates demonstrated longer OS, which is consistent with the increased antitumor effect observed in PDAC-bearing mice treated with GVAX in combination with PD-1/PD-L1-targeted immune modulation (Soares and colleagues; submitted for publication).

This study also aimed to address the role of Tregs in altering T-cell responses within the TME. Prior reports from our group and others have shown that Treg infiltration in PDAC and other cancers is associated with T-cell suppression within the TME in human patients and in mouse models (16, 21–29). Some reports have also correlated baseline Treg-infiltration with poorer prognosis in patients with PDAC (20, 43). Preclinical data have indicated that the Teff:Treg ratio, rather than the absolute numbers of Tregs infiltrating the TME, correlates with antitumor immunity (59). This is the first study conducted in human patients confirming that it is the balance between the infiltrating effector T cells and Tregs that correlates with treatment outcomes. This is also the first study to show in patients that cyclophosphamide given with vaccination does indeed alter the balance in favor of an effector T-cell response. However, not all patients who did not receive cyclophosphamide had a lower Teff:Treg ratio, supporting differences in the natural inflammatory composition of PDACs among patients, which will require further exploration.

The complex nature of the TME is demonstrated by the functional and signaling differences between the lymphoid aggregates and the TIL that were evaluated in a smaller subset of patients. The lymphoid aggregates expressed signals of T-cell activation and regulation; TIL expressed signals of activation predominantly. Notably, the aggregates expressed mixed Th1 and Th17 signals, whereas TIL mostly expressed Th1-like signals. In fact, differentially expressed genes in the five signaling pathways (Th17/Treg, NF-kB, ubiquitin–proteasome, chemokines/chemokine receptors, and integrins/adhesion molecules) within the intratumoral lymphoid aggregates were associated with improved postvaccination responses. These pathways have known effects on immune responses and the development of lymphoid structures. In particular, the activation of the noncanonical NF-kB pathway following lymphotoxin/lymphotoxin receptor engagement is critical for lymphoid structure development (60). Our results are consistent with prior studies showing that Tregs can suppress the development of tertiary lymphoid structures (61, 62), whereas cells with Th17 subsignatures can promote their development (63, 64). Procancer inflammatory signals, including IL6 and STAT3 (65) that are also positive regulators of Th17 responses (55, 66), were not upregulated in the vaccine responders. Our data suggest that the upregulation of the Th17 pathway is associated with improved responses to GVAX, but the exact role of IL17-producing cells in the postvaccination TME is not yet known. Given that Th17 cells can possess both Treg and Th1-like effector functions (56, 67–70), it is possible that particular Th17 subsignatures are correlated more closely with improved postvaccination immune responses (65, 71–73). Although it is not clear how the vaccine-induced lymphoid aggregates identified in this study compare with naturally occurring tertiary lymphoid structures, several of the 12 chemokines (including CCL2, CCL18, CCL19, CCL21, and CXCL9) that are associated with melanoma (31) were expressed but downregulated in postvaccination aggregates in patients who demonstrated prolonged survival and elevated intratumoral Teff:Treg ratios. These chemokines may affect the formation of vaccine-induced lymphoid aggregates by attracting both effector T cells and Tregs; our data suggest that at higher levels, these chemokines may favor Tregs and a more immunosuppressive TME. Additional studies are required to refine these signatures and determine which members of these pathways are critical for regulating the trafficking of lymphocytes into the tumor, the formation of lymphoid aggregates, and the evolution of the PDAC TME following vaccination.
On the basis of our findings, we propose a new model in which traditionally "nonimmunogenic" tumors can be converted into "immunogenic" tumors responsive to combination immune-based therapies. In this model (Fig. 7D), a systematically administered vaccine is required to initiate the induction of immune responses that can traffic into tumors and create an inflammatory environment. This environment has the potential to support an anticancer immune response, but it contains numerous immunosuppressive components, such as infiltrating Tregs and other suppressive cell populations, and the upregulation of PD-L1, which is a natural response to the initial inflammatory "invasion" of the TME. At this stage, the organized inflammatory infiltrates are sites of immune regulation. In some patients, this initial inflammation is enough to shift the balance from immune suppression to immune activation, particularly when a vaccine is given with Treg-depleting therapy. However, in most patients, additional immune modulation is needed, which may include targeting MDSC, tumor-associated macrophages (TAM), and tumor-associated neutrophils (TAN), to more effectively shift the balance from predominantly cumulative T-cell downregulatory signals toward a majority of T-cell–activating signals. Additional studies are required to determine the role of these immunosuppressive cell subsets in modulating the postvaccination TME.

On the basis of the evidence available, this model explains why vaccination and immune modulation as single-agent therapies have failed in patients with PDAC. This model is further supported by our recent study demonstrating objective responses in patients with metastatic PDAC treated with the combination of GVAX + ipilimumab but not with ipilimumab alone (12). Our model provides the strongest rationale so far for combining immuno-modulating agents with vaccination in patients whose tumors do not naturally contain abundant effector T cells.

Disclosure of Potential Conflicts of Interest
R.A. Anders has received commercial research support from Bristol-Myers Squibb. No potential conflicts of interest were disclosed by the other authors.

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