Targeting Cytokine Therapy to the Pancreatic Tumor Microenvironment Using PD-L1–Specific VHHs


Abstract

Cytokine-based therapies for cancer have not achieved widespread clinical success because of inherent toxicities. Treatment for pancreatic cancer is limited by the dense stroma that surrounds tumors and by an immunosuppressive tumor microenvironment. To overcome these barriers, we developed constructs of single-domain antibodies (VHHs) against PD-L1 fused with IL-2 and IFNγ. Targeting cytokine delivery in this manner reduced pancreatic tumor burden by 50%, whereas cytokines fused to an irrelevant VHH, or blockade of PD-L1 alone, showed little effect. Targeted delivery of IL-2 increased the number of intratumoral CD8+ T cells, whereas IFNγ reduced the number of CD11b+ cells and skewed intratumoral macrophages toward the display of M1-like characteristics. Imaging of fluorescent VHH–IFNγ constructs, as well as transcriptional profiling, demonstrated targeting of IFNγ to the tumor microenvironment. Many tumors and tumor-infiltrating myeloid cells express PD-L1, rendering them potentially susceptible to this form of targeted immunotherapy.

Introduction

Targeted cytokine delivery to the tumor microenvironment can have a powerful effect on the immune landscape of tumors (1). Cytokines act in autocrine or paracrine fashion and have short half-lives. The concentration of cytokines at the right location is thus critical, yet the few cytokine-based therapies used in the clinic, such as IL-2 and IFNγ, are given systemically, often resulting in severe dose-limiting toxicities (1, 2). Efficacy of cytokine-based therapies is limited by an inability to deliver them to the proper location and an incomplete knowledge of the effects of particular cytokines in various cancer types.

The construction of antibody–cytokine fusions is an established preclinical approach to target cytokine therapy to the tumor microenvironment. However, the size of these adducts results in persistence in circulation and comparatively poor tissue penetra-

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on pancreatic ductal adenocarcinoma (PDAC), a tumor type unresponsive to checkpoint blockade (13, 17, 18).

PDAC is one of the deadliest cancers, with the 5-year survival rate of 8% (19). The disease is rapidly metastatic, and the majority of primary pancreatic tumors are inoperable due to invasion of the surrounding vasculature. Pancreatic tumors are dense, fibrotic masses that preclude adequate drug delivery and may limit accessibility for full-sized antibodies (20). The dense stroma creates a nutrient-poor, immunosuppressive environment. Approximately 60% of human PDAC tumors express PD-L1 (staining >10% by immunohistochemistry; ref. 21). The majority of immune cells, in both human tumors and mouse models, are cells of the myeloid lineage, with both granulocytic and monocytic myeloid-derived suppressor cells (MDSC), as well as tumor-associated macrophages (TAM) contributing to local immunosuppression. Many human and mouse PDAC tumors are devoid of CD8+ T-cell infiltrates at baseline, suggesting that T cells are either not primed against PDAC antigens, fail to reach the tumor at all, or are rendered nonfunctional due to early establishment of an immunosuppressive microenvironment (22). Strategies to reduce infiltration of myeloid cells or to reprogram these cells to an alternative fate can enhance CD8+ T-cell infiltration and synergize with checkpoint blockade therapy (23–25). Reprogramming of myeloid cells can lead to a restructuring of extracellular matrix and allow for more effective drug delivery (26, 27). Tumor cell death releases antigens and can further prime protective antigen-specific T-cell responses (28). Pancreatic tumors, even in the absence of neoantigens, contain self-antigens that, under certain circumstances, can be recognized by CD8+ T cells to mediate tumor regression (29, 30). We propose that effective therapy for pancreatic cancer will incorporate both myeloid reprogramming of myeloid cells can lead to a restructuring of extracellular matrix and allow for more effective drug delivery (26, 27). Tumor cell death releases antigens and can further prime protective antigen-specific T-cell responses (28). Pancreatic tumors, even in the absence of neoantigens, contain self-antigens that, under certain circumstances, can be recognized by CD8+ T cells to mediate tumor regression (29, 30). We propose that effective therapy for pancreatic cancer will incorporate both myeloid targeting and a T-cell response as part of a multigrounded regimen.

Here, we use an anti–PD-L1-VHH to target the tumor microenvironment. We show that radiolabeled anti–PD-L1-VHH accumulates in orthotopically implanted pancreatic tumors. We then generated fusions of the anti–PD-L1 VHH with IL-2 or IFNγ to direct these fusions to the tumor. Treatment with the IL-2 fusion showed a 50% reduction in overall tumor burden in the Panc02 model and an increase in the intratumoral ratio of CD8+ T cells to CD4+ Tregs, whereas the IFNγ fusion caused a profound reduction in the size of Panc02, RPC, and M19 (KPC organoid) orthotopic tumors, largely by reprogramming intratumoral macrophages.

Materials and Methods
Cloning and expression of B3-IL2
B3 and A12 are mAbs specific for PD-L1 (3). The B3-IL2 coding sequence was subcloned into the E. coli periplasmic expression vector pET22b, with the inclusion of a C-terminal sortase motif and His6 tag. BL21(DE3) E. coli containing the plasmid was grown in mid-log phase at 37°C in TB plus ampicillin and induced with 0.5 mmol/L IPTG overnight at 25°C. Cells were harvested by centrifugation at 5,000 × g for 15 minutes at 4°C, then resuspended in 25 mL 1× TES buffer (200 mmol/L Tris, pH 8, 0.65 mmol/L EDTA, 0.5 mol/L sucrose) per liter culture, and incubated for 1 hour at 4°C with agitation. Resuspended cells were then submitted to osmotic shock by diluting 1:4 in 0.25× TES and incubating overnight at 4°C. The periplasmic fraction was isolated by centrifugation at 8,000 rpm for 30 minutes at 4°C, and then loaded onto Ni-NTA (Qiagen) in 50 mmol/L Tris, pH 8, 150 mmol/L NaCl, and 10 mmol/L imidazole. Protein was eluted in 50 mmol/L Tris, pH 8, 150 mmol/L NaCl, 500 mmol/L imidazole, and 10% glycerol, then loaded onto a Superdex 75 16/600 column (GE Healthcare) in 50 mmol/L Tris, pH 8, 150 mmol/L NaCl, 10% glycerol. Recombinant VHH purity was assessed by SDS-PAGE, and peak fractions were recovered and concentrated with an Amicon 10,000 KDa MWCO filtration unit (Millipore), and stored at –80°C.

Cloning and expression of VHH–IFNy
The PD-L1–specific VHH A12- and B3-, and irrelevant specificity VHHCTR(1B7)-IFNy coding sequences were subcloned into the mammalian expression vector pVRC and transiently transfected using polyethyleneimine into HEK293F cells cultured in Freestyle media (ThermoFisher; ref. 3). Media containing secreted protein were harvested 6 days following transfection by centrifugation at 8,000 × g for 20 minutes at 4°C, then loaded onto a HiTrap NiNTA column (GE Healthcare) and washed with 50 mmol/L Tris, pH 8, 150 mmol/L NaCl, and 10 mmol/L imidazole. Protein was eluted in 50 mmol/L Tris, pH 8, 150 mmol/L NaCl, 500 mmol/L imidazole, and 10% glycerol, then loaded onto a Superdex 200 16/600 column (GE Healthcare) in 50 mmol/L Tris, pH 8, 150 mmol/L NaCl, 10% glycerol. Recombinant VHH purity was assessed by SDS-PAGE, and peak fractions were recovered and concentrated with an Amicon 10,000 KDa MWCO filtration unit (Millipore), and stored at –80°C.

C-terminal labeling of VHHs with biotin or Alexa647
A heptamutant (“7M”) variant of S. aureus sortase A, 7M-SrtA (10, 11), was used to label B3, B3-IFNy, or VHHCTR(1B7)-IFNy by incubating 30 μmol/L purified VHH protein with 5 μmol/L 7M-SrtA and 100 μmol/L GGK-biotin or GGK-Alexa647 nucleophiles in 50 mmol/L Tris, pH 8, 150 mmol/L NaCl for 2 hours at room temperature. Unreacted VHH and 7M-SrtA were removed by adsorption onto Ni-NTA agarose beads (Qiagen). The unbound fraction was concentrated and excess nucleophile with an Amicon 3,000 KDa MWCO filtration unit (Millipore) and stored at –80°C.

Lipopolysaccharide (LPS) removal
All therapeutics were depleted of LPS (<2 IU/mg) or purchased LPS-free from the manufacturer. To remove LPS, VHHs were immobilized on HiTrap HP 1 mL columns (GE Healthcare), in PBS, washed with 40 column volumes PBS + 0.1% TritonX-114, and eluted in 2.5 column volumes endotoxin-free PBS (Teknova) with 500 mmol/L imidazole. Imidazole was removed by PD10 column (GE Healthcare), eluting in LPS-free PBS. LPS content was tested using the LAL Chromogenic Endotoxin Quantiﬁcation Kit (Pierce) according to the manufacturer's instructions.

Animal care
Animals were housed at either the Whitehead Institute for Biomedical Research (B16 experiments) or the Dana-Farber Cancer Institute (pancreatic tumor experiments, CT26 experiments, and PET imaging) and were maintained according to protocols approved by the MIT Committee on Animal Care or the DFCI IACUC, respectively. C57BL/6 and RAG2−/− mice were purchased from The Jackson Laboratories.

Orthotopic pancreatic tumors
Orthotopic surgeries were performed as described (31). Briefly, C57BL/6 and RAG2−/− mice were anesthetized with a ketamine/
xylazine cocktail, shaved on the left flank, and the surgical site cleaned with ethanol and betadine. An incision was made in the skin and peritoneum, and the pancreas were externalized with forceps. Panco2, KPC cells, or KPC organoids (M19 line, kindly provided by David Tuveson, ref. 31) were resuspended in PBS and mixed 1:1 by volume with Matrigel (Corning) for a total of 100,000 cells per 30 μL. The cell suspension was kept on ice and drawn into a chilled insulin syringe. Cells were then injected into the tail of the pancreas, and a bubble was observed. Mice that showed signs of leakage were removed from the experiment. The pancreas was left external to the body cavity for 1 minute with the mice on a warming pad to solidify the Matrigel. The pancreas was then reinserted, peritoneum sutured with one stitch of absorbable suture, and the skin stapled with a sterile wound clip. Mice were given analgesia (Buprenex) and monitored after surgery according to protocols approved by the Dana-Farber IACUC. Mice were sacrificed 21 days after surgery unless otherwise indicated. Tumors were weighed at the time of sacrifice. In some cases, we combined multiple smaller experiments that were started at slightly different times. In these cases, we normalized tumor masses to the mean mass of the control tumors in order to compare across experiments. These values are reported as ‘relative tumor mass.’

**Tumor-infiltrate analysis**

Pancreatic tumors were excised, weighed, minced, and incubated in RPMI-containing collagenase and antitrypsin at 37 °C for 1 hour. Tumors were filtered through a 40-μm cell strainer, washed with PBS, and centrifuged. The resulting cell pellet containing tumor debris and infiltrating immune cells were resuspended in FACS buffer (PBS with 2% fetal calf serum) and stained with a master mix of antibodies. Cells were incubated with staining mix for 30 minutes at 4 °C, washed with PBS, and centrifuged. The resulting cell pellet was resuspended in FACs buffer (PBS with 2% fetal calf serum) and stained with a master mix of antibodies. Cells were incubated with staining mix for 30 minutes at 4 °C, washed once in PBS, and resuspended in 1% formalin prior to analysis on a BD Fortessa flow cytometer. All tumor infiltrates were first gated on CD45+ cells.

**Flow cytometry.** Cells were harvested from spleen, or draining lymph nodes were dispersed into PBS through a 40-μm cell strainer using the back of a 1-ml syringe plunger. Cell preparations were subjected to hypotonic lysis to remove erythrocytes, stained, and analyzed using a Flowcytometry (BD). Flow cytometry fluorescence dye-conjugated antibodies to the following proteins were purchased from Biolegend [B220-PacificBlue (clone RA3-682), CD107a-Fitc (1D4B), CD11b-AlexaFluorophore 488 (M1/70), CD11c-APC (N418), CD11c-PE/Cy7 (N418), CD25-AlexaFluorophore 488 (PC61), CD45-Bv711 (30-F11), CD4-APC (RM4-5), CD4-PacificBlue (RM4-5), CD69-PE (H1.2F3), CD8-Bv500 (53-6.7), FoxP3-PE (MF-14), Gr1-PE (RB6-8C5), Gr1-PE/Cy7 (RB6-8C5), MHC I-APC/PE-Cy5 (M5/114.15.2), NK1.1-FITC (PK136), NK1.1-PE/Cy7 (PK136), PD-1-PE/Cy7 (29.F1A12), Tim3-APC (RMT3-23), PD-L1-APC (10F.9G2)], and Allophycocyanin [CD19-PE (MB19-1)].

**Two-photon confocal microscopy.** After implantation of pancreatic tumors (after 7 days) organoid KPC (post 6 weeks; as described above) and 1 hour after intraperitoneal injection of B3-IFNγ or VHHCRT conjugated to Alexa Fluor 488, the entire pancreas bearing implanted tumors were harvested from adult mice and immediately fixed in phosphate PBS containing 1% paraformaldehyde, 1.5% t-lysine, 0.2% sodium periodate, and sodium dibasic 0.1 mol/L. NaHPO4 solution overnight at 4 °C on a rotisserie. Samples were washed three times for 30 minutes each at 4 °C in wash buffer (PBS, 0.2% BSA, 0.1% Triton × 100) and then blocked in blocking buffer (PBS, 0.5% BSA, 0.3% Triton × 100) containing 2.4G2 antibody to block nonspecific binding of immunoglobulin to Fcγ receptors. The tissue samples were stained for 3 days at 4 °C in blocking buffer containing DAPI, PE-conjugated anti-CD11b clone M1/70 (Biolegend; cat #101208) and Alexa Fluor 647–conjugated anti-CD31 clone 390 (Biolegend cat #102416). Samples were washed three times for 30 minutes each at 4 °C in wash buffer and incubated overnight in the same buffer at 4 °C. The whole pancreas bearing tumors were mounted on SuperFrost Plus Slides (VWR; cat # 48311-703) in Fluoroshield mounting medium (Abcam; cat #ab104135) and then covered with a number 1 cover glass (VWR; cat #48393081).

Pancreatic samples were imaged on an Ultima Two-Photon Microscope (Prairie Technologies/Bruker) equipped with two Tsunami Ti:sapphire lasers with a 10-W MileniaX pump laser (Spectra-Physics) and a 20 × /0.95-NA water-immersion objective (Olympus). The two-photon excitation wavelength was set between 815 and 826 nm for optimal fluorescence excitation. Fluorescence emission was detected with 665/65-, 590/50-, 525/50-, and 450/50-nm bandpass filters for 4-color imaging. Z-stacks of the pancreas were captured and displayed as maximum projection. Raw image sequences were processed using Imaris 7.4.2 software (Bitplane Scientific). Each image channel was assigned a pseudocolor according to emitted light wavelengths (bp 665/65 nm, cyan; bp 590/50 nm, green; bp 525/50 nm, red; bp 455/70 nm). Scale bars were either 100 μm or 5 μm in length and were displayed at the bottom right corner of the image.

**Synthesis of (Gly)3-Cys–NOTA.** (Gly)3-Cys–NOTA was synthesized as described (32, 33). In brief, maleimide–NOTA (macromolecules) was dissolved in 0.1 mol/L NaHCO3, pH 8.3. The tetrapeptide GGGC was added at room temperature for 30 minutes until LC-MS analysis indicated almost complete conversion to the product. The product was purified by RP-HPLC on a semipreparative column (C18 column, Gemini, 5 μm, 10 Å × 250 mm; Phenomenex) at a flow rate of 5.0 mL/min: solvent A, 0.1% TFA in H2O; solvent B, 0.1% TFA in CH3CN. The desired product eluted from 15% to 20% (vol/vol) solvent B. Fractions containing pure product were collected and lyophilized. LC-MS calculated for C23H34N6O2Cl15S (M+H)+ was 717.298; found = 717.305.

**Enzymatic incorporation of NOTA chelator onto single-domain antibodies using sortase.** The pentamutant sortase A, with an improved Kcat was used. Reaction mixtures (0.5 mL) contained Tris–HCl (50 mmol/L, pH 7.5), CaCl2 (10 mmol/L), NaCl (150 mmol/L), tritylglycine–NOTA (800 μmol/L), VHH (300 μmol/L), and sotase (5 μmol/L). After incubation at 4 °C with agitation for 1 hour, reaction products were analyzed by LC-MS, with yields generally >80%. When the yield was below 80%, the reaction was allowed to proceed for an additional hour, with addition of sortase to 10 μmol/L and tritylglycine–NOTA to 1 mmol/L. The reaction mixture was loaded into a prequillibrated PD-10 columns and eluted with 1× PBS, removing excess of NOTA substrate and GGHiis2 byproduct. Ni-NTA beads were added to the collected fractions with agitation for 5 minutes at 25 °C, followed by centrifugation to remove sotase and any remaining unreacted His-tagged VHH. The labeled VHH was...
stored at −20°C with 5% glycerol and was stable for at least six months.

**Synthesis of ⁶⁴Cu–VHHs.** ⁶⁴Cu–VHHs were synthesized as described before (34, 35). In brief, a 1.5 mL centrifuge tube was loaded with purified NOTA labeled-VHHH (50 µL, 1 mg/mL), PBS buffer (300 µL, pH 7.3), and ⁶⁴CuCl₂ (~1 mCi) in 200 mmol/L NH₄OAc buffer (75 µL, pH 6.5). The tube was sealed and shaken at 37°C for 20 minutes. The mixture was analyzed by radio TLC (ITLC, 50 mmol/L EDTA pH 7, Rf = 0.0) showing >95% conversion to ⁶⁴Cu–VHH. At this time, the mixture was loaded onto a PD-10 size-exclusion cartridge and elution with 1 x PBS provided radiolabeled ⁶⁴Cu–VHH, ready to be used for imaging. The amount injected to each mouse was in the range of 50 to 100 µCi.

**PET imaging.** ⁶⁴Cu–VHHs PET imaging was carried out on a dedicated small animal PET/CT scanner (Siemens Multimodality Inveon, Siemens Medical Solutions USA, Inc.). The mice were anesthetized using 2% sevoflurane/medical air inhalation prior to the radiotracer injection and throughout the scan duration. Warming was used to maintain healthy core body temperature of the mice during periods of unconsciousness. Following a bolus intravenous injection (via the lateral tail vein) of ⁶⁴Cu–VHH–B3 (~3.3 MBq), ⁶⁴Cu–VHH–A12, or ⁶⁴Cu–VHH–96GM (~3.3 MBq), and an uptake period of 120 minutes, a low dose CT scan was first acquired (80 kVp, 0.5 mA) for anatomical reference and to provide guidance for the delineation of selected tissue volume of interest (VOI). Static PET emission scans were then acquired (80 kVp, 0.5 mA) for 15 minutes and corrected for decay and dead time. The acquired data were then sorted into 0.5 mm bin size and time frame for image reconstruction using FORE/3D-OSEM-MAP image reconstruction. The reconstructed PET/CT images were analyzed with the Siemens Inveon Research Workplace software.

**Melanoma models**

B16 cells were purchased from ATCC. B16 GM-CSF and B16-ova cells were a gift from Glenn Dranoff (currently at Novartis Institute for Biomedical Research, Cambridge, MA). For in vivo challenge experiments, 5 × 10⁵ B16 cells were inoculated by subcutaneous injection in 500 µL of Hank’s Balanced Salt Solution (HBSS). For vaccinations, 5 × 10⁶ irradiated (3,500 rad) GM-CSF secreting B16 cells (GVAX), secretory injection in 250 µL HBSS. VHHs, anti-PD-L1 (10F.9G2, BioXCell), and TA99 (gift from K. Dane Wittrup) were administered in 200 µL LPS free PBS (TekNovo) by intraperitoneal injection or intravenous injection (via the lateral tail vein) of ⁶⁴Cu–VHH–B3 (~3.3 MBq), ⁶⁴Cu–VHH–A12, or ⁶⁴Cu–VHH–96GM (~3.3 MBq), and an uptake period of 120 minutes, a low dose CT scan was first acquired (80 kVp, 0.5 mA) for anatomical reference and to provide guidance for the delineation of selected tissue volume of interest (VOI). Static PET emission scans were then acquired (80 kVp, 0.5 mA) for 15 minutes and corrected for decay and dead time. The acquired data were then sorted into 0.5 mm size bins and time frame for image reconstruction using FORE/3D-OSEM-MAP image reconstruction. The reconstructed PET/CT images were analyzed with the Siemens Inveon Research Workplace software.

**Results**

**Anti-PD-L1 VHH can penetrate dense pancreatic tumors**

Pancreatic tumors have a dense stroma and are often poorly vascularized (20, 36–38). We inoculated either Panc02 cells or KPC cells (line derived from a K-rasLSL.G12D/+; p53R172H/+; PdxCre mouse, gift of A. Maitra) orthotopically into the pancreas (39). Orthotopic implants recapitulate many of the essential features of spontaneous pancreatic tumors, including the dense stromal infiltrate, the presence of suppressive myeloid cells, and poor vascularization (39). To assess whether the small size of VHHs would favor penetration into the stroma, we used a previously reported VHH against CD47, a widely expressed surface protein (6, 7). An anti–CD47–VHH (15 KDa) or a version of CD47–VHH fused to mouse IgG2a Fc (80 kDa) were conjugated to Alexa⁴⁸⁴ and injected intraperitoneally into mice bearing orthotopic KPC tumors (Fig. 1A). Tumors were resected 2 hours later and used to create a single-cell suspension, which was then stained with excess labeled reagent to determine the maximal attainable binding. The anti–CD47–VHH showed greater binding after in vivo injection.
than did the larger Fc fusion. We conclude that the small size of VHHs indeed favors penetration of dense pancreatic tumors (Fig. 1A).

To target the tumor microenvironment, we used high-affinity VHHs (B3 and A12), both of which recognize murine PD-L1 and inhibit interactions with its ligands, B7-1 and PD-1 (Fig. 1B and ref. 3). Both VHHs recognize PD-L1 with similar subnanomolar affinities, binding more strongly than the commercial mAb 10F.9G2 by ~100-fold and efficiently competing for binding with the mAb (Fig. 1B; ref. 3). B3 recognizes only mouse PD-L1, whereas A12 cross-reacts with human PD-L1. B3 recognizes IFNγ-inducible PD-L1 on multiple murine cancer cell lines (Supplementary Fig. S1; ref. 40). Consistent with its ability to block PD-L1 interactions with its targets, B3 augments in vivo cytotoxicity by OT-I cells against B16-OVA or by melanoma-specific TRP1 CD8 T cells cultured with B16 melanoma cells (Fig. 1C and D; Supplementary Fig. S2; ref. 41). B3 delays growth of B16 melanoma in vivo after vaccination with GVAX to a similar extent as the full-sized antibody against PD-L1 (Fig. 1E and Supplementary Fig. S3)

B3 binds to the pancreatic cancer cell lines Panc02 and KPC in vitro,

Figure 1.

PD-L1-blocking VHHs penetrate dense pancreatic tumors. A, C57BL/6 mice were inoculated orthotopically with KPC tumors. Fourteen days later, mice were injected i.p. with 100 μg of Alexa647 labeled A4 (VHH) or the molar equivalent of an Fc-conjugated version (VHH-Fc). Two hours after injection, tumors were harvested, and digested, and single-cell suspensions were either analyzed directly or incubated with excess labeled reagent prior to analysis by flow cytometry. In vivo staining is calculated as the percentage of maximum mean fluorescence intensity achieved by in vivo injection alone. B, Plate-bound PD-L1 was incubated with (left) anti–PD-L1-biotin in the presence of increasing concentrations of unlabeled VHH or antibody as indicated; right, B3-biotin incubated in the presence of increasing concentrations of unlabeled VHH or antibody as indicated. C, B16-ova cells treated for 18 hours with IFNγ (20 ng/mL) were washed to remove the IFNγ and were incubated with CD5/CD28 activated OT-I T cells and cultured for 48 hours in the presence of B3 or anti–PD-L1 as indicated. B16 survival was assessed using CellTiterGlo. D, IFNγ-treated B16 wild-type cells were incubated with activated melanoma-specific TRP1 cells for 48 hours with VHH or antibody as indicated. Tumor cell survival was measured by CellTiterGlo. E, C57BL/6 mice inoculated with B16 and were vaccinated with GM-CSF secreting B16 cells (GVAX) on day 0 and treated every other day with 250 μg B3, anti–PD-L1, or CTR as indicated. F, Panc02 or KPC cells either from tissue culture or isolated from resected orthotopic tumors were stained with B3-Alexa647 and analyzed by flow cytometry. Gray histograms, staining with VHHCTR-Alexa647. G, Mice bearing orthotopic KPC (n = 2) or Panc02 (n = 2) tumors were injected i.v. with 80 μCi 64Cu-B3 (top) or 80 μCi 111In-A12 or 111In-VHHCTR (bottom). Mice were imaged 2 hours later, sacrificed, and scintillation counts obtained from tumor vs. normal pancreas. Graphs are from two different experiments, where scintillation counts were obtained on 24-hour frozen samples (left) or freshly isolated tissue (right). All experiments are representative of two independent replicates.
and ex vivo on cells from resected orthotopic tumors (Fig. 1F). B3, A12, and a control VHH were conjugated to 64Cu and injected intravenously into tumor-bearing mice 2 hours before harvesting the tumor (3, 8, 32). In B3- and A12-injected mice, accumulation of label was detected in the orthotopic pancreatic tumor, but not in adjacent normal pancreas (Fig. 1G). Neither B3 nor A12 shows significant staining in spleen, lymph nodes, nor liver, although significant staining is found in brown adipose tissue (3).

Anti–PD-L1 VHH delivers IL-2 to the tumor microenvironment without inducing systemic toxicity. Although effective in a small subset of patients, systemic delivery of IL-2 for cancer treatment is limited by its toxicity (14, 40, 42–45). In order to use a low, nontoxic dose of IL-2 while maintaining a therapeutic effect, we conjugated murine IL-2 to B3 (B3-IL2, Fig. 2A). Through its PD-L1 binding domain, we reasoned that the B3–IL2 fusion would be retained by the tumor, allowing for higher local doses of cytokine with lower systemic concentrations, preventing toxicity, yet achieving intratumoral concentrations sufficient for therapeutic benefit. B3–IL2 is considerably smaller (~32 kDa) than similar antibody conjugates (~170 kDa), and retained its affinity for PD-L1 (Fig. 2A and B). Likewise, the fusion retains IL-2 activity, as shown by its ability to increase killing of B16-ova cells by OT-I cells (Fig. 2C). It also supported T-cell survival and CD8 skewing in cell culture to an extent comparable with that of recombinant IL-2 (Fig. 2D). When administered at a low dose in vivo, (15 μg twice weekly over two weeks), B3–IL2 caused a modest increase in spleen size (Fig. 2E) with minimal toxicity, comparable with similar doses of recombinant IL-2 or IL-2 fused to a control VHH (Fig. 2F–I).

Adding a VHH domain to IL-2 is predicted to extend its serum half-life (5, 45). To control for possible effects of extended half-life IL-2, we generated a control VHH fused to IL-2 (VHHCTR–IL2), which has a similar size and serum half-life to B3–IL2, but without the PD-L1—targeting domain. When used in combination with the antimalanoma antibody TA99 (13, 17, 45), B3–IL2 slowed tumor growth and prolonged survival compared with treatment with TA99 and anti–PD-L1 alone (Fig. 2I). Equimolar amounts of VHHCTR–IL2 and B3 admixed did not confer a survival advantage, indicating that IL-2 conjugation to B3 is critical for efficacy (Fig. 2I).

Effective treatment of orthotopic pancreatic cancer and enhanced CD8+ T-cell accumulation. Having first validated B3–IL2 in B16 melanoma, we next tested B3–IL2 in orthotopic pancreatic
B3-IL2 augments response to orthotopic Panc02 tumors. 100,000 Panc02 cells were orthotopically implanted into C57BL/6 mice. Mice were treated i.p. with VHHCTR, B3, or B3-IL2 at 1 μg/mouse daily for 18 days. Tumors were harvested at day 21 after implantation, weighed, and digested, and infiltrates were analyzed by flow cytometry. A, Spleen weights from Panc02-inoculated mice treated with the indicated constructs. B, Tumor weights from mice treated with the indicated constructs. Results are pooled from multiple experiments, where each tumor weight is normalized to the mean of the VHHCTR-treated tumors in that experiment to obtain relative tumor mass. VHHCTR n = 10, B3 n = 8, B3-IL2 n = 22. C, CD8+ cells as a percentage of total tumor infiltrates (TILs). D, 100,000 Panc02 cells were orthotopically implanted into C57BL/6 mice. Mice were treated i.p. with VHHCTR, B3, or B3-IL2 at 1 μg/mouse daily starting on day 5 after inoculation. Mice were given 250 μg of anti-CD4, anti-CD8, or isotype by i.p. injection on days 5 and 11 after inoculation. Absence of CD4 or CD8 T cells was confirmed by flow cytometry of spleen cells at time of sacrifice (day 18 after inoculation). VHHCTR and B3-IL2, n = 3; anti-CD4 and anti-CD8, n = 5. E, 100,000 Panc02 cells were orthotopically implanted into C57BL/6 mice. Mice were treated i.p. with B3-IL2 or VHHCTR-IL2 at 1 μg/mouse daily for 18 days. Tumors were harvested at day 21 after implantation. N = 8 mice per group. F, Tumors from E were digested with collagenase and stained with antibodies to the indicated cell populations. Tregs were identified by intracellular Foxp3 staining. G, Draining lymph nodes were isolated from mice treated as in A–C, and Tregs were identified by intracellular Foxp3 staining. n = 5 mice per group. Error bars, SEM (A–C) or SD (D–F). Results are combined from 4 experiments for (A–C), representative of two independent experiments for (D–G). *P < 0.05, using the Student t test.

cancer. PD-1 blockade has so far proven ineffective against pancreatic cancer in both mice and humans (13, 29). Treatment of mice bearing Panc02 orthotopic tumors with B3–IL2 caused a small increase in spleen weight relative to a control VHH and significantly reduced tumor size (Fig. 3 A and B), which was accompanied by an increase in tumor-infiltrating CD8+ T cells in B3–IL2-treated animals (Fig. 3C). Depletion of CD8+ T cells resulted in increased growth of Panc02 tumors, confirming previous reports of CD8+ T-cell dependence in this model (Fig. 3D, ref. 46). Treatment with B3 alone was without effect, showing that PD-L1 blockade was insufficient to confer a meaningful antitumor response in this model (Fig. 3B and C). We also saw improved responses of B3–IL2 when compared with treatment with IL-2 fused to an irrelevant and similarly sized control VHH (VHHCTR–IL2), with a concomitant increase in CD8+ T-cell infiltration (Fig. 3E and F). The lack of efficacy of the control VHH–IL2 shows the importance of the B3 component for targeting IL-2 to the tumor microenvironment. Foxp3+ Tregs express CD25 and expand in response to low doses of IL-2 (47). Indeed, B3–IL2 enhanced the fraction of Tregs in the tumor microenvironment (Fig. 3F) and in the tumor draining lymph nodes (Fig. 3G), possibly counteracting some of the antitumor activity of the conjugate.

KPC tumors are less heavily infiltrated by CD8+ T cells. Although B3 penetrates into KPC tumors (Fig. 1G), B3–IL2 treatment of mice bearing orthotopic KPC tumors showed no increase in CD8+ T cells into the tumors, nor derived any therapeutic benefit in terms of tumor size, consistent with a minor role for CD8+ T cells in the KPC model (Supplementary Fig. S4A and S4B). An anti–PD-L1 VHH fusion with GM-CSF showed no reduction in tumor size either (Supplementary Fig. S4C and S4D), consistent with reports that pancreatic tumor cells themselves produce GM-CSF, which supports an immunosuppressive microenvironment in these tumors (35, 48, 49).
Fusion of PDL1–VHH to IFNγ enhanced antitumor responses

IFNγ plays a central role in the antitumor immune response in both mice and humans (50, 51). Mutations in IFNγ signaling are a major pathway of resistance to immunotherapy, further elucidating its clinical importance (50). Both A12 and B3 were fused to IFNγ and expressed as a secreted protein in mammalian cells. Minimal differences between A12–IFNγ and B3–IFNγ were observed, and these two PDL1–VHH constructs were used interchangeably. A12–IFNγ retains its affinity for surface PD-L1 (Fig. 4A), stimulates class I and class II MHC surface expression on B16 to a similar extent as recombinant IFNγ (Fig. 4B), and increases survival as a monotherapy in the CT26 colon cancer model (Fig. 4C). Admixing VHHCCTR–IFNγ and B3 had no effect, indicating that targeting IFNγ via conjugation to B3 was superior to merely giving IFNγ plus PD-L1 blockade (Fig. 4C). Similarly, combination treatment with A12–IFNγ and TA99 extended survival in B16 melanoma compared with giving VHHCCTR–IFNγ admixed with equimolar anti–PD-L1 (A12; Fig. 4D).

A12–IFNγ decreased growth of Panc02 orthotopic tumors, leading to a reduction in tumor size relative to treatment with a control VHH or treatment with anti–PD-L1 admixed with IFNγ (Supplementary Fig. S5). We combined A12–IFNγ and B3–IL2 treatment of mice bearing orthotopic Panc02 tumors and observed an additive benefit of the two treatments leading to reduced tumor burden. The B3–IL2-mediated increase in Foxp3+ Treg populations in the draining lymph node was obviated by inclusion of A12–IFNγ in the treatment regimen (Supplementary Fig. S5). Combination targeted therapy appeared to provide modestly increased efficacy over single agents alone, however, determining the optimal dose and schedule of delivering multiple agents is complicated by the disparate mechanisms of action of the two cytokines and by the fact that A12 and B3 bind to overlapping epitopes on PD-L1, possibly leading to decreased delivery of each cytokine fusion. We therefore chose to pursue the mechanism of action of targeted IFNγ as a single agent.

Pancreatic tumor organoids—when injected orthotopically—grow more slowly than similarly derived flat-cultured cells and develop more extensive fibrosis (31). We implanted KPC organoids (M19 line, and treated mice with control VHH1, VHHCCTR–IFNγ, or A12–IFNγ. A12–IFNγ-treated tumors were significantly smaller than either control group (Fig. 5A). As further evidence that A12 successfully delivered IFNγ to the tumor microenvironment, we observed an increase in surface expression of class II MHC on the pancreatic tumor cells themselves, but only in mice that received targeted A12–IFNγ, and not VHHCCTR–IFNγ. MDSC populations were likewise decreased (Supplementary Fig. S6A and S6B).

Similar to Panc02 cells and the KPC organoids, mice implanted orthotopically with a KPC pancreatic cancer cell line also showed a reduction in tumor growth with A12–IFNγ compared with VHHCCTR–IFNγ (Fig. 5B and C). We noted a decrease in the numbers of tumor-infiltrating granulocytic MDSCs in KPC tumors treated with A12–IFNγ, and a slight decrease in monocytic MDSCs (Fig. 5D), resulting in an overall loss of CD11b+ cells (Fig. 5E). The remaining myeloid cells showed increased surface expression of class II MHC, consistent with exposure to IFNγ (Fig. 5F). Several groups have reported immune control of pancreatic tumors mediated entirely by myeloid cells (26, 27). We therefore tested whether adaptive immunity was required for the efficacy of targeted IFNγ treatment. Although B3–IFNγ significantly reduced tumor burden in wild-type mice, it failed to do so in RAG2−/− mice that lack both T and B cells (Fig. 5G and H). Consistent with
these findings, both CD8 and CD4 effector T-cell populations increased in tumor infiltrates from mice treated with B3–IFNγ and trended toward an increase in Ki67 positivity, suggesting increased proliferation of effector T-cell populations (Supplementary Fig. S6C).

**PDL1–VHH delivers IFNγ to the tumor microenvironment**

PD-L1 is expressed at low levels on pancreatic tumor cells and can be expressed on tumor-infiltrating myeloid cells. To determine the cell types affected by targeted IFNγ delivery, we generated fluorescently labeled VHH–IFNγ fusions and
administered them to mice with midstage orthotopic tumors. One hour later, we resected the tumors for imaging by two-photon microscopy (Fig. 6A and B). B3–IFNγ was present on both CD11b+ and CD11b− cells dispersed throughout the tumor microenvironment, whereas VHHCTR–IFNγ was not detected. Thus, a nontargeted IFNγ conjugate was not retained in the tumor microenvironment.

Given that B3–IFNγ colocalized with CD11b+ cells (Fig. 6A), and that myeloid cells were the major cell types affected by therapeutic dosing of B3–IFNγ, we performed transcriptional analysis on myeloid cells isolated from treated KPC tumors. Global analysis of gene expression showed minimal transcriptional changes between VHHCTR- and VHHCTR–IFNγ-treated tumors. The IFNγ response gene Gbp2β was the only significantly upregulated transcript (cutoff of >1.5 log, fold change). B3–IFNγ induced far greater transcriptional changes than did VHHCTR, consistent with its having activity in the tumor microenvironment (Fig. 6C). When comparing B3–IFNγ with VHHCTR–IFNγ, we saw a signature of increased antigen processing and presentation (Fig. 6D). Across a large panel of IFNγ-regulated genes, B3–IFNγ consistently displayed increased transcriptional activity, evidence that B3 successfully targeted IFNγ to the tumor microenvironment, whereas VHHCTR did not (Fig. 6E).

**Discussion**

PD-L1–specific alpaca-derived antibody fragments readily penetrate into the tumor microenvironment, entering not only melanoma but also the dense stroma of pancreatic tumors. These anti–PD-L1 VHHs can deliver attached payloads to the tumor microenvironment and improve antitumor activity. Melanomas are generally sensitive to immunotherapy, and we show enhanced antitumor activity with anti–PD-L1 VHH–cytokine fusions in established murine melanoma models (7, 41, 52, 53). Immunotherapy for pancreatic cancer has so far been less successful (54).

Delivery of either IL-2 or IFNγ VHH fusions as single agents can produce pancreatic tumors size by 50% in orthotopic models. IL-2 and IFNγ act through distinct mechanisms, with IL-2 treatment expanding intratumoral CD8 T cells and IFNγ decreasing MDCs and enhancing class II MHC presentation. These two examples demonstrate the utility of anti–PD-L1 VHH-mediated delivery and could be used as part of combination therapy for pancreatic cancer.

Pancreatic cancer is unusually resistant to treatment, and any single agent alone is unlikely to show sustained efficacy. Indeed, the rapidly progressing nature of the disease requires that patients receive chemotherapy, either gemcitabine/Abraxane or FOLFIRINOX, as standard of care. These cytotoxic agents can not only release tumor antigens to prime favorable T-cell responses, but can also have a negative impact on the immune response by killing rapidly dividing immune cells (55). Combination of immunotherapy with chemotherapy, particularly for pancreatic cancer, must take into account how each component part interacts with the other. The two immunotherapeutic agents described here show changes in tumor-infiltrating cell populations that correlate with efficacy of treatment. Increase in intratumoral CD8 T cells or increased class II MHC expression on intratumoral macrophages are potential biomarkers for efficacy of these two agents, respectively. The distinct mechanisms of action of IL-2 and IFNγ also provide a rationale for combining these agents, or with immunotherapeutics known to exploit yet other pathways.

The role of IFNγ in the tumor microenvironment is complex. Genes regulating IFNγ signaling in tumor cells are frequently mutated in patients who fail to respond to immunotherapy, suggesting that successful immunotherapy may involve direct growth-inhibitory effects of IFNγ on malignant cells (50). In pancreatic cancer, agnostic antibodies to CD40 stimulate systemic IFNγ production, leading to increased intratumoral Ly6C+ inflammatory monocytes that secrete matrix metalloproteinases (27). Although we observed no effects of targeted IFNγ on the extracellular matrix in our models, we did occasionally observe modest efficacy of VHHCTR–IFNγ treatment compared with VHHCTR alone, consistent with a minor role for systemic IFNγ in mimicking the mechanism of action of anti-CD40. Intratumoral IFNγ may increase antigen processing and presentation by myeloid cells, as well as skew the phenotype of intratumoral macrophages. However, IFNγ also induces negative regulatory pathways, including production of indoleamine 2,3-dioxygenase (IDO), and upregulation of PD-L1 and other inhibitory ligands on tumor cells (56). Here, we combine targeting of IFNγ with concurrent blockade of PD-L1, thereby partially negating the negative regulatory effects of IFNγ. Nevertheless, targeted IFNγ may be more efficacious if targeted exclusively to myeloid cells, or if combined with other agents, such as IDO inhibitors.

VHHs are versatile tools that can be expressed cheaply and are easily conjugated to a variety of agents (3, 7, 8, 11, 32, 57). AVHH can be equipped with a sortase recognition motif (LPETG) that can then be used to covalently attach any moiety with an N-terminal glycine (11, 58–64). In this manner, “click” handles can be conjugated to the site-specific attachment of monoprotein payloads such as radioisotopes without compromising the binding properties of the VHH (3, 8, 32). We are thus able to use one and the same reagent for detection of PD-L1 expression in vivo, blockade of PD-L1 interaction with PD-1, and delivery of therapeutic compounds, not limited to the two examples reported here (3).

Alpaca VHHs are potentially immunogenic when administered to a heterologous recipient. In mice that were dosed for 3 weeks or more with B3, we observed low titer antibodies to VHH in approximately 30% of mice, similar to previously reported anti-VHH responses in mice (3, 7). Substitution of particular amino acids in the VHH framework region renders alpaca antibodies more similar to their human orthologs, which enables repeated dosing without altering the anti-VEH immune response (65). Indeed, in a phase II trial of the humanized VHH caplacizumab (specific for von Willebrand factor), patients were dosed for 60 days with caplacizumab, and nonneutralizing antibodies to the alpaca VHH were observed in only 9% of patients (65). Thus VHH-based therapies can be safe and relatively nonimmunogenic.

In healthy individuals, PD-L1 expression is confined to a subset of myeloid dendritic cells and brown adipocytes, two cell types that are neither abundant nor critical for survival (40, 66). In cancer patients, PD-L1 is expressed predominantly in the tumor microenvironment. PD-L1 expression can be heterogeneous, with expression on tumor cells, myeloid cells, on both or on neither (42, 67). However, by delivering compounds to the tumor microenvironment, there is no need to invoke homogeneous expression of PD-L1 on tumor cells as a precondition for therapeutic efficacy. Indeed, pancreatic tumor cells resected from orthotopic KPC tumors showed weak staining of PD-L1, with a significant fraction of the cells altogether negative for expression. Myeloid cell...
Figure 6.
B3 effectively targets IFNγ to myeloid cells in the pancreatic tumor microenvironment. A, C57BL/6 mice were inoculated with either 100,000 KPC cells and harvested 7 days after tumor implantation or B, implanted with KPC organoids and harvested 6 weeks after implantation. One hour prior to harvest, mice were injected i.p. with 50 μg of Alexa Fluor 488 labeled of either B3-IFNγ or VHHCTR-IFNγ. Pancreas bearing tumors were processed and stained with mAb against CD31 (cyan), CD11b (green), and the labeled B3-IFNγ or VHHCTR-IFNγ (red). Confocal micrographs were captured on a two-photon microscope using a 20× objective (scale bars either 100 μm or 5 μm). C–E, CD11b+ cells were sorted by FACS from day 12 orthotopic KPC tumors of mice that had been treated with VHHCTR (n = 2), VHHCTR-IFNγ (n = 3), or B3-IFNγ (n = 2) as in Fig. 5F. RNA was prepared and used for RNA-seq analysis. C, Volcano plots of differentially expressed genes when comparing VHHCTR with VHHCTR-IFNγ or with B3-IFNγ. D, Gene set enrichment score analysis for genes involved in antigen processing and presentation. E, Fold change above VHHCTR for FPKM values of known IFNγ-regulated genes. Error bars, SEM. Results are representative of two independent experiments for A–B. RNA-seq in C–E was performed once.
expression of PD-L1 may be adequate for targeted cytokine delivery with anti-PD-L1 VHH, suggesting the broad potential for this approach.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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**References**

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