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



Cancer

Immunology

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Michael Dougan¹, Jessica R. Ingram², Hee-Jin Jeong², Munir M. Mosaheb³, Patrick T. Bruck², Lestat Ali², Novalia Pishesha⁴, Olga Blomberg⁴, Paul M. Tyler², Mariah M. Servos², Mohammad Rashidian⁴, Quang-De Nguyen⁵, Ulrich H. von Andrian³, Hidde L. Ploegh⁴, and Stephanie K. Dougan^{2,3}

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 singledomain antibodies (VHHs) against PD-L1 fused with IL-2 and IFN γ . 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 IFN γ reduced the number of CD11b⁺ cells and skewed intratumoral macrophages toward the display of M1-like characteristics. Imaging of fluorescent VHH–IFN γ constructs, as well as transcriptional profiling, demonstrated targeting of IFN γ to the tumor microenvironment. Many tumors and tumor-infiltrating myeloid cells express PD-L1, rendering them potentially susceptible to this form of targeted immunotherapy. *Cancer Immunol Res;* 6(4); 389–401. ©2018 AACR.

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 IFN α , are given systemically, often resulting in severe dose-limiting toxicities (1, 2). Efficacy of cytokinebased 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-

M. Dougan and J.R. Ingram contributed equally to this article.

Corresponding Author: Stephanie K. Dougan, Harvard Medical School, 450 Brookline Ave., Sm770A, Boston, MA 02215. Phone: 617-582-9609; E-mail: Stephanie_dougan@dfci.harvard.edu

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tion. As an alternative to full-sized antibodies as fusion partners, we developed alpaca-derived heavy chain-only antibody fragments (VHHs) against programmed death-ligand 1 (PD-L1; ref. 3). In cancer patients, PD-L1 expression is confined largely to the tumor microenvironment, being expressed on a wide variety of tumor cell types and tumor-infiltrating myeloid cells (4). VHHs, at \sim 15 kDa, more readily penetrate tumors than fullsized antibody fusions. Antibody-cytokine fusions designed for delivery of IL-2 to the tumor microenvironment have been tried before (5). Unfortunately, the high affinity of IL-2 for its receptors and the abundant expression of IL-2 receptors in peripheral blood, spleen, and liver lead to a distribution of the antibodycytokine fusion that is dictated primarily by the cytokine partner rather than the antibody to which it is attached for targeted delivery. Mathematical modeling predicts that smaller sized protein fusions would allow IL-2 to concentrate in the tumor microenvironment (5). Although VHHs have a short circulatory half-life of ~30 minutes, they can remain bound to their targets in vivo for more than 24 hours (6, 7). The rapid clearance of VHHs from the circulation, combined with their high affinities and long tissue half-lives, means that it should be possible to concentrate VHHs and attached payloads in the tumor microenvironment while minimizing systemic exposure. VHHs accept a variety of payloads, including radioisotopes and cytokines, which can be installed in a straightforward manner (3, 8-11). Disruption of immune checkpoint interactions by monoclonal antibodies (mAb) has replaced chemotherapy as the standard of care for metastatic melanoma and is similarly promising in the treatment of other cancers (4, 12–14). Checkpoint blockade largely augments a preexisting T-cell response and has had far less efficacy against tumors that are poorly infiltrated by T cells at baseline (15, 16). Cytokine-based therapies may be particularly promising as a means of manipulating the immune microenvironment. We have chosen to focus



¹Division of Gastroenterology, Department of Medicine, Massachusetts General Hospital, Boston, Massachusetts. ²Department of Cancer Immunology and Virology, Dana-Farber Cancer Institute, Boston, Massachusetts. ³Department of Microbiology and Immunobiology, Harvard Medical School, Boston, Massachusetts. ⁴Program in Cellular and Molecular Medicine, Children's Hospital Boston, Boston, Massachusetts. ⁵Lurie Family Imaging Center, Dana-Farber Cancer Institute, Boston, Massachusetts.

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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 tumorassociated 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 targeting and a T-cell response as part of a multipronged 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 IFN γ 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 IFN γ fusion caused a profound reduction in the size of Panc02, KPC, 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 to 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 $8,000 \times g$ 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-IFN γ , or VHHCTR(1B7)-IFN γ by incubating 30 µmol/L purified VHH protein with 5 µmol/L 7M-SrtA and 100 µmol/L GGGK-biotin or GGGK-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 manufacture. To remove LPS, VHHs were immobilized on HisTrap 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 Quantitation 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 or 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. Panc02, 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 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 Fortessa cytofluorimeter (BD). Flow cytometry fluorescent dye-conjugated antibodies to the following proteins were purchased from Biolegend [B220-PacificBlue (clone RA3-6B2), 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-Bv650 (53-6.7), FoxP3-PE (MF-14), Gr1-PE (RB6-8C5), Gr1-PE/Cy7 (RB6-8C5), MHC I-A/I-E-Bv510 (M5/114.15.2), NK1.1-FITC (PK136), NK1.1-PE/Cy7 (PK136), PD-1-PE/Cy7 (29F.1A12), Tim3-APC (RMT3-23), PD-L1-APC (10F.9G2)], and Affymetrix [CD19-PE (MB19-1)].

Two-photon confocal microscopy. After implantation of pancreatic tumors (after 7 days) organoid KPC (post 6 weeks; as describe above) and 1 hour after intraperitoneal injection of B3-IFN γ or VHHCTR conjugated to Alexa Fluor 488, the entire pancreas bearing implanted tumors were harvested from adult mice and immediately fixed in phosphate PBS containing 1% paraformal-dehyde, 1.5% L-lysine, 0.2% sodium periodate, and sodium dibasic 0.1 mol/L Na₂HPO₄ 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 \times 100) and then blocked in blocking buffer (PBS, 0.5% BSA, 0.3% Triton \times 100) containing 2.4G2 antibody to block nonspecific binding of immunoglobulin to Fc γ 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. 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 MilleniaXs pump laser (Spectra-Physics) and a 20 \times /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 C₂₇H₄₅N₁₀O₁₁S (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 k_{cat} was used. Reaction mixtures (0.5 mL) contained Tris-HCl (50 mmol/L, pH 7.5), CaCl₂ (10 mmol/L), NaCl (150 mmol/L), triglycine-NOTA (800 µmol/L), VHH (300 µmol/L), and sortase (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 triglycine-NOTA to 1 mmol/L. The reaction mixture was loaded into a preequilibrated PD-10 columns and eluted with $1 \times$ PBS, removing excess of NOTA substrate and GGHis₆ byproduct. Ni-NTA beads were added to the collected fractions with agitation for 5 minutes at 25°C, followed by centrifugation to remove sortase and any remaining unreacted His-tagged VHH. The labeled VHH was

stored at $-20^\circ\mathrm{C}$ 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-VHH (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, R_f^{64} Cu–VHH = 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× 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 (~3.3MBq), ⁶⁴Cu–VHH–A12, or ⁶⁴Cu–VHH–96GM (~3.3MBq), 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 in list-mode format over 15 minutes and corrected for decay and dead time. The acquired data were then sorted into 0.5 mm sinogram bins and 1 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 B16ova cells were a gift from Glenn Dranoff (currently at Novartis Institute for Biomedical Research, Cambridge, MA). For *in vivo* challenge experiments, 5×10^5 B16 cells were inoculated by subcutaneous injection in 500 µL of Hank's Balanced Salt Solution (HBSS). For vaccinations, 5×10^5 irradiated (3,500 rad) GM-CSF secreting B16 cells (GVAX) were administered as a subcutaneous 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 (TekNova) by intraperitoneal injection. Tumor size was measured in two dimensions using precision calipers. Mice were euthanized when the total tumor volume exceeded 125 mm³.

Competition ELISAs

High binding microtiter plates (Corning) were coated with recombinant PD-L1-Fc fusion protein overnight at 25 ng/mL in carbonate buffer. Biotinylated VHHs or antibodies (BD Biosciences) were incubated on the coated plates in 10% inactivated fetal calf serum in phosphate-buffered saline for 1 hour in the presence or absence of unlabeled VHH B3 or mAb to PD-L1 (10F.9G2) across a range of concentrations, washed in 0.5% Tween in PBS, and then developed using streptavidin-HRP (BD Biosciences) and tetramethylbenzidine.

In vitro cytotoxicity assays

Spleen cells were isolated from OT-I;RAG1^{-/-} mice or from TRP1;RAG2^{-/-} mice and were stimulated with α CD3/CD28 beads (Dynabeads) for 48 hours at 2×10^6 cell/mL in RPMI according to the manufacturer's instructions. Beads were then removed by magnetic separation, and cells were incubated for an additional 24 hours in the culture supernatant. In parallel, 2.5 \times 10³ B16-ova or WT cells were incubated for 18 hours in 250 µL RPMI containing 20 ng/mL IFNy. IFNy containing media were exchanged for fresh media; T cells were then cocultured with the B16 cells for 48 hours at a ratio of 2:1. VHHs or antibodies were added at the start of the culture. After 48 hours, T cells were removed from the cultures by washing, and B16 viability was assessed using the CellTiterGlo (CTG) bioluminescence assay according to the manufacturer's instructions. Percent survival was calculated using untreated B16 cells cultured without T cells for normalization.

IL2 toxicity measurements. Blood was collected in heparin-coated tubes by cheek bleed. Hemoglobin was determined using a commercial complete blood counter available through Boston Children's Hospital. ALT was measured in plasma using a commercially available NADH and LDH based method (Infinity ALT, Thermo). Mice were euthanized using CO_2 asphyxiation, lungs were dissected and flash frozen in liquid nitrogen and weighed. Frozen tissue was then lyophilized at -80° C overnight and weighed again. The pulmonary wet weight was then calculated as the pulmonary weight at autopsy – the dry weight of the lungs after overnight lyophilization.

Statistical analysis

Two sample comparisons used the Student t test with pooled variance if there was no evidence of inhomogeneity of variances between groups. If the variances were unequal, the exact Wilcoxon rank sum test, a nonparametric alternative to the t test, was used. Every effort was made to keep testing consistent across related experiments. For comparisons of more than two groups, analysis of variance (ANOVA) was used if there was no evidence of inhomogeneity of variance; the Kruskal–Wallis test was the nonparametric alternative. Tumor growth studies were analyzed using mixed-model ANOVA.

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-ras^{LSL.G12D/+}*; *p*53^{*R*172H/+}; 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 Alexa⁶⁴⁷ 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 IFNγinducible 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 vitro* 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 fullsized 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 μ g 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; right, B3-obiotin incubated. **C**, B16-ova cells treated for 18 hours with IFNY (20 ng/mL) were washed to remove the IFNY and were incubated with CD3/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**, IFNY-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 μ g B3, anti-PD-L1, or CTR as indicated. CTR (*n* = 10), B3 (*n* = 10), anti-PD-L1 (*n* = 5). **F**, PancO2 or KPC cells either from tissue culture or isolated from resected orthotopic KPC (*n* = 2) or PancO2 (*n* = 2) tumors were injected i.v. with 80 μ Ci ⁶⁴Cu-B3 (top) or 80 μ Ci ⁶⁴Cu-A12 or ⁶⁴Cu-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 sam



Figure 2.

B3-IL2 fusion protein preserves PD-L1 binding and IL2 cytokine activity. **A**, Diagram of B3-IL2. **B**, Flow cytometry using the indicated VHH fusions on B16 cells treated with IFN γ or left untreated (–). **C**, OT-I T cells were cultured with B16-ova cells, treated, and analyzed as in 1B. **D**, WT spleen cells were cultured with equimolar concentrations (100 ng/mL IL2 equivalents) for 72 hours and immune subsets were analyzed by flow cytometry. **E-I**, WT mice were treated twice weekly with CTR (7.5 µg), B3 (7.5 µg), IL2 (7.5 µg), VHHCTR-IL2 (15 µg), or B3-IL2 (15 µg) as indicated for 2 weeks. **E**, Spleen weight. **F**, Percentage of change in body weight from baseline. **G**, Serum Hb or (**H**) ALT on day 14 measured using Infinity ALT (Thermo). **I**, Pulmonary wet weight, calculated as the pulmonary weight at autopsy – the dry weight of the lungs after overnight lyophilization at -80° C. Lungs were flash frozen in liquid nitrogen prior to analysis. **J**, C57BL/6 mice were inoculated with B16 and treated with the indicated antibodies or VHH constructs for 2 weeks. VHHCTR-IL2, B3-IL2 n = 10, other groups n = 5. TA99 is an antibody specific for the melanoma antigen TRPI. Results are combined from two experiments for **J**, representative of two independent experiments for **A-I**. **, P < 0.01; ***, P < 0.001, using the Student *t* test.

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 μ g 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 antimelanoma antibody TA99 (13, 17, 45), B3–IL2 slowed tumor growth and prolonged survival compared with treatment with TA99 and anti–PD-L1 alone (Fig. 2J). 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. 2J).

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



Figure 3.

B3-IL2 augments response to orthotopic PancO2 tumors. 100,000 PancO2 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 PancO2-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* = 19, B3 *n* = 8, B3-IL2 *n* = 22. **C**, CD8⁺ cells as a percentage of total tumor infiltrates (TILs). **D**, 100,000 PancO2 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 PancO2 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. 3A 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).



Figure 4.

Fusion to PDL1-VHH augments IFN γ responses *in vivo*. **A**, Flow cytometry using the indicated VHH fusions on B16 cells treated with IFN γ or left untreated (–). **B**, MHC class II (I-A/E) and MHC class I (K^b) flow cytometry on B16 cells treated with IFN γ constructs. **C**, BALB/c mice were inoculated subcutaneously with CT26 colon carcinoma cells. On day 4, mice began once weekly treatment with the indicated VHH constructs for 2 weeks. **D**, C57BL/6 mice were inoculated with B16 by subcutaneous injection. On day 4, mice began once weekly treatment with the indicated VHH constructs for 2 weeks with or without twice weekly TA99, an antibody specific for the melanoma antigen TRP1. A12-IFN γ and VHHCTR-IFN γ *n* = 5, all other groups *n* = 10. **E**, Survival of mice from **D**. Results are representative of five independent experiments for **A-B** and show combined data from two experiments for (**C-E**). *, *P* < 0.05, using the Student *t* test.

Fusion of PDL1–VHH to IFN γ enhanced antitumor responses

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

A12–IFN γ 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 IFN γ (Supplementary Fig. S5). We combined A12–IFN γ 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–IFN γ 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 IFN γ 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 VHH, VHHCTR– 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 VHHCTR–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–IFN γ compared with VHHCTR–IFN γ (Fig. 5B and C). We noted a decrease in the numbers of tumor-infiltrating granulocytic MDSCs in KPC tumors treated with A12–IFN γ , 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 express of class II MHC, consistent with exposure to IFN γ (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 IFN γ treatment. Although B3–IFN γ 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



Figure 5.

PDL1VHH–IFN γ decreases tumor burden in PancO2 and KPC orthotopic models of pancreatic cancer. **A**, Organoids derived from KPC mice (line M19) were inoculated orthotopically into C57BL/6 mice and treated with the indicated VHH constructs 5 µg/mouse i.p. daily. Five weeks after implantation, tumors were harvested. **B**, C57BL/6 mice were inoculated with 100,000 KPC cells orthotopically and treated with VHHCTR, VHHCTR–IFN γ , or A12–IFN γ . Compounds were administered daily for 18 days at 5 µg per mouse i.p. Tumors were harvested at 21 days after implantation. VHHCTR *n* = 22, VHHCTR–IFN γ , or A12–IFN γ . Compounds were administered daily at 5 µg per mouse i.p. Tumors were harvested at 21 days after implantation. VHHCTR, VHHCTR–IFN γ , or A12–IFN γ . Compounds were administered daily at 5 µg per mouse i.p. Experiment was performed once. **D** and **E**, KPC tumors from **B** were analyzed by flow cytometry for the indicated populations. *N* = 5 mice per group. **F**, C57BL/6 mice were inoculated with orthotopic KPC tumors and treated with VHHCTR, VHHCTR–IFN γ , or B3–IFN γ . Compounds were administered twice weekly for 18 days at 50 µg per mouse i.p. Tumors were harvested at 21 days after implantation, digested, and analyzed by flow cytometry for surface PD-L1 expression on tumor cells (CD45⁻). **G**, Weights of tumors from **F**. **H**, RAG2^{-/-} mice were inoculated with 100,000 KPC cells subcutaneously and orthotopically, for a total of 2 tumors were harvested at 21 days after implantation. Results are representative of three independent experiments for **A**, show combined data from two experiments for **B**, and are representative of two independent experiments for **D**-**H**. *, *P* <0.05, using the Student *t* test.

these findings, both CD8 and CD4 effector T-cell populations were increased in tumor infiltrates from mice treated with B3–IFN γ 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 IFN γ delivery, we generated fluorescently labeled VHH–IFN γ 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–IFN γ was present on both CD11b⁺ and CD11b⁻ cells dispersed throughout the tumor microenvironment, whereas VHHCTR–IFN γ was not detected. Thus, a nontargeted IFN γ conjugate was not retained in the tumor microenvironment.

Given that B3–IFN γ colocalized with CD11b⁺ cells (Fig. 6A), and that myeloid cells were the major cell types affected by therapeutic dosing of B3-IFNy, 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-IFNy-treated tumors. The IFNγ response gene *Gbp2b* was the only significantly upregulated transcript (cutoff of >1.5 log₂ 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-IFNy with VHHCTR-IFNy, we saw a signature of increased antigen processing and presentation (Fig. 6D). Across a large panel of IFNy-regulated genes, B3-IFNy consistently displayed increased transcriptional activity, evidence that B3 successfully targeted IFNy 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 IFNy VHH fusions as single agents can reduce pancreatic tumor size by 50% in orthotopic models. IL-2 and IFNy act through distinct mechanisms, with IL-2 treatment expanding intratumoral CD8 T cells and IFNy decreasing MSDCs 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 FOLFIR-INOX, 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 others. 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 IFNy also provide a rationale for combining these agents, or with immunotherapeutics known to exploit yet other pathways.

The role of IFNy in the tumor microenvironment is complex. Genes regulating IFNy 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 IFNy on malignant cells (50). In pancreatic cancer, agnostic antibodies to CD40 stimulate systemic IFNy production, leading to increased intratumoral Ly6C⁺ inflammatory monocytes that secrete matrix metalloproteinases (27). Although we observed no effects of targeted IFNy on the extracellular matrix in our models, we did occasionally observe modest efficacy of VHHCTR-IFNy treatment compared with VHHCTR alone, consistent with a minor role for systemic IFNy in mimicking the mechanism of action of anti-CD40. Intratumoral IFNy may increase antigen processing and presentation by myeloid cells, as well as skew the phenotype of intratumoral macrophages. However, IFNy 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 IFNy with concurrent blockade of PD-L1, thereby partially negating the negative regulatory effects of IFNy. Nevertheless, targeted IFNy 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). A VHH 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 enable site-specific attachment of nonprotein 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 while avoiding a neutralizing anti-VHH 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 IFN γ 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–IFN γ or VHHCTR–IFN γ . Pancreas bearing tumors were processed and stained with mAb against CD31 (cyan), CD11b (green), and the labeled B3–IFN γ or VHHCTR–IFN γ (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–IFN γ (*n* = 3), or B3–IFN γ (*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–IFN γ or with B3–IFN γ . **D**, Gene set enrichment score analysis for genes involved in antigen processing and presentation. **E**, Fold change above VHHCTR for FPKM values of known IFN γ -regulated genes. Error bars, SEM. Results are representative of two independent experiments for **A–B**. RNA-seq in **C–E** was performed once.

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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.

Authors' Contributions

Conception and design: M. Dougan, J.R. Ingram, H.L. Ploegh, S.K. Dougan Development of methodology: M. Dougan, J.R. Ingram, M. Rashidian, U.H. von Andrian, H.L. Ploegh

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Dougan, H.-J. Jeong, M.M. Mosaheb, P.T. Bruck, L. Ali, N. Pishesha, O. Blomberg, P.M. Tyler, M.M. Servos, Q.-D. Nguyen, S.K. Dougan

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Dougan, J.R. Ingram, M.M. Mosaheb, M.M. Servos, Q.-D. Nguyen, U.H. von Andrian, S.K. Dougan

Writing, review, and/or revision of the manuscript: M. Dougan, J.R. Ingram, H.L. Ploegh, S.K. Dougan

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): P.T. Bruck, L. Ali Study supervision: J.R. Ingram

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