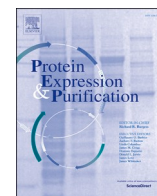




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# Characterization of a single chain variable fragment of nivolumab that targets PD-1 and blocks PD-L1 binding

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## ABSTRACT

Activated T-cells express Programmed cell Death protein 1 (PD-1), a key immune checkpoint receptor. PD-1 functions primarily in peripheral tissues, where T cells may encounter tumor-derived immunosuppressive ligands. Monoclonal antibodies that disrupt the interaction between T-cell derived PD-1 and immunosuppressive ligands, such as PD-L1, have revolutionized approaches to cancer therapy. For instance, Nivolumab is a monoclonal Ab that targets human PD-1 and has played an important role in immune checkpoint therapy. Herein we report the purification and initial characterization of a ~27 kDa single chain variable fragment (scFv) of Nivolumab that targets human PD-1 and blocks binding by PD-L1. The possibility that the anti-PD-1 scFv can serve as both an anti-tumor agent and as an anti-viral agent is discussed.

**Importance:** The clinical significance of anti-PD-1 antibodies for treatment of a range of solid tumors is well documented (reviewed in [1–4]). In this report, we describe the results of studies that establish that an anti-PD-1 scFv purified from *E. coli* binds tightly to human PD-1. Furthermore, we demonstrate that upon binding, the anti-PD-1 scFv disrupts the interaction between PD-1 and PD-L1. Thus, the properties of this scFv, including its small size, stability and affinity for human PD-1, suggest that it has the potential to be a useful reagent in subsequent immunotherapeutic, diagnostic and anti-viral applications.

## 1. Introduction

Activation of “immune checkpoints” is a fundamental event that limits collateral tissue damage during T cell responses to infection (reviewed in Refs. [5–7]). However, activation of immune checkpoints also enables cancer cells to avoid destruction by the human immune system. These negative checkpoints are established when immunosuppressive ligands on cancer cells interact with receptors on immune effector cells [1–3]. As a result of these activated checkpoints, the human immune system does not mount a full attack on many tumors.

One critical form of checkpoint-based inhibition is due to the interaction of the inhibitory receptor Programmed cell Death protein-1 (PD-1), located on the surface of a number of cell types (including activated T cells, T regs, activated B cells and natural killer cells) with ligands such as PD1 ligand 1 (PD-L1) (e.g., Ref. [8–12]; reviewed in Refs. [13]).

Normally, the PD-1/PD-L1 interaction plays a critical role in the suppression of CD8<sup>+</sup> cytotoxic T cells and related cells of the immune system [2]. As a result, the activity of T cells in inflamed peripheral tissues is regulated and thereby autoimmunity is limited (e.g. Ref. [5]). Unfortunately, many human cancer cells have up-regulated levels of PD-L1 and the PD-1/PD-L1 dependent suppression of immune cells enables cancer cells to escape the immune system and to further proliferate ([14–16]; reviewed in Ref. [16]).

A seminal discovery in the immunotherapy field was that monoclonal antibodies that bind PD-1, and thereby disrupt PD-1's interactions with ligands such as PD-L1, promote T cell proliferation and activation (e.g., Ref. [17]; reviewed in Ref. [4,18,19]). This led to the observation that broad-spectrum antitumor activity can result from antibody-based inhibition of the interaction between PD1 and its ligands (e.g. Refs. [3,5,8,16,20]). Anti-PD1 monoclonal antibodies include Nivolumab

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(OPDIVO [21–25]); Bristol-Myers Squibb) and Keytruda (Pembrolizumab [26]; Merck). It is anticipated that further characterization of these and related molecules will lead to additional strategies to overcome tumor dependent immune checkpoints.

Of interest, single chain derivatives of monoclonal antibodies have been used as surrogates for full-length antibodies ([27,28]; reviewed in Ref. [29,30]). Among the advantages of scFvs is that they have relatively high tumor penetration [31,32] and decreased immunogenicity in humans [33]. Furthermore, they are relatively inexpensive to prepare and are easily modified by genetic manipulations. In view of these and related considerations, we elected to design, purify and characterize an scFv antibody targeting human PD-1.

## 2. Materials and methods

### 2.1. Purification of the anti-PD-1 scFv

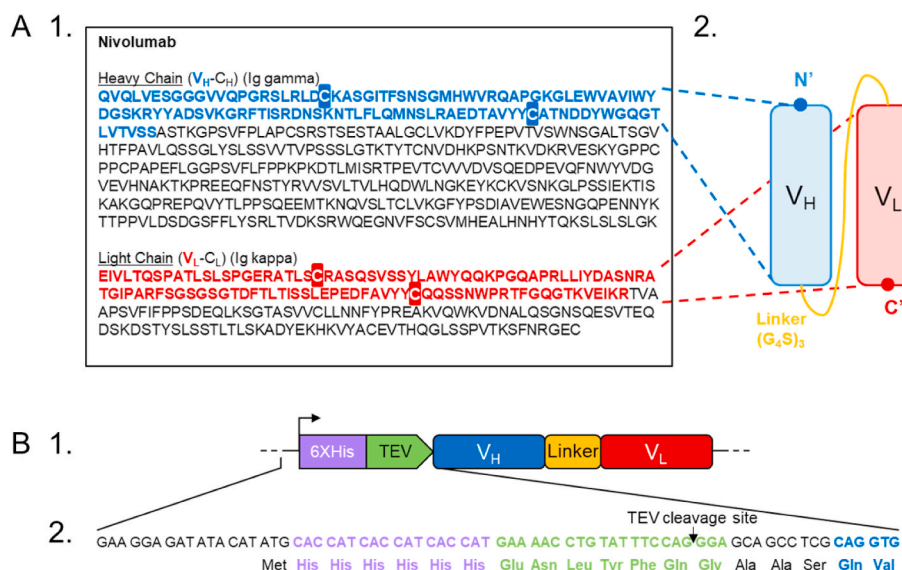
An inexpensive method for the production of scFv antibodies is via expression in *E. coli* (e.g. Ref. [34]). Therefore, the following steps were taken to clone, express and purify the anti-PD-1 scFv from *E. coli*.

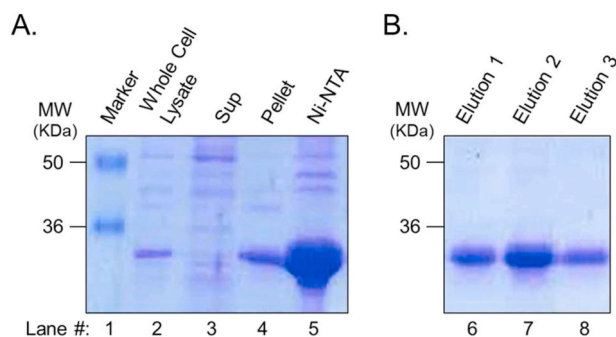
**1. Molecular Cloning:** The amino-acid sequence for the full-length anti-PD-1 mAb Nivolumab was obtained from the ChEMBL website. Using the Clustal W program, the  $V_H$  and  $V_L$  regions were identified (Fig. 1A1). DNA encoding the  $V_H$  and  $V_L$  regions, and a segment of DNA encoding a (Gly<sub>4</sub>S)<sub>3</sub> repeat used to link the  $V_H$  and  $V_L$  regions together (Fig. 1A2 [35]), was purchased from IDT as a gBlock. The anti-PD-1 scFv fragment also contained flanking regions that were homologous to the pLIC plasmid and utilized for plasmid assembly by homologous recombination. Prior to molecular cloning by Gibson Assembly [36], the pLIC-His plasmid was linearized by PCR (the forward primer was 5'CGAGGCTGCTCCCTGGAAATACAGG3', while the reverse primer was 5'CGAGGCCGGTGTCTTGCAGGATCCG3'). Following fragment assembly, the DNA was transformed into competent *E. coli* cells (NEB5-alpha) and colonies harvested following ampicillin selection. DNA was purified from selected colonies using a Qiagen Miniprep Kit and candidate clones identified by size following agarose gel electrophoresis. The sequence encoding the anti-PD-1 scFv was confirmed by Sanger DNA sequencing. The resulting vector was termed pLIC-His-anti-PD-1. It is noted that the gBlock fragment encoding the anti-PD-1 scFv was cloned into the pLIC plasmid in frame with a 6xHis tag and a TEV protease cleavage site (Fig. 1B1). Thus, following the initial methionine at its N-terminus, the anti-PD1 scFv has residues that form both the 6xHis motif and a TEV protease cleavage site (Fig. 1B2).

**2. Expression and Solubilization of the Anti-PD-1 scFv:** Plasmid pLIC-His-anti-PD1 was transformed into *E. coli* (BL21 DE3; pLysS). Protein expression was induced at an OD<sub>600</sub> of ~0.6 by addition of IPTG (0.1 mM) and the cells were grown for 8 h at 30 °C. The cells were then harvested by centrifugation at 4500 RPM in a Sorvall RC-3B Plus. To initiate the isolation of the scFv, 4 g of cell pellet was re-suspended in 100 mls of Buffer A (50 mM Tris.HCl pH 8.0, 0.3 M NaCl, 2 mM EDTA, 10% glycerol, 1 mM PMSF, 1% Igepal CA-630 and 0.1% β-mercaptoethanol) and the cells lysed by passage two times through an Avestin homogenizer. To remove cell debris and insoluble proteins, the lysate was centrifuged for 30 min at 18,000 rpm (38,700 g) at 4 °C in a Sorvall SS34 rotor. SDS-PAGE revealed that the anti-PD-1 scFv was in the pellet (Fig. 2A; lane 4). Therefore, a urea/high pH-based protocol [37] was used to extract the anti-PD-1 scFv. In brief, the pellet was re-suspended in 100 ml of Buffer B (100 mM Tris.HCl pH 12.5, 2 M urea, 20 mM imidazole, 10% glycerol and 0.02% Tween 80) and incubated at room temperature for 30 min with gentle rocking on a “nutator”. Following incubation, the pH was lowered to pH 8.0 via addition of 1 N HCl, while stirring. The suspension was then centrifuged for 20 min at 18,000 rpm at 4 °C in a Sorvall SS34 rotor.

**3. Refolding and Ni-NTA Column Chromatography:** The solubilized anti-PD1 scFv was mixed with QIAGEN Ni-NTA agarose beads (~2 ml of beads) for 1 h at 4 °C with gentle rocking. The Ni-NTA beads, bound to the unfolded anti-PD1 scFv, were then pelleted in a Beckman GS-6R centrifuge at 280 RPM for 10 min; non-bound material was removed by aspiration. As an initial wash, the Ni-NTA agarose resin was re-suspended in Buffer B (5X column volume), incubated for 5 min and then re-pelleted in the GS-6R centrifuge. To refold the anti-PD-1 scFv, the Ni-NTA beads were re-suspended in 10X the bead volume of Buffer C (20 mM Tris.HCl pH 8.0, 100 mM NaCl, 0.1% Triton X-100, 4 mM oxidized glutathione and 4 mM reduced glutathione) and incubated for 30 min at 4 °C. Upon centrifugation at 280 RPM for 10 min, and aspiration of Buffer C, the pellet was re-suspended in 10X column volumes of Buffer D (20 mM Tris.HCl pH 8.0, 100 mM NaCl, 5 mM β-cyclodextrin, 4 mM oxidized glutathione and 4 mM reduced glutathione) and incubated for 1 h at 4 °C. After an additional cycle of centrifugation and aspiration, the pellet was re-suspended in 10X the bead volume of Buffer E (20 mM Tris.HCl pH 8.0 and 0.5 M NaCl). The beads bound to the scFv were then divided into two 1 ml polypropylene disposable columns (QIAGEN) and the resin allowed to settle by gravity.

Prior to elution, the Ni-NTA agarose resin containing the bound anti-PD-1 scFv was washed in ten column volumes of low imidazole “Wash buffer” (20 mM Tris.HCl pH 8.0, 300 mM NaCl, 10% glycerol, 20 mM





**Fig. 2.** Protein induction and purification of the anti-PD-1 scFv from *E. coli* BL21 cells. **A.** Representative SDS-PAGE gel showing the distribution of the anti-PD-1 scFv during stages of purification. Pre-stained “SeeBlue Plus 2” protein size markers (Thermo Fisher) were run in Lane 1. The whole cell lysate following induction with IPTG (20  $\mu$ l) is shown in Lane 2; a key feature is the prominent band running at the expected location of the anti-PD-1 scFv (~27 kDa). Lane 3, the lysate supernatant (20  $\mu$ l) following centrifugation at 18,000 RPM. Lane 4, the cell pellet following solubilization in Buffer B (20  $\mu$ l). Lane 5, an aliquot of the loaded Ni-NTA resin (~5  $\mu$ l) after *in situ* renaturation of the anti-PD-1 scFv and washing with ten column volumes of low imidazole “wash buffer”. **B.** To elute the anti-PD-1 scFv from the Ni-NTA column, 10 mls of “elution buffer” was applied and 1 ml fractions were collected. The peak fractions of the anti-PD-1 scFv were identified by spotting aliquots onto Whatman filter paper and staining with Coomassie Brilliant Blue, as well as subsequent analyses of 20  $\mu$ l aliquots by SDS-Page (lanes 6–8). Finally, our yield of purified anti-PD-1 scFv was lower than expected (i.e., ~1 mg from 2.5 g of bacterial cell pellet). It is noted that during elution with 300 mM imidazole not all of the anti-PD-1 scFv was removed from the Ni-NTA column. In addition, not all of the scFv was recovered from the pellet during the urea/high pH-based extraction (data not shown). While we have yet to determine why these purification steps are sub-optimal, a likely contributor is insoluble aggregation of some fraction of the scFv.

imidazole and 0.02% Tween 80). The bound anti-PD-1 scFv was then eluted with 5 ml of “Elution buffer” (20 mM Tris.HCl pH 8.0, 0.3 M NaCl, 10% glycerol, 0.02% Tween 80 and 300 mM imidazole) and ten 0.5 ml fractions were collected. To immediately lower the pH away from the pI of the scFv (~8.65; determined using the ExPASy WEB site), each Eppendorf collection tube contained 0.5 ml of 2X collection buffer (25 mM Hepes, pH 6.8, 0.4 M NaCl, 100 mM Na citrate dihydrate (pH 6.0), 25% glycerol and 0.02% Tween 80). Following elution, the peak fractions of the anti-PD-1 scFv were detected by Coomassie Blue staining of aliquots spotted onto Whatman filter paper. Fractions containing the highest concentrations of the anti-PD-1 scFv were then dialyzed overnight at 4 °C against 2 L of “Storage buffer” (25 mM HEPES, pH 6.8, 0.125 M NaCl, 0.2 M Mannitol, 21.5  $\mu$ M EDTA, 0.02% Tween 80, 40 mM Na citrate dihydrate and 20% glycerol). The purified anti-PD-1 scFv (~0.2 mg/ml) was stored at –80 °C until needed. Regarding the determination of the concentration of the purified anti-PD-1 scFv; using the ExPASy portal, the extinction coefficient for the 6X His containing anti-PD-1 scFv at 280 nm was determined to be 51,130 M<sup>–1</sup> cm<sup>–1</sup>. After obtaining the spectra of the purified anti-PD-1 on an Agilent 8453 UV–visible spectrophotometer, and subtracting the spectra of the Storage buffer, the concentration of anti-PD-1 scFv samples was determined using Beer’s Law. The accuracy of this approach was confirmed by SDS-PAGE of aliquots of the purified anti-PD-1 scFv and subsequent quantitation using ImageJ.

## 2.2. Modeling the structure of the anti-PD-1 scFv

The program SWISS-MODEL [38] was used to predict the structure of the 27.1 kDa anti-PD-1 scFv; this structure was then displayed in PyMol [39]. To superimpose the predicted structure of the anti-PD-1 scFv with the corresponding region of Nivolumab, the coordinates for the co-structure of human PD-1 bound to the Fab region of Nivolumab [40]

were obtained from the PDB (5WT9). The internal alignment function of PyMol was then used to align the predicted structure of the anti-PD-1 scFv with the corresponding region of Nivolumab (calculation of alignment was placed with a restriction to alpha carbons of the backbone of the proteins).

## 2.3. A molecular model demonstrating that the anti-PD-1 scFv bound to PD-1 will block binding to PD-L1

The residues on PD-1 that bind to the Fab region of nivolumab [40, 41] and those that bind to PD-L1 [42], were previously reported. The anti-PD-1 scFv was positioned opposite the nivolumab-binding site via standard procedures. In brief, starting with the nivolumab/PD-1 co-structure [40], the predicted structure of the anti-PD-1 scFv (described herein) was aligned to nivolumab using the alignment function of PyMol [43].

## 2.4. Western blotting

Human PD-1 was purchased from ACROBiosystems and the indicated amounts were separated by SDS-PAGE on a 10% acrylamide gel. The PD-1 protein, and BSA control, was then transferred to a PVDF membrane (Millipore). After overnight blocking at 4 °C with 5% dry milk in TBST (20 mM Tris (pH 7.5), 137 mM NaCl and 0.1% Tween-20) the membrane was incubated for 1 h at RT with the purified anti-PD-1 scFv (0.25  $\mu$ g/ml) in ~20 ml of TBST containing 2% dry milk. The 6xHis tag on the scFv was subsequently detected using a horseradish peroxidase-conjugated anti-polyhistidine monoclonal antibody ((Abcam: ab49781 diluted 1:2500) and the blot was developed using an enhanced chemiluminescence detection kit (Millipore).

## 2.5. Determining the IC<sub>50</sub> of the anti-PD-1 scFv for PD-1

BPS Bioscience has developed a FRET-based high-throughput assay that measures the effectiveness of molecules at inhibiting the PD-1/PD-L1 interaction <http://bpsbioscience.com/pd-1-pd-l1-tr-fret-72038>. In brief, to identify a PD-1/PD-L1 binding inhibitor, a 384-well time-resolved fluorescence resonance energy transfer (TR-FRET)-based assay was used that measures the binding of biotinylated PD-L1 (PD-L1-biotin) to europium-labeled PD-1 (PD-1-Eu). To conduct the assay, PD-L1-biotin, PD-1-Eu and streptavidin-allophycocyanin (SA-APC) are incubated in the presence of a range of compound concentrations. In the absence of inhibition, the complex PD-L1-biotin/SA-APC is bound to PD-1-Eu and allows the resonance energy transfer from the donor Eu to the acceptor APC; as a result, the final fluorescence emission is high. In the presence of an inhibitor, the interaction between PD-L1-biotin/SA-APC and PD-1-Eu is blocked; therefore, the energy transfer is disrupted and fluorescence emission from the acceptor is low.

## 3. Results

### 3.1. The isolation of an anti-PD-1 scFv from *E. coli*

The residues from the V<sub>H</sub> and V<sub>L</sub> chains of Nivolumab used to form the ~27 kDa anti-PD-1 scFv are presented in Fig. 1A1 (blue and red sequences, respectively). The regions comprising the V<sub>H</sub> and V<sub>L</sub> chains were connected via the (Gly<sub>4</sub>S)<sub>3</sub> linker [35] that is depicted in Fig. 1A2 (yellow line; C-terminus of V<sub>H</sub> linked to the N-terminus of V<sub>L</sub>). The sequences encoding the anti-PD-1 scFv were purchased as a gBlock from IDT and cloned into plasmid pLIC (Materials and Methods). A schematic of the region of plasmid pLIC-His-anti-PD-1, that encodes the V<sub>H</sub> and V<sub>L</sub> regions of the anti-PD-1 scFv, as well as the N-terminal 6xHis and TEV protease sites, is presented in Fig. 1B1. Furthermore, the DNA sequences of the 6xHis and TEV protease regions of the vector are presented in Fig. 1B2. Immediately below the DNA sequence are the amino acids encoded by these residues, including the Met used to initiate the 6xHis



and TEV protease site-containing anti-PD-1 scFv.

The procedure used to express the anti-PD-1 scFv in *E. coli* BL21, and the Ni-NTA resin-based protocol used to purify the molecule, are also described in the Materials and Methods section. It is stressed that the purified anti-PD-1 scFv contains the N-terminal 6xHis tag and the TEV protease cleavage site. SDS-PAGE analysis of aliquots taken during key stages of purification are presented in Fig. 2A (lanes 2–5). Additional SDS-PAGE analyses (Fig. 2B; lanes 6–8) established that the isolation procedure resulted in the purification to apparent homogeneity of the anti-PD-1 scFv. Finally, using this approach, approximately 1 mg of the anti-PD-1 scFv was purified from 4 g of bacterial cell pellet.

### 3.2. The predicted structure of the anti-PD-1 scFv

**A. Phyre 2 based model of the scFv:** A model of the structure of the anti-PD-1 scFv was obtained using the program Phyre 2 [43]. A ribbon diagram of the predicted structure of the scFv that includes the V<sub>H</sub> (blue), V<sub>L</sub> (red) and (Gly<sub>4</sub>S)<sub>3</sub> linker (yellow) regions of the molecule, is presented in Fig. 3A. The side chains for the C-terminal arginine (Fig. 1A1) are shown as red spheres. Residues of the molecule including the N-terminal 6xHis [44] and TEV protease site (Fig. 1B1) are, however, likely to be unstructured (see legend) and therefore, these residues were not included in the modeling studies. Thus, the structure presented in Fig. 3A depicts only the predicted location of the N-terminus of the V<sub>H</sub> chain. Finally, to help locate the region of the anti-PD-1 scFv involved in binding to PD-1 [40,41], Fig. 3A also depicts where PD-1, symbolized by the dotted circle, would dock to the surface of anti-PD-1 scFv.

**B. Superposition of the scFv model onto the structure of full-length nivolumab:** The co-structure of the nivolumab-Fab complex bound to human PD-1 has been reported [41,45]. Therefore, as an initial test of the validity of the predicted structure of the anti-PD-1 scFv, the program

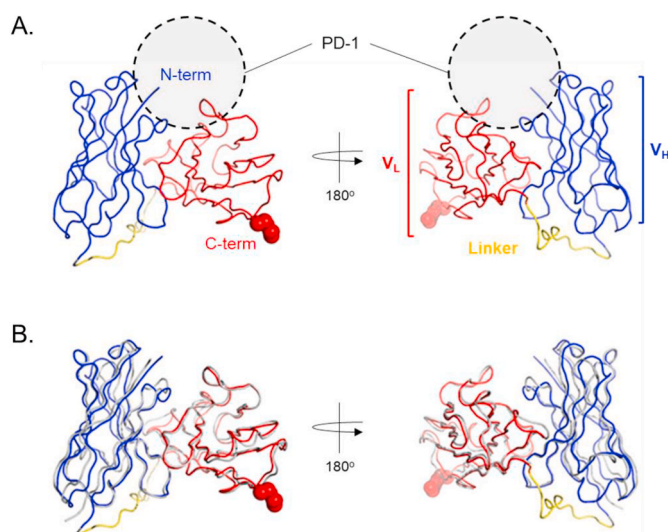
Coot [46] was used to superimpose the model of the anti-PD-1 scFv (Fig. 3A) onto the co-structure of nivolumab-Fab bound to PD-1 (Fig. 3B; the nivolumab-Fab/PD-1 co-structure is shown in gray). These analyses establish that there is extensive alignment between the two structures (the RMSD for the superimposed structures was 1.049), suggesting that the predicted structure is a reasonable working model of the anti-PD-1 scFv.

### 3.3. The purified anti-PD-1 scFv selectively binds to human PD-1 and blocks interactions with PD-L1

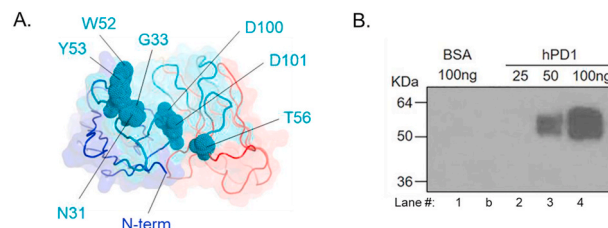
**A. The antigen-binding site on the anti-PD-1 scFv:** Fig. 4A presents a space-filling model of the anti-PD-1 scFv that depicts both the predicted antigen-binding region (shown in cyan) and residues in nivolumab (shown in teal) that are known to interact with PD-1 [40,41]. Importantly, residues derived from the (Gly<sub>4</sub>S)<sub>3</sub> linker are not predicted to be in a position to obscure the residues used to bind to PD-1. However, given the previously discussed uncertainties regarding the location(s) of the N-terminal 6xHis and TEV protease regions, these N-terminal sequences might be situated on the surface of the anti-PD-1 scFv in a manner that could interfere with binding to PD-1. Therefore, to eliminate this possibility, and to confirm the hypothesis that we had identified the amino acid residues needed for a functional anti-PD-1 scFv, we tested if the 6xHis and TEV protease site-containing anti-PD-1 scFv binds to purified PD-1.

**B. The anti-PD-1 scFv binds to human PD-1:** To determine if the purified anti-PD-1 scFv binds to PD-1, full-length human (h) PD1 was purchased from ACROBiosystems and subjected to SDS-PAGE. A Western blot was then performed, using the 6xHis anti-PD-1 scFv as the primary antibody and an anti-polyhistidine monoclonal Ab conjugated to HRP as the secondary Ab (materials and methods). Inspection of Fig. 4B establishes that the anti-PD-1 scFv bound to either 50 or 100 ng of purified hPD1, but not to 100 ng of BSA (compare lane 1 with lanes 3–4). Therefore, it was concluded that the anti-PD-1 scFv is active and that the N-terminal 6xHis and TEV protease regions do not obscure residues needed for binding to PD-1. Finally, it is noted that the full-length human PD1 from ACROBiosystems is heavily glycosylated; therefore, this molecule does not run as a distinct species.

**C. Determining the half maximal inhibitory concentration of the anti-PD-1 scFv needed to disrupt the PD-1/PD-L1 interaction:** The IC<sub>50</sub> of molecules that bind to purified PD-1 can be determined using a FRET-



**Fig. 3.** Models depicting the predicted structural features of the anti-PD-1 scFv. **A.** A model of the anti-PD-1 scFv antibody generated using the program Phyre 2 [43]. The V<sub>H</sub> domain is shown in blue, the C-terminal V<sub>L</sub> region is shown in red and the (Gly<sub>4</sub>S)<sub>3</sub> linker is shown in yellow. The location of the side chain of the C-terminal (abbreviated C-term) arginine is indicated. Regarding sequences in the N-terminal region, TEV protease recognizes a linear epitope and His tags are known to be unstructured; therefore, the 6X His and TEV cleavage sites are not included in this model. As a result, the depicted N-terminal (abbreviated N-term) is only an approximation. Finally, the circular dotted line symbolizes PD-1 and is used to establish the relative location of the surface on the anti-PD-1 scFv that binds to PD-1 (based on the nivolumab-Fab complex bound to human PD-1 [41]). **B.** Superimposition of the predicted structure of the anti-PD-1 scFv (blue, red and yellow ribbons) onto the corresponding region of the nivolumab-Fab co-structure [41]; shown in gray) that was bound to human PD-1.



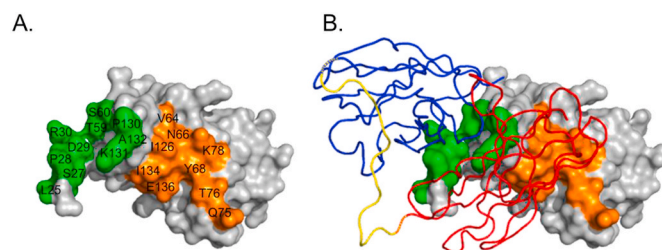
**Fig. 4.** A surface representation of the anti-PD-1 scFv showing residues needed for binding to PD-1 and evidence that the anti-PD-1 scFv binds to PD-1. **A.** A view of the anti-PD-1 scFv showing the locations of residues (in teal) needed for binding to PD-1 (determined using the nivolumab-Fab/PD-1 co-structure [41]). Based on this model, it was predicted that the PD-1 binding surface on the anti-PD-1 scFv is not blocked by the (G<sub>4</sub>S)<sub>3</sub> linker. However, since the location (s) of the 6X His and TEV cleavage sequences are unknown, the modeling studies could not address whether residues from the N-terminus of the anti-PD-1 scFv obscure the residues needed for PD-1 binding. **B.** Western blot-based evidence that the anti-PD-1 scFv binds to human PD-1. The Western blot was conducted with the indicated amounts (25–100 ng) of human PD-1 (lanes 3–5). In contrast, binding was not detected to 100 ng of the control BSA sample (lane 2). The secondary Ab was an anti-polyhistidine monoclonal Ab conjugated to HRP (Abcam: ab49781). Protein size markers were also loaded on the gel; the relative mobilities of the individual markers are indicated. Finally, “b” stands for blank.

based competition assay developed by BPS Bioscience. A depiction of this FRET assay is presented in Fig. 5A and the individual symbols are defined in the figure legend. It is stressed that this assay measures the extent to which a molecule that binds to PD-1 is able to disrupt the PD-1/PD-L1 interaction. Using this assay, it was established that the anti-PD-1 scFv disrupted the PD-1/PD-L1 interaction with an IC<sub>50</sub> of 26 nM (Fig. 5B1). Additional evidence for tight binding is the observation that the anti-PD-1 scFv maintained ~15% inhibition at 6 nM. As a control, a second reaction was conducted with the nonpeptidic PD-1/PD-L1 complex inhibitor BMS 202 (Fig. 5B2). In this assay, the IC<sub>50</sub> for BMS202 was determined to be 8.2 nM, which compares favorably to the previously published value of 18 nM [47]. In summary, the anti-PD-1 scFv binds tightly to human PD-1 and this association blocks the further interaction between PD-1 and PD-L1.

### 3.4. Evidence that the disruption of the PD-1/PD-L1 interaction by the anti-PD-1 scFv is due to steric exclusion

Nivolumab is known to block the PD-1/PD-L1 interaction owing to a steric clash resulting from residues in the V<sub>L</sub> region of nivolumab obscuring the PD-L1 binding site ([41]; reviewed in Refs. [48,49]). As a test of the hypothesis that the V<sub>L</sub> region present in the anti-PD-1 scFv blocks PD-L1 binding in a similar manner, we modeled the docking of the anti-PD-1 scFv to PD-1 and then analyzed whether the residues needed for binding by PD-L1 are obscured.

In Fig. 6 (left panel), the residues on the surface of PD-1 that comprise the binding site for PD-L1 [42] are shown in orange, while those used to bind nivolumab are colored in green. Inspection of Fig. 6 (right panel) reveals that upon binding of the anti-PD-1 scFv (depicted as the blue, yellow and red ribbon diagram) to PD-1 that the PD-L1 binding site is blocked. Thus, our modeling studies indicate that the same mechanism is used by the anti-PD-1 scFv, and full-length nivolumab, to block the PD-1/PD-L1 interaction.

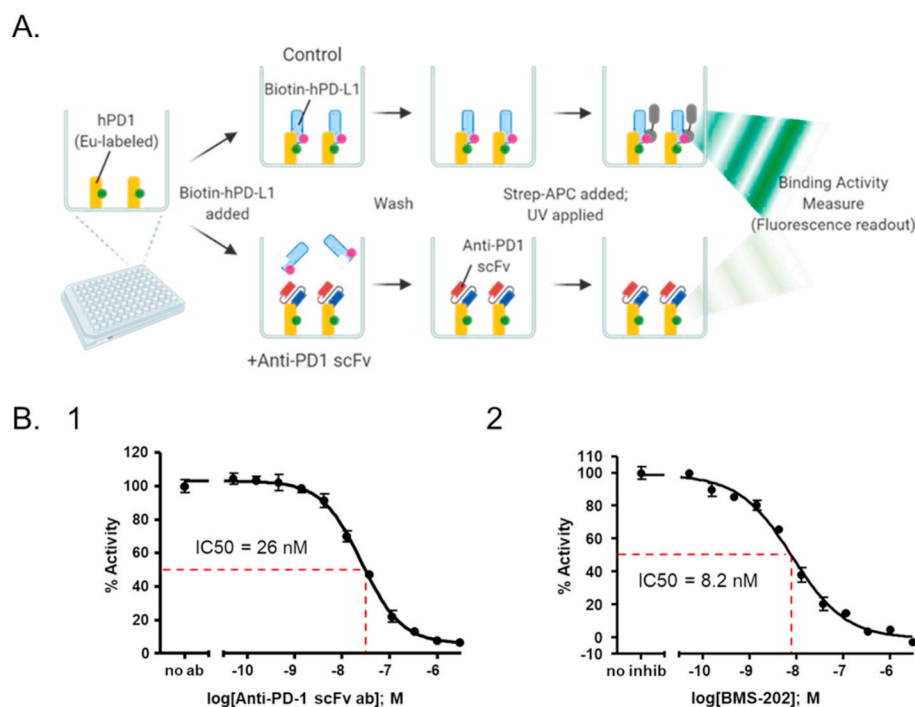


**Fig. 6.** A model of the surface of PD-1 illustrating that the binding site for PD-L1 would be obscured upon binding of the anti-PD-1 scFv to PD-1. **A.** Depiction of the surface of PD-1 showing residues utilized for binding to Nivolumab ([40]; shown in green) and those required for binding to PD-L1 ([42]; shown in orange). **B.** Upon docking of the anti-PD-1 scFv to PD-1, using the residues needed to bind Nivolumab, it is apparent that the V<sub>L</sub> region of the anti-PD-1 scFv (in red) would obscure the PD-1 residues needed for binding to PD-L1.

## 4. Discussion

Full-length anti-PD1 monoclonal Abs have been used with remarkable success to treat certain cancers (reviewed in Refs. [4,48,50–53]). However, there are limitations associated with full-length Ab based immune checkpoint therapies (reviewed in Ref. [54]). For instance, the majority of patients will not respond, or will respond only incompletely, to PD-1 or PD-L1 inhibitors (reviewed in Refs. [3,4,7,16]). Furthermore, intact immunoglobulins are too large for effective tumor penetration and their slow clearance from the blood can result in high retention in multiple organs, including the liver [55]. Therefore, there is a need to pursue the development of additional reagents for immune checkpoint therapies, such as the anti-PD-1 scFv described herein.

That scFvs can be used as therapeutic agents in checkpoint and cancer therapy has been previously established (reviewed in Ref. [56]). For instance, an scFv derivative of the PD-L1 targeting antibody Avelumab has been reported [57], as has an scFv targeting human CTLA-4 [58]. Furthermore, Rafiq et al. reported that CAR-T cells that secrete a PD-1-blocking scFv enhanced the survival of mice harboring several



**Fig. 5.** Determining the IC<sub>50</sub> for the anti-PD1 scFv. **A.** An overview of the BPS Bioscience competition assay, which was used to determine the concentration of the anti-PD-1 scFv needed for 50% inhibition of binding of PD-L1 to human PD-1. The anti-PD-1 scFv is symbolized by the linked red and dark blue rectangles, human PD-1 by the yellow rectangle and PD-L1 by the light blue images. Note that in this assay, PD-1 is linked to europium (Eu) (green dot); PD-1-Eu, human PD-L1 is conjugated to biotin (purple dot) and the reaction takes place in the presence of streptavidin-allophycocyanin ((SA-APC); gray structure bound to biotin). In the absence of inhibitor, PD-L1 biotin forms a complex with SA-APC and PD-1-Eu. Upon excitation by U.V. light, there is an energy transfer from the donor Eu to the SA-APC acceptor and thus high fluorescence emission (top row). In the presence of an inhibitor that binds to PD-1 (e.g., the anti-PD-1 scFv), the interaction between PD-L1-biotin/SA-APC and PD-1-Eu is blocked. Therefore, the U.V. dependent energy transfer is disrupted and fluorescence emission from the acceptor is low (bottom row). **B.** Use of the BPS Bioscience competition assay to determine the IC<sub>50</sub> of the anti-PD-1 scFv for human PD-1. The IC<sub>50</sub> of the anti-PD-1 scFv, derived from the three separate experiments shown in the plot, was determined to be 26 nM (Fig. 5B1). As a control, the competition assay was repeated in the presence of the small molecule BMS-202; based on three separate assays, the IC<sub>50</sub> was determined to be 8.2 nM (Fig. 5B2).

different types of tumors [59]. They also reported that the scFvs secreted by CAR-T cells remain locally in the tumor microenvironment. In addition, a novel method was used to generate libraries of scFvs targeting PD-1, that were subsequently re-engineered into full-length monoclonal Abs for additional validation [60].

The clinical significance of targeting the PD-1/PD-L1 axis by full-length Abs, or Ab fragments, is further illustrated by the fact that a broad range of antiviral immune responses is regulated by the PD-1/PD-L1 interaction. For instance, exhausted CD8 T cells present in mice chronically infected with lymphocytic choriomeningitis virus (LCMV) had up-regulated PD-1 levels and antibodies that blocked the PD-1/PD-L1 interaction restored T-cell responses [61]. Furthermore, a wide range of viruses, including Hepatitis B and C [62] and HIV [63], are known to up-regulate PD-1 ligands on both hematopoietic and non-hematopoietic cells (reviewed in Ref. [64,65]). The possibility of treating SARS-CoV-2 with immune checkpoint inhibitors has also been discussed [66]. Of additional interest, treatment with anti-PD-1 antibodies led to durable tumor regression in patients with Merkel Cell Carcinoma (e.g. Refs. [67–70]) and positive responses in PML patients (reviewed in Ref. [65, 71]). In view of these reports, the anti-PD-1 scFv, or second-generation derivatives, may have additional efficacy as both anti-viral agents and reagents to combat virally induced diseases.

In terms of the mechanism utilized by the anti-PD-1 scFv to disrupt the PD-1/PD-L1 interaction, the purified anti-PD-1 scFv has an IC50 of 26 nM, while the IC50 of full-length nivolumab is 2.52 nM (as determined by surface plasmon resonance [72]). Thus, the IC50s, obtained using two separate methods, are not identical. Whether this difference is because the anti-PD-1 scFv is actually 10-fold weaker than full-length nivolumab, or if only 10% of the scFv molecules are active, remains to be determined. Nevertheless, the tight binding of the anti-PD-1 scFv to PD-1 is in keeping with previous experiments that demonstrated that scFv fragments that contain the complete antigen-binding site of an antibody, and have not undergone reorientation of the two domains, have the same monomeric binding affinity as the parental monoclonal antibody [73]. Furthermore, based on the design of the BPS Bioscience FRET-based assay, it is apparent that once tightly bound to PD-1, the anti-PD-1 scFv blocks the ability of PD-L1 to interact with PD-1. Indeed, our modeling studies indicate that the anti-PD-1 scFv blocks the PD-1/PD-L1 interaction via the same steric exclusion mechanism utilized by full-length Nivolumab (reviewed in Ref. [48]).

Regarding possible clinical advantages of the purified anti-PD-1 scFv, high tumor penetrance is an established clinical advantage of relatively small scFv fragments [31]. An additional property of the scFvs is that owing to their high dissociation rates, they are cleared relatively rapidly from the body [55,74]. This has been suggested to be an advantage [6], since agents that clear rapidly from the circulation, relative to intact IgGs, have the potential for lower toxicities. However, if rapid clearance is demonstrated to be a limitation of the anti-PD-1 scFv, the half-life and stability of the anti-PD-1 scFv could be increased by various modifications, including linkage to polyethylene glycol ([75]; reviewed in Ref. [30]). Alternatively, its size could be increased to ~60 kDa using techniques such as peptide linker dimerization [74,76]. Dimerization would be expected to increase the avidity of the anti-PD-1 scFv for PD-1 (e.g. Refs. [30,77]), while its increased size would likely reduce clearance by the kidney [74].

A related consideration is that owing to Fc dependent interactions with a range of cellular receptors [78], many full-length Abs have the potential to induce cellular effector functions (including targeted cell lysis and phagocytosis (e.g. Ref. [79,80])). However, the IgG4 Ab subclass is an exception. They have a low affinity for Fc and C1q receptors; thus, they poorly activate host effector functions [81]. Indeed, the IgG4 isotype is the preferred class for immunotherapy when host effector function(s) are undesirable [81]. As a result, the anti-tumor effectiveness of nivolumab, an IgG4 monoclonal Ab, is not dependent upon the induction of host effector functions. Similarly, since the anti-PD-1 scFv does not contain an Fc region, it is unlikely to induce cellular effector

functions. A related advantage of the anti-PD-1 scFv is that full-length anti-PD-1 mAbs are captured within minutes from the surface of T-cells by tumor-associated macrophages and the Fc domains of the mAbs play a critical role in this interaction [82]. Since the anti-PD-1 scFv does not contain an Fc domain, its binding to T-cells should be less prone to disruption by tumor-associated macrophages.

An additional concern is that checkpoint therapies utilizing full-length antibodies are associated with “immune-related adverse effects” (IRAEs; ([83,84]; reviewed in Ref. [85]). For instance, IRAEs occur in up to 70% of patients treated with PD-1/PD-L1 antibodies (reviewed in Ref. [85]) and serious (i.e., grade 3 or 4) adverse events were associated with many patients being treated with nivolumab ([49, 51,86,87]; reviewed in Ref. [50]). In addition, the frequency of adverse events was much higher when full-length nivolumab was used in combination therapy with CTLA-4 targeting ipilimumab [88]. Addressing IRAE related concerns, it was proposed that CAR-T based localized delivery of the much smaller anti-PD-1 scFvs to tumors may decrease the IRAEs associated with checkpoint blockades [59]. Likewise, scFvs, including the anti-PD-1 scFv described herein, may reduce IRAEs owing to related properties, such as their inherently lower immunogenicity [33].

Given the results presented herein, and the properties of scFvs summarized above, it is apparent that continued characterization and modification of the anti-PD-1 scFv is warranted. As noted previously, the anti-PD-1 scFv could serve as the building block for the production of larger derivatives, including bi-specific and multi-variant antibody fragments (e.g., Refs. [89–92]; reviewed in Ref. [93]). Moreover, to enhance diagnostic tests for the presence of PD-1 in tumor biopsies, the anti-PD-1 scFv could be coupled to fluorescent protein domains. The anti-PD-1 scFv could also be coupled to radioactive tracers and used in whole-body imaging studies (e.g. Ref. [94]). Regarding the fact that the anti-PD-1 scFv is purified from *E. coli*, since the 1980's there have been a number of recombinant biopharmaceuticals produced in *E. coli* (reviewed [95]) and biopharmaceuticals produced in *E. coli* include antibodies (e.g. Ref. [96]). Finally, the high cost of production of therapeutic antibodies in mammalian cell culture can limit their availability to patients (e.g. Ref. [80]); therefore, a significant incentive for further studies of the anti-PD-1 scFv purified from *E. coli* is that it can be manufactured at greatly reduced costs.

## CRedit authorship contribution statement

**Jong Shin:** Investigation, Software, Data curation, Methodology. **Paul J. Phelan:** Investigation, Software, Methodology, Writing - review & editing. **Ole Gjoerup:** Conceptualization, Methodology. **William Bachovchin:** Conceptualization. **Peter A. Bullock:** Conceptualization, Methodology, Supervision, Writing - review & editing.

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