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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+ 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-PD-1 monoclonal antibodies include Nivolumab
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<sub>H</sub> and V<sub>L</sub> regions were identified (Fig. 1A1). DNA encoding the V<sub>H</sub> and V<sub>L</sub> regions, and a segment of DNA encoding a (Gly<sub>2</sub>S<sub>2</sub>) repeat used to link the V<sub>H</sub> and V<sub>L</sub> 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’ CGAGGCCGGTGCTTTGCAGGATCCG3’ and the reverse primer was 5’ CGAGGCTGCTCCCTGGAAATACAGG3’). 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 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’ CGAGGCCGGTGCTTTGCAGGATCCG3’ and the reverse primer was 5’ CGAGGCTGCTCCCTGGAAATACAGG3’). 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 ml 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 though 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-PD1 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 polystyrene disposable columns (QIAGEN) and the resin allowed to settle by gravity.

Prior to elution, the Ni-NTA agarose resin containing the bound anti-PD1 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 (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 scFv 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.
3. Results

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

The residues from the V_H and V_L 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_H and V_L chains were connected via the (Gly_3Ser) linker [35] that is depicted in Fig. 1A2 (yellow line; C-terminus of V_H linked to the N-terminus of V_L). 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_H and V_L regions of the anti-PD-1 scFv, as well as the N-terminal 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
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 $V_H$ (blue), $V_L$ (red) and (Gly$_S$)$_3$ 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 $V_H$ 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-PD1 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 is 1.049), suggesting that the predicted structure is a reasonable working model of the anti-PD1 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$_S$)$_3$ 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-PD1 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-polylhistidine monoclonal Ab conjugated to HRP as the secondary Ab (materials and methods). Inspection of Fig. 4B establishes that the anti-PD1 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-PD1 scFv needed to disrupt the PD-1/PD-L1 interaction: The IC$_{50}$ of molecules that bind to purified PD-1 can be determined using a FRET-
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scFv disrupted the PD-1/PD-L1 interaction with an IC50 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 IC50 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\textsubscript{\alpha} region of nivolumab obscuring the PD-L1 binding site ([41]; reviewed in Refs. [48,49]). As a test of the hypothesis that the V\textsubscript{\alpha} 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. 5. Determining the IC50 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 thus high fluorescence emission (top row). 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 IC50 of the anti-PD-1 scFv for human PD-1. The IC50 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 IC50 was determined to be 8.2 nM (Fig. 5B2).

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 Avolumab 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
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 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 scFv 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 scFv 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. [92]). 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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