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Soluble production of a biologically active single-chain antibody against murine PD-L1 in *Escherichia coli*

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ABSTRACT

Programmed death ligand 1 (PD-L1), is an important regulator of T-cell activation and has emerged as an important target for cancer immunotherapy. Single chain variable fragments (scFvs) have several desirable characteristics and are an attractive alternative to monoclonal antibodies for experimental or therapeutic purposes. Three chickens were immunized against murine PD-L1, and mRNA isolated from their spleens was used to generate an immunized immunoglobulin variable region library. Using splice-overlap extension PCR, variable region cDNAs were combined to generate full-length scFvs. M13 phage display of the resulting scFv library identified a functional scFv against PD-L1 (αPD-L1 scFv). The scFv was expressed as soluble protein in the periplasm and culture supernatant of recombinant *Escherichia coli* and purified with a 6×-His tag using immobile metal affinity chromatography. The dissociation constant of αPD-L1 scFv was determined to be 7.11×10^{-10} M, and the scFv demonstrated inhibitory biological activity comparable to an antagonistic monoclonal antibody, providing an alternative agent for blocking PD-1/PD-L1 signaling.

Introduction

Programmed death 1 (PD-1)[1] is a key inhibitory receptor that affects T-cell function. PD-1 is expressed on T-cells upon activation and is increased when the co-stimulatory molecule CD28 binds to its ligands B7.1 or B7.2 on antigen presenting cells. PD-1 primes T-cells for regulatory signals and limits their activation. Mice deficient in this receptor develop severe autoimmune disorders soon after birth [1]. PD-1, upon engagement with its ligands PD-L1 and PD-L2, inhibits T-cell activation in response to antigen [2]. While important in peripheral tolerance, PD-1 attenuates anti-tumor immune responses, thereby limiting the efficacy of cancer immunotherapies such as immunostimulatory cytokine treatment. Additionally, tumor cells in several cancers will exploit PD-1-mediated inhibition by surface expression of PD-L1 [3]. Because of these properties, immunostimulation by PD-L1 blockade has received considerable attention as cancer treatment in preclinical [4–7] and clinical [8] studies.

The primary agents used to block PD-L1 are monoclonal antibodies (mAb), but mAb production is costly and requires maintenance of genetically unstable hybridoma cell cultures. In addition, immunogenicity and interactions of the Fc domains of mAbs can lead to compliment fixation or phagocytosis of bound cells, complicating the interpretation of experimental results and interfering with therapeutic effects [9,10]. Furthermore, the toxicities associated with systemic antibody therapies [10] has warranted the investigation of viral and bacterial delivery systems that specifically target the tumor microenvironment; however these systems are generally incapable of expressing complex antibody molecules. To overcome some of the problems associated with full-length mAbs, smaller antibody formats have been developed [11].

An scFv is an antibody fragment that consists of the variable heavy (V_H) and variable light (V_L) domains of one arm of an immunoglobulin (Ig) protein bound together via a flexible polypeptide linker. scFvs can retain specificity and affinity for their antigens with the reduced complexity of a single polypeptide chain without the Fc or other constant region [12]. The simple structure of scFvs eliminates the complications associated with whole antibodies. Moreover, they have demonstrated superior tissue-penetrating capabilities when compared to whole antibodies [9,13,14]. Additionally, the reduced complexity of scFvs allows for production in prokaryotic cells, which is often desirable due to the lower maintenance required and the rapid growth of prokaryotic expression systems when compared to eukaryotic hosts [15,16].

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[1] *Abbreviations used:* PD-1, programmed death 1; PD-L1, programmed death ligand 1; mAb, monoclonal antibodies; Ig, immunoglobulin; IgY, yolk immunoglobulin; HA, hemagglutinin; MHC, major histocompatibility complex.

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Typically, scFvs are cloned from the variable regions of existing high-affinity B-cell hybridomas, which often results in scFvs that are unstable, of low affinity, or fold incorrectly due to the absence of stabilizing constant regions. These limitations have restricted their use as blocking agents [17]. Selection for scFvs by phage display can overcome some of these limitations but is most often performed on suboptimal variable region libraries from naïve mammalian hosts, preventing the isolation of high-affinity scFvs [18]. Alternative hosts, such as chickens, have been shown recently to be more effective for scFv development [18]. The avian germline sequences encoding Ig variable regions are well suited for scFv library development. Consisting of a single set of V_H and V_L regions, the entire Ig repertoire can be easily captured via PCR [19]. Additionally, scFvs generated from chicken variable-region libraries typically have higher affinities due to the greater immune response to mammalian proteins generated in a non-mammalian host. Other advantages include greater inherent stability of polypeptides generated from chicken variable regions [18] and the reported successes of multi-antigen immunizations [20–22]. For these reasons, we constructed an immunized chicken scFv library and used phage display to successfully isolate a biologically active, high affinity antagonistic scFv against mouse PD-L1 that is readily expressed as soluble protein in *Escherichia coli*, is easily purified and antagonizes PD-1/PD-L1 signaling, serving as an attractive alternative reagent to mAbs for blocking PD-L1 signaling.

Materials and methods

Chicken immunizations and IgY purification

Immunizations were based on a protocol developed by Finlay et al. [23], and were performed at Aves Labs (Oregon). Three female chickens of egg-laying age were immunized intramuscularly with purified recombinant mouse PD-L1 (Sino Biological). The immunization regime included 4 intramuscular injections three weeks apart: (1) 50 μg PD-L1 emulsified in complete Freund's adjuvant; (2) 50 μg PD-L1 in incomplete Freund's adjuvant; (3 and 4) 25 μg PD-L1 in incomplete Freund's adjuvant. Eggs were collected for 10 days before immunization and after final the immunization. IgY from egg yolk was purified at Aves Labs (Oregon).

IgY ELISA

High protein-binding 96-well plates (Costar: 3590) were coated with 1.25 μg/ml recombinant PD-L1 (Sino biological: 50010-M08H) in 0.1 M sodium carbonate pH 9.5 overnight at 4 °C. Plates were washed three times in PBS + 0.1% Tween-20. Plates were blocked using 5% milk for 2 h at room temperature and washed as before. Varying dilutions of IgY from each chicken were added to plates and incubated for 90 min at room temperature. Plates were washed, and bound IgY was detected using an anti-IgY-HRP conjugate (Aves Labs: H-1004) diluted 1:5000 in 5% milk for 90 min at room temperature followed by washing and detection with OptEIA substrate (BD: 555214) for 30 min according to manufacturers' protocol. Absorbance at 450 nm was corrected for background by subtracting absorbance at 570 nm.

RNA isolation and cDNA generation

Chickens were sacrificed and their spleens were frozen in RNAlater (Qiagen). Total RNA was isolated from 200 mg of each spleen using an RNeasy Midi Kit (Qiagen) according to manufacturers' instructions. cDNA was generated from total RNA using SuperScript™ 1st Strand Synthesis System for RT-PCR (Life Technologies) with random hexamers according to manufacturers' instructions.

PCR synthesis of scFv library

Library synthesis was performed according to methods published by Finlay et al. [23], including PCR primers and temperature parameters. Briefly, V_L and V_H libraries were amplified from cDNA from each chicken using PCR primers flanking the variable regions. PCR reactions were repeated 10 times with new cDNA to in order to retain as much library diversity as possible. Resulting DNA fragments were purified from an agarose gel using a Qiaquick Gel Extraction Kit (Qiagen) and pooled for each chicken. Individual V_L and V_H region libraries were randomly combined with the addition of a flexible peptide linker using splice overlap extension PCR with defined primers [23], and 4 μg of gel-purified scFv DNA from each chicken was combined to generate the final scFv library.

Ligation and transformation of TG1 cells for phage display

The scFv library was ligated into pComb3× phagemid vector (from Dr. Barbas, Scripps Research Inst.). Before ligation, 40 μg of pComb3× vector and 10 μg of the combined scFv library were digested with Sfi1 in a 200 μl final reaction volume (New England Biolabs) according to manufacturers' instructions for 8 h at 50 °C. Digested fragments were separated by gel electrophoresis and extracted from agarose using a Gel Extraction Kit (Qiagen). The pComb3× vector was treated with Antarctic phosphatase (New England Biolabs: M0289S) to prevent self-ligation according to manufacturers' instructions. Phosphatase was removed before ligation using a PCR cleanup Kit (Qiagen). The pComb3× vector and scFv library were ligated with T4 DNA ligase (New England Biolabs) using 1.5 μg of vector and 1 μg scFv library for a 1:3 vector to insert molar ratio in 200 μl total reaction volume for 16 h at 16 °C. Following ligation, the DNA library was freed of enzyme and ligation buffer using a PCR Cleanup Kit and eluted in 40 μl sterile deionized water. The entire ligation was added to 300 μl of electrocompetent *E. coli* TG1 cells (Agilent Technologies), and cells were electroporated according to manufacturers' instructions, rescued in 5 ml of pre-warmed S.O.C. medium, plated on 2× yeast-tryptone agar containing 100 μg/ml carbenicillin in a 22 × 22 cm Qtray (Genetix) and incubated overnight at 37 °C. A 100 μl aliquot of the transformation was serially diluted and plated for counting to determine the theoretical library diversity. After incubation, colonies were scraped from Qtrays in 20 ml 2× yeast-tryptone broth + 15% glycerol and stored in aliquots at −80 °C for phage display and panning.

Phage display and panning for binding sequences

Phage display and binding screens were performed according to methods published by Finlay et al. [23]. Briefly, the *E. coli* TG1 cells containing the scFv library were infected with M13K07 helper phage (New England Biolabs). Infected cultures were grown overnight and phage displaying the scFvs were precipitated and allowed to bind immobilized PD-L1 (Sino Biological: 50010-M08H). Unbound phages were removed by washing 10 times with PBS − 0.1% Tween-20 and 10 times with PBS. Antigen-bound phage were eluted with 0.1 M glycine pH 2.2 and used to infect *E. coli* strain ER2738 (Agilent Technologies), which were spread onto Qtrays containing 50 μg/ml carbenicillin. The selection and re-infection procedure was repeated for four total rounds. An aliquot of input and output phage was titered for each panning round to monitor enrichment of binding sequences.

ELISA screen for binding sequences

Several colonies from the output of round 4 of panning were tested for antigen binding. Colonies were inoculated into