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Evaluation of plant-expressed human PD-1 as an immunogen capable of inducing native PD-1 recognizing antibodies

Hong Ju Moon^{1†}, Gyeongik Ahn^{1†}, Seong-Ah Shin², Chang Sup Lee², Woe-Yeon Kim^{1*}  and Min Gab Kim^{2*}

Abstract

Programmed cell death protein-1 (PD-1) is a key immune checkpoint receptor and an important target for therapeutic antibody development. A critical factor in the development of therapeutic monoclonal antibodies is the selection and preparation of the target antigen. Here, we established a plant-based platform for human PD-1 production using *Nicotiana benthamiana* and evaluated its biochemical properties and immunogenic potential in comparison with bacterial (*E.coli*) and mammalian (HEK293T cells) expression systems. Codon optimization and promoter selection significantly enhanced transient PD-1 expression, with the pFM'M vector producing the highest yield. Although plant-derived PD-1 predominantly accumulated in the insoluble fraction and required denaturing purification, sufficient quantities were obtained for immunological evaluation. Mice immunized with plant-produced PD-1 exhibited strong splenic and lymph-node responses. ELISA and flow cytometry analyses demonstrated that antibodies generated from plant-derived PD-1 recognized native, cell-surface PD-1, although with lower potency than antibodies induced by mammalian cell-derived antigen. While further optimization is required to improve solubility and preserve native protein conformation, this study highlights the potential for generating antigens for therapeutic antibody discovery. Thus, *N. benthamiana* is a viable platform for producing immunogenic human PD-1 capable of eliciting functional antibodies.

Keywords Recombinant protein production, Plant-based antigen, Human PD-1, Antibody development, Immunogenicity

Introduction

Monoclonal antibodies (mAbs) are highly specific immunoglobulins produced by B cells that recognize and bind to distinct antigenic epitopes. Since the establishment of hybridoma technology, the ability to generate monoclonal antibodies has transformed both basic research and therapeutic applications [1]. In parallel, advances in genetic engineering, protein expression platforms, and large-scale purification technologies have enabled the production of therapeutic antibodies with improved affinity, stability, effector functions, and in vivo half-life [2]. In the clinical field, monoclonal antibodies have emerged as one of the most successful classes of biopharmaceuticals,

[†]Hong Ju Moon and Gyeongik Ahn contributed equally.

*Correspondence:

Woe-Yeon Kim

kim1312@gnu.ac.kr

Min Gab Kim

mgk1284@gnu.ac.kr

¹Division of Applied Life Science (BK21 Four), PBRR, PMBBRC, RILS & IALS, Gyeongsang National University, Jinju 52828, Republic of Korea

²College of Pharmacy and Research Institute of Pharmaceutical Sciences, Gyeongsang National University, Jinju 52828, Republic of Korea

demonstrating remarkable efficacy in the treatment of cancers, autoimmune disorders, infectious diseases, and metabolic disorders [3]. Their precise targeting capabilities allow modulation of specific signaling pathways and immune responses, offering therapeutic advantages with reduced off-target effects. Consequently, the global market for therapeutic antibodies has expanded rapidly and continues to represent one of the largest and fastest-growing sectors in the biopharmaceutical industry [4].

Among these therapeutics, programmed cell death protein 1 (PD-1) has become a major target in immunoncology. PD-1 is an immune checkpoint receptor expressed on activated T cells that maintains immune tolerance by suppressing excessive immune activation [5, 6]. However, many cancer cells exploit this pathway by expressing its ligands, PD-L1 or PD-L2, thereby suppressing anti-tumor immune responses and enabling immune evasion [7]. Therapeutic monoclonal antibodies targeting PD-1, such as pembrolizumab and nivolumab, have been developed to block this inhibitory interaction, effectively reactivating cytotoxic T cell responses against tumors [8–10]. These immune checkpoint inhibitors have demonstrated remarkable clinical success across various cancer types, transforming treatment strategies and significantly improving patient outcomes [11].

The successful development of therapeutic monoclonal antibodies critically depends on the selection and preparation of appropriate antigens [12, 13]. The antigen determines the specificity and functional properties of the resulting antibody, and its structural fidelity is essential for effective screening, affinity maturation, and downstream clinical performance [14, 15]. For this reason, strategies for antigen preparation, such as recombinant protein expression, peptide synthesis, or cell-based antigen display, play an essential role in generating functional and clinically useful antibodies [16]. To produce these antigens, a variety of expression hosts have been utilized depending on the desired structural and functional features. Bacterial systems including *Escherichia coli* allow rapid and cost-effective antigen production but often lack proper post-translational modifications, which can be essential for structural integrity. Yeast and insect cell expression systems provide improved folding and some glycosylation capacity, offering a balance between efficiency and protein quality [17]. Mammalian cell hosts, such as CHO or HEK293 cells, are commonly used for antigens that require complex and human-like glycosylation patterns, particularly in cases where precise epitope presentation is critical to antibody function [18]. More recently, plant-based expression platforms have emerged as an attractive alternative, offering scalability, reduced contamination risks from human pathogens, and unique glycoengineering potential [19, 20]. Therefore, the choice of host system should be carefully aligned with the

structural characteristics of the antigen and the intended therapeutic application.

Plant-based expression systems have undergone significant advancements, establishing them as a sophisticated platform for biopharmaceutical production. A notable example is the COVID-19 vaccine candidate developed by Medicago, a Canadian biotechnology company, which utilized *Nicotiana benthamiana* as a host to produce virus-like particle (VLP) antigens [21, 22]. These plant-derived VLPs structurally mimicked the SARS-CoV-2 virion while lacking infectious genetic material, enabling strong immune activation with a favorable safety profile [23–25]. Clinical studies demonstrated that the plant-produced antigen elicited robust neutralizing antibody responses and protective immunity in humans, ultimately progressing to late-stage clinical trials. This case illustrates that plant expression systems are capable of generating structurally complex and immunogenic antigens suitable for large-scale vaccine and therapeutic antibody development [26, 27].

Despite these advances, challenges remain in optimizing plant-based expression systems to improve productivity and purity, as well as in reducing manufacturing costs to ensure broader accessibility [28]. To address these limitations, this study aimed to establish a codon-optimized PD-1 gene for efficient expression in *N. benthamiana*, purify the recombinant protein under denaturing conditions, and compare its immunogenic properties with those of PD-1 produced in *E. coli* and HEK293T cells [29]. Through this approach, we demonstrate that plant-derived PD-1 can serve as a functional antigen suitable for antibody discovery pipelines. Overall, this work provides a framework for antigen production in plants and highlights the potential of plant-based systems to serve as an alternative platform for therapeutic antibody development [30].

Materials and methods

Plant materials and growth conditions

Nicotiana benthamiana plants (wild type) were grown in soil under controlled growth chamber conditions at 26–28 °C and 50% relative humidity with a 16 h light/8 h dark photoperiod (light intensity, 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$). Five- to seven-week-old plants were used for *Agrobacterium*-mediated transient expression experiments.

Codon optimization and vector construction

The human PD-1 gene sequence was optimized according to the codon usage bias of *N. benthamiana* using Gene Universal software (<https://www.geneuniversal.com>), resulting in a codon adaptation index (CAI) of 0.95. The codon-optimized PD-1 gene was synthesized commercially and amplified by overhang PCR to introduce BamHI or NdeI and SpeI restriction sites. The amplified

fragment was ligated into pRI101 and pFM'M binary vectors for plant expression under the control of the CaMV 35S and FM'M promoters, respectively. Both constructs contained an ER signal peptide (BiP), a Cor1 targeting sequence, a LysM domain, and a C-terminal 6×His tag for purification. The integrity of the plasmids was confirmed by colony PCR and Sanger sequencing.

Agrobacterium-mediated transient expression in *N. benthamiana*

Agrobacterium tumefaciens strain GV3101 was transformed with the recombinant vectors by electroporation. Transformed cells were cultured in LB medium containing kanamycin, rifampicin, and gentamicin at 30 °C with shaking (180 rpm) for 24 h. Bacterial cells were harvested by centrifugation at 4000×g for 20 min and resuspended in infiltration buffer (10 mM MgCl₂, 10 mM MES, and 100 μM acetosyringone) to an OD₆₀₀ of ~1.0. The suspension was incubated in the dark for 2 h at room temperature and subsequently infiltrated into the abaxial side of *N. benthamiana* leaves using a needleless syringe. Leaves were collected at 3 and 5 days post-infiltration.

Protein extraction and purification under denaturing conditions

Harvested leaf tissue was frozen in liquid nitrogen and homogenized using a mortar and pestle. Crude extracts were prepared using two extraction buffers: buffer 1 [0.5% (v/v) Triton X-100 and 1 mM PMSF in PBS] and buffer 2 [0.3% (v/v) Tween-20 and 1 mM PMSF in PBS]. Extracts were centrifuged at 12,000 rpm for 20 min at 4 °C to separate soluble and insoluble fractions. The resulting pellets were resuspended and solubilized in 6 M guanidine HCl. Denatured proteins were purified using Ni-NTA affinity chromatography under denaturing conditions following the manufacturer's protocol (Invitrogen). Eluted protein was subjected to dialysis prior to immunization to remove residual denaturant and associated contaminants. Dialysis was performed using SnakeSkin™ Dialysis Tubing with a 10 kDa molecular weight cutoff (Thermo Scientific) against phosphate-buffered saline (PBS) at 4 °C overnight. Following dialysis, the protein was used for immunization and ELISA assay.

Immunoblot analysis

Protein expression and purification were verified by SDS-PAGE followed by immunoblotting. Proteins were transferred onto PVDF membranes and probed with mouse anti-His monoclonal antibody (Invitrogen), followed by horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG secondary antibody. Signals were visualized using the Super Signal™ Pico chemiluminescent substrate (Thermo Fisher Scientific).

Expression and purification of PD-1 in *E. coli* and HEK293T cells

The PD-1 expression plasmid was transformed into *E. coli* BL21 (DE3) cells and cultured in LB medium containing antibiotics at 37 °C. Protein expression was induced with 0.5 mM IPTG at OD₆₀₀=0.8 and incubated for 5 h. Cells were harvested and resuspended in 6 M guanidine HCl. Denatured proteins were purified using Ni-NTA affinity chromatography under denaturing conditions following the manufacturer's protocol (Invitrogen). The purity of the protein was analyzed by SDS-PAGE, and the concentration was determined by the Bradford assay. The HEK293T cell-derived PD-1 protein was obtained as a commercially available recombinant protein (Sino Biological). According to the manufacturer's specifications, PD-1 (residues 1–167) was expressed in HEK293T cells and purified by high-performance liquid chromatography (HPLC). This protein was used directly in subsequent experiments.

Animal ethics statement

All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Gyeong-sang National University (Approval No. GNU-2022–0177). All experiments were conducted in accordance with the Korean Animal Protection Act and institutional guidelines under SPF conditions.

Mouse immunization

Female BALB/c mice (4–5 weeks old) were immunized subcutaneously with 25, 75, or 125 pmol of purified PD-1 protein formulated with an equal volume of adjuvant (complete Freund's adjuvant for the primary immunization and incomplete Freund's adjuvant for boosters). Boosts were administered at 10-day intervals for a total of three immunizations. Blood samples were collected 10 days after each boost.

Collection of spleen and lymph-node

Following the final immunization, mice were anesthetized, and spleens and lymph-nodes were aseptically dissected. Spleen enlargement was evaluated as an indicator of antigen-specific immune activation. All surgical procedures were performed under sterile conditions.

Enzyme-linked immunosorbent assay

To assess serum antibody titers, 96-well microplates were coated with purified PD-1 proteins (100 ng/well) overnight at 4 °C. For Fig. 5, *E. coli*-derived processed PD-1 (residues 25–167) was used as the coating antigen, while HEK293T cell-derived PD-1 (residues 1–167) was used for Fig. 6. Plates were washed with TBST and blocked with 5% (w/v) skim milk for 1 h at room temperature. Serially diluted sera (starting at 1:200) were added and

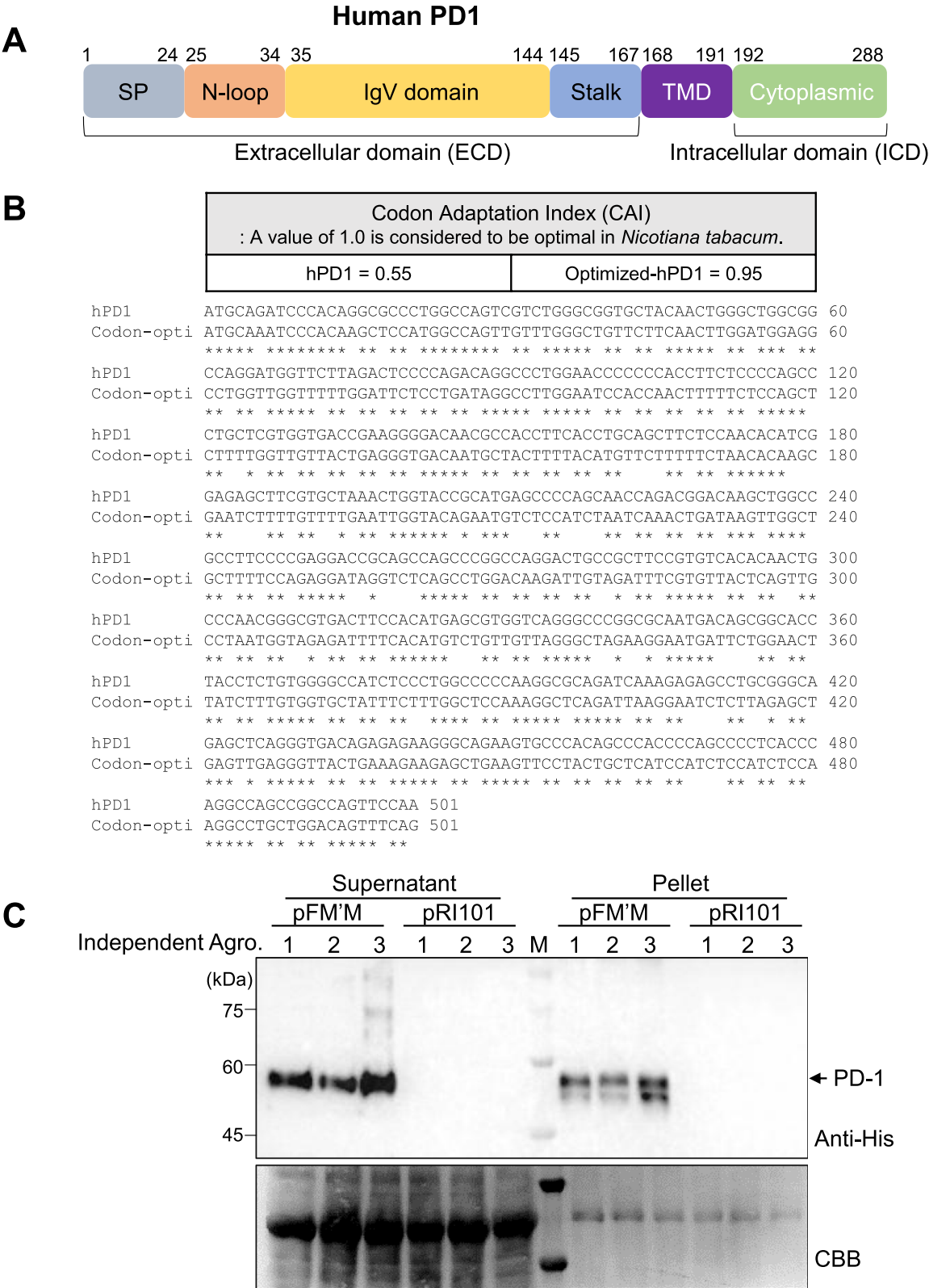


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Fig. 1 Structural features, codon optimization, and expression of human PD-1 antigen in *Nicotiana benthamiana*. **(A)** Schematic representation of the human PD-1 protein domains. SP (signal peptide), N-loop, IgV domain, stalk, transmembrane domain (TMD), and cytoplasmic region are illustrated. The extracellular (ECD) and intracellular (ICD) domains are indicated. **(B)** Codon optimization of the human PD-1 gene for expression in plants. Alignment between native (hPD-1) and plant-optimized (Codon-opt) sequences is shown. Codon Adaptation Index (CAI) improved from 0.55 to 0.95, suggesting enhanced compatibility with *N. tabacum* codon usage. **(C)** Expression analysis of PD-1 recombinant protein in *N. benthamiana*. Immunoblot using anti-His antibody confirmed expression in independent agrobacterium-infiltrated samples carrying pFM'M and pRI101 constructs. PD-1 signal was mainly observed in the supernatant fraction; CBB staining shows total protein loading

incubated for 3 h. After washing, HRP-conjugated anti-mouse IgG (1:2000; Invitrogen) was applied for 2 h. TMB substrate was added for color development, and absorbance was measured at 450 nm using a microplate reader (PerkinElmer). EC_{50} values were calculated by nonlinear regression.

Flow cytometric analysis of antibody binding

LR73 cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin–streptomycin at 37 °C with 5% CO₂. Cells were seeded at 5×10^4 cells/well in 24-well plates and were transfected the next day with pCMV3::PD-1-GFP (0.5 µg/well) using Lipofectamine 3000 (Invitrogen). After 24 h, cells were detached with PBS/EDTA, washed twice with FACS buffer (PBS containing 5% horse serum (HS)), and used for antibody staining. For blocking, cells ($\sim 1 \times 10^5$) were incubated in 5% HS for 30 min at 4 °C. Mouse antisera raised against plant-, *E. coli*-, or mammalian-derived PD-1 were diluted 1:200 in HS buffer and incubated with cells for 1 h at 4 °C. A commercial anti-PD-1 antibody (BioLegend) was included as a positive control (1:800), and pre-immune serum (1:200) served as the negative control. Following three washes, cells were incubated with Alexa Fluor 647–conjugated goat anti-mouse IgG (Invitrogen, 1:1000) for 30 min at 4 °C in the dark, washed again, and resuspended in FACS buffer. Flow cytometry data were acquired on a BD FACSSVerse, collecting at least 10,000 events per sample. GFP (FITC channel) was used to gate PD-1-expressing cells, and antibody binding was analyzed based on Alexa 647 fluorescence intensity. Data was processed in FlowJo software.

Statistical analysis

All experiments were performed in triplicate. Data are presented as means $\pm$ standard deviation (SD) and analyzed using GraphPad Prism (version 8.0, GraphPad Software, San Diego, CA, USA). Differences between two groups were evaluated using one-way analysis of variance with Tukey's post hoc test. A p -value < 0.05 was considered statistically significant.

Results

Transient expression of codon-optimized human PD-1 in *N. benthamiana*

Human PD-1 consists of an extracellular domain (ECD) containing an N-terminal signal peptide (SP), N-loop,

IgV-like domain, and stalk region, followed by a transmembrane domain (TMD) and a short intracellular domain (ICD) (Fig. 1a). Since the ECD is responsible for ligand recognition and serves as the primary antigenic determinant, this region was selected as the immunogen for therapeutic antibody development. To enhance expression efficiency in plants, the PD-1 coding sequence was redesigned to match the codon usage preferences of *Nicotiana* species. This optimization increased the codon adaptation index (CAI) relative to *N. tabacum* from 0.55 (native sequence) to 0.95 (optimized sequence), indicating a substantial improvement in predicted compatibility with the plant expression (Fig. 1b). Transient expression of codon-optimized PD-1 gene was carried out in *N. benthamiana* leaves using two *Agrobacterium*-mediated plant expression vectors, pFM'M and pRI101, driven by the FM'M and CaMV 35S promoters, respectively [31]. The pFM'M construct produced higher PD-1 expression levels compared to pRI101 construct (Fig. 1c). These results demonstrate that codon optimization combined with an appropriate expression vector enhances the transient expression of human PD-1 in *N. benthamiana*. This supports the utility of plant transient expression systems as a suitable host for the rapid production of human therapeutic protein candidates.

Optimization of human PD-1 expression and extraction in *N. benthamiana*

To further improve PD-1 expression in *N. benthamiana*, we systematically evaluated factors affecting protein yield and solubility (Fig. 2). Co-expression with RNA-silencing suppressors revealed that p19 suppressor significantly increased PD-1 levels compared to p38 (Fig. 2a). Under p19 co-expression, PD-1 abundance peaked at 3 days post-infiltration (dpi) and declined by 5 dpi. Leaf developmental stage also strongly influenced expression levels (Fig. 2b). Among leaves positioned 2 to 5 from the basal region, intermediate-aged leaves (leaf position 3) exhibited the highest PD-1 levels, whereas more mature leaves showed a pronounced decline in expression. These findings indicate that physiological age of leaf tissue is a critical determinant of protein productivity in plant transient expression systems.

Protein solubility is a key factor in determining appropriate purification strategies. To assess solubilization efficiency, various concentrations of non-ionic detergents Triton X-100 and Tween 20 were tested (Fig. 2c).

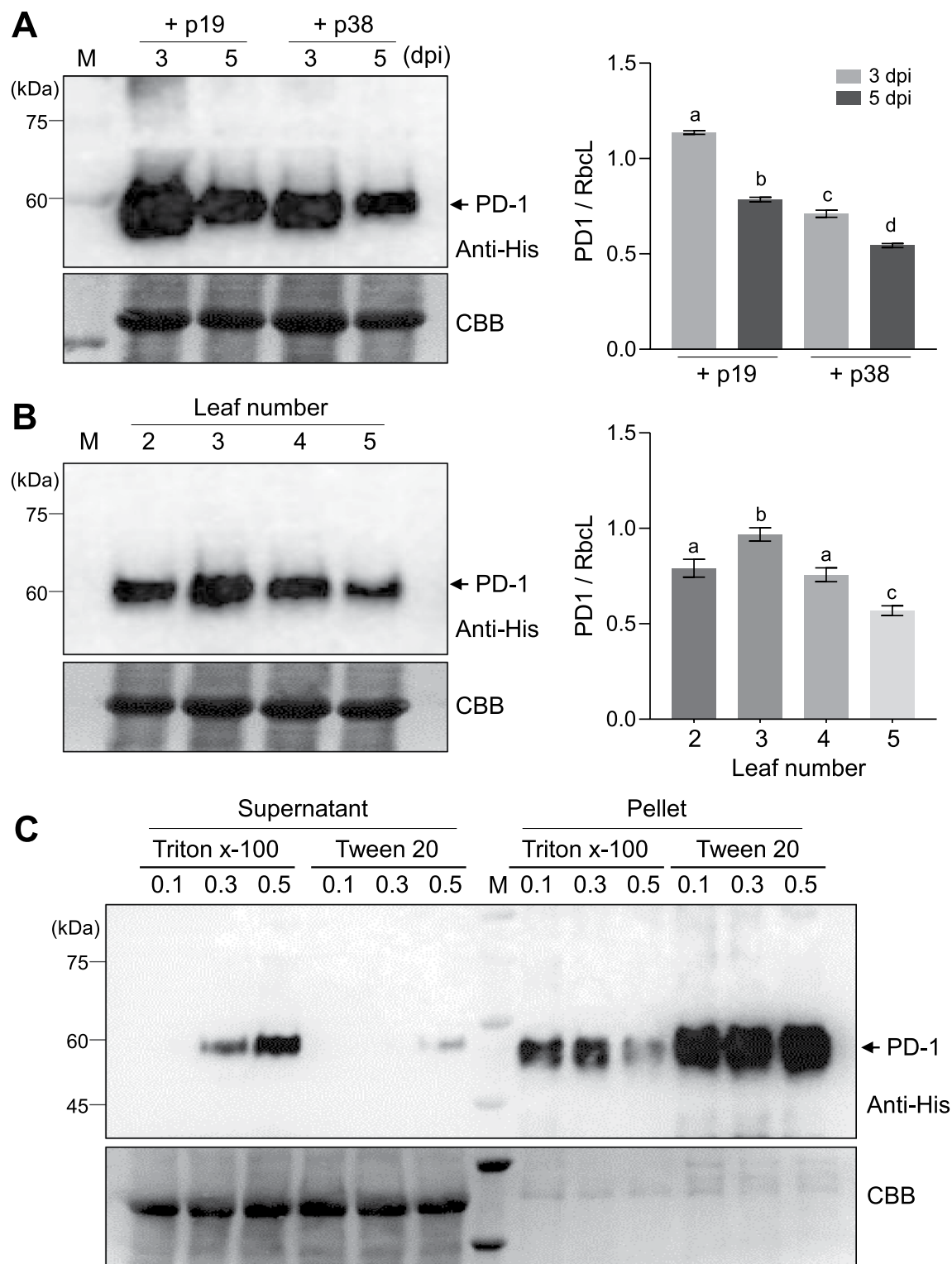


Fig. 2 (See legend on next page.)

Across all conditions, PD-1 was predominantly detected in the insoluble pellet fraction, indicating a tendency for the protein to aggregate or form inclusion-like bodies in plant cells. Only minimal solubilization was achieved even at 0.5% Triton X-100, demonstrating that native extraction conditions are insufficient. These results imply that purification under partially or fully denaturing conditions may be required for efficient PD-1 production.

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Fig. 2 Optimization of PD-1 recombinant protein expression in *Nicotiana benthamiana*. **(A)** Effect of viral suppressors on PD-1 expression. Leaves co-infiltrated with *Agrobacterium* carrying pFM'M-PD-1 or pRI101-PD-1 together with p19 or p38 suppressor constructs were harvested at 3 and 5 days post-infiltration (dpi). Immunoblot analysis using anti-His antibody revealed that PD-1 accumulation was higher with p19 co-expression at 3 dpi. Quantification of PD-1/Rubisco L band intensity is shown on the right. **(B)** Influence of leaf developmental stage on PD-1 accumulation. Leaves numbered 2–5 from *N. benthamiana* plants were sampled at 5 dpi. The highest PD-1 signal was detected in middle-aged leaves (No. 3–4). The bar graph indicates relative PD-1 abundance normalized to Rubisco L. **(C)** Solubilization efficiency of PD-1 under different detergent conditions. Plant-expressed PD-1 was mainly detected in the pellet fraction even after treatment with 0.1–0.5% Triton X-100 or Tween 20, indicating that a large proportion of PD-1 accumulated in an insoluble/aggregated form. This result suggested that purification under denaturing conditions would be required for recovery of plant-derived PD-1. CBB staining was used as a loading control

Purification and productivity of PD-1 protein

Since plant-expressed PD-1 predominantly accumulated in the insoluble fraction (Fig. 2c), the protein was purified under denaturing conditions using Ni–NTA affinity chromatography (Fig. 3a). To estimate production yield, densitometric quantification was performed using BSA as a standard (Fig. 3b). Approximately 50 µg of purified PD-1 was obtained from 10 g of fresh leaf tissue, corresponding to a yield of about 5 µg per gram fresh weight. Furthermore, to compare the immunogenic properties of plant-derived PD-1 with those of a bacterial expression system, the PD-1 extracellular domain (residues 1–167) was also expressed and purified from *E. coli*. In addition, a processed form of PD-1 corresponding to the mature protein lacking the signal peptide (residues 25–167) was produced to represent the physiologically relevant cell-surface form (Fig. 3c). This processed PD-1 was prepared for subsequent ELISA assays to evaluate antibody binding to the native-like form.

Comparable immune activation by PD-1 antigens produced in plants, bacteria, and mammalian cells

To evaluate immunogenicity, BALB/c mice were immunized with PD-1 proteins produced in plants, bacteria, or mammalian cells (Fig. 4a). Each group received equivalent molar doses of purified antigen (25, 75, or 125 pmol) formulated with Freund's adjuvant, and sera were collected at 10-day intervals for immunological analysis (Fig. 4b). Mice immunized with plant-derived PD-1 exhibited pronounced splenic enlargement at all antigen doses, comparable to those immunized with bacteria-derived PD-1 and exceeding that observed for the mammalian cell-derived PD-1 (Fig. 4c). Lymph-node enlargement was also observed in plant-derived PD-1, indicating that plant-derived PD-1 effectively stimulates the adaptive immune response (Fig. 4d). Collectively, these results demonstrate that plant-derived PD-1 induces immune responses comparable to antigens produced in conventional bacterial or mammalian expression systems, supporting the suitability of plant-derived PD-1 as a functional immunogen for therapeutic antibody development.

Immunogenicity evaluation of plant-derived PD-1 antigen in a mouse model

To compare the humoral immune responses elicited by PD-1 antigens produced in plant, bacterial, and mammalian expression systems, serial serum samples collected after each immunization were analyzed by ELISA. Antibody binding activity was assessed using a processed PD-1 fragment (residues 25–167) purified from *E. coli* as the coating antigen (Fig. 5). Following the first immunization with 25 pmol of antigen, plant-derived PD-1 induced strong antibody generation, comparable to that elicited by bacteria-derived PD-1. In contrast, mammalian cell-derived PD-1 showed a markedly weaker antibody response (Fig. 5A). These results are consistent with the observed enlargement of the spleen and lymph-node (Fig. 4b and c), supporting the induction of a systemic immune response following immunization with antigens produced in plants or bacteria. After booster immunization, sera from mice immunized with plant-derived PD-1 continued to exhibit high binding activity, exceeding that of the mammalian cell-derived PD-1, although slightly lower than, the responses induced by bacteria-derived PD-1 (Fig. 5b and c). Quantitative curve fitting of ELISA absorbance values was used to determine EC₅₀ titers for each group (Fig. 5d). plant-derived PD-1 showed an EC₅₀ value of 1.8×10^{-4} (25 pmol antigen), 9.0×10^{-5} (75 pmol antigen), 6.7×10^{-5} (125 pmol antigen), whereas bacteria-derived PD-1 exhibited a substantially lower EC₅₀ value of 3.5×10^{-5} (25 pmol antigen), 3.5×10^{-5} (75 pmol antigen), 2.2×10^{-5} (125 pmol antigen), indicating approximately three-fold higher binding potency compared to the plant-derived PD-1.

Recognition of native PD-1 by antibodies induced by plant, bacterial, or mammalian-derived antigens

To test whether antibodies induced by plant-derived PD-1 can recognize native PD-1 protein, serum samples collected after the third immunization were analyzed by ELISA and flow cytometry (Fig. 6). Antibody binding activity was examined using mammalian cell-derived PD-1 as the coating antigen (Fig. 6a and b). To quantitatively compare the humoral immune responses elicited by PD-1 antigens produced in plant, bacterial, and mammalian systems, EC₅₀ values were determined from ELISA binding curves generated using serially diluted

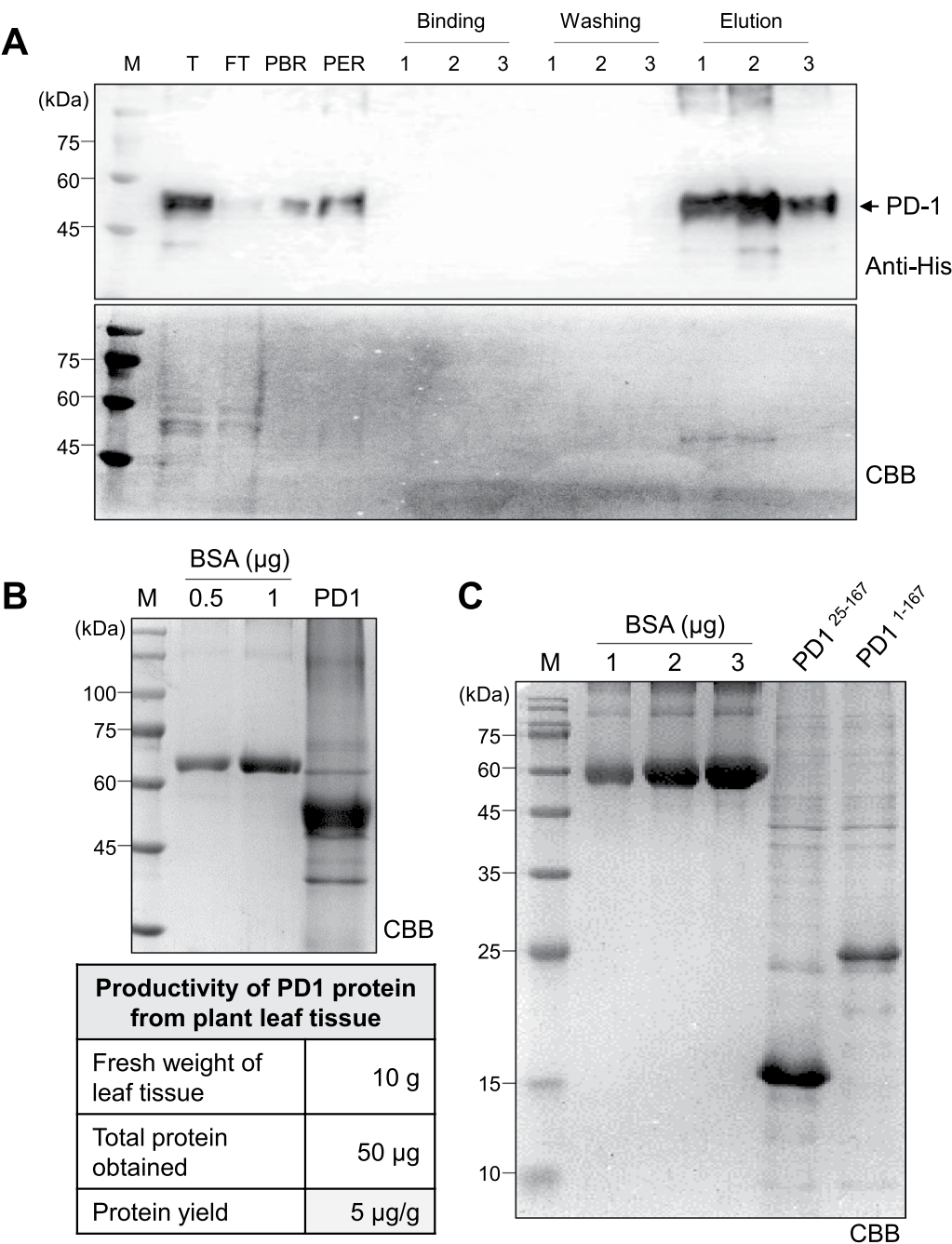


Fig. 3 Denaturing Ni-NTA purification of plant-derived PD-1 and comparison with *E. coli*-expressed PD-1 domain variants. **(A)** Purification of insoluble plant-expressed PD-1 under denaturing conditions using Ni-NTA affinity chromatography. Total extract (T), flow-through (FT), protein-binding resin (PBR), post-elution residue (PER), and the sequential binding/washing/elution fractions were analyzed by immunoblotting with anti-His antibody. **(B)** Estimation of yield of denatured/purified plant PD-1. CBB-stained SDS-PAGE comparing purified PD-1 with BSA standards (0.5 and 1 μg) was used for densitometric quantification. From 10 g of fresh leaf tissue, ~50 μg of PD-1 was obtained, corresponding to an overall yield of ~5 μg/g fresh weight (see inset table). **(C)** SDS-PAGE analysis of PD-1 domain constructs expressed in *E. coli*. To validate the expected molecular sizes and domain organization, PD-1(25–144) and PD-1(25–167) were produced in *E. coli* and analyzed by SDS-PAGE alongside BSA standards

sera. The EC₅₀ titers varied depending on both the antigen source and the immunization dose. For mice immunized with plant-derived PD-1, the EC₅₀ values were 1.6 × 10⁻⁴, 1.2 × 10⁻⁴, and 8.9 × 10⁻⁴ at antigen doses of 25, 75, and 125 pmol, respectively. Sera from mice immunized with bacteria-derived PD-1 exhibited EC₅₀ values of 1.0 × 10⁻⁴, 1.4 × 10⁻⁴, and 9.2 × 10⁻⁴ at the corresponding doses. As expected, immunization with mammalian cell-derived PD-1 resulted in substantially lower EC₅₀ values, 6.9 × 10⁻⁵, 4.8 × 10⁻⁵, and 4.8 × 10⁻⁵, reflecting

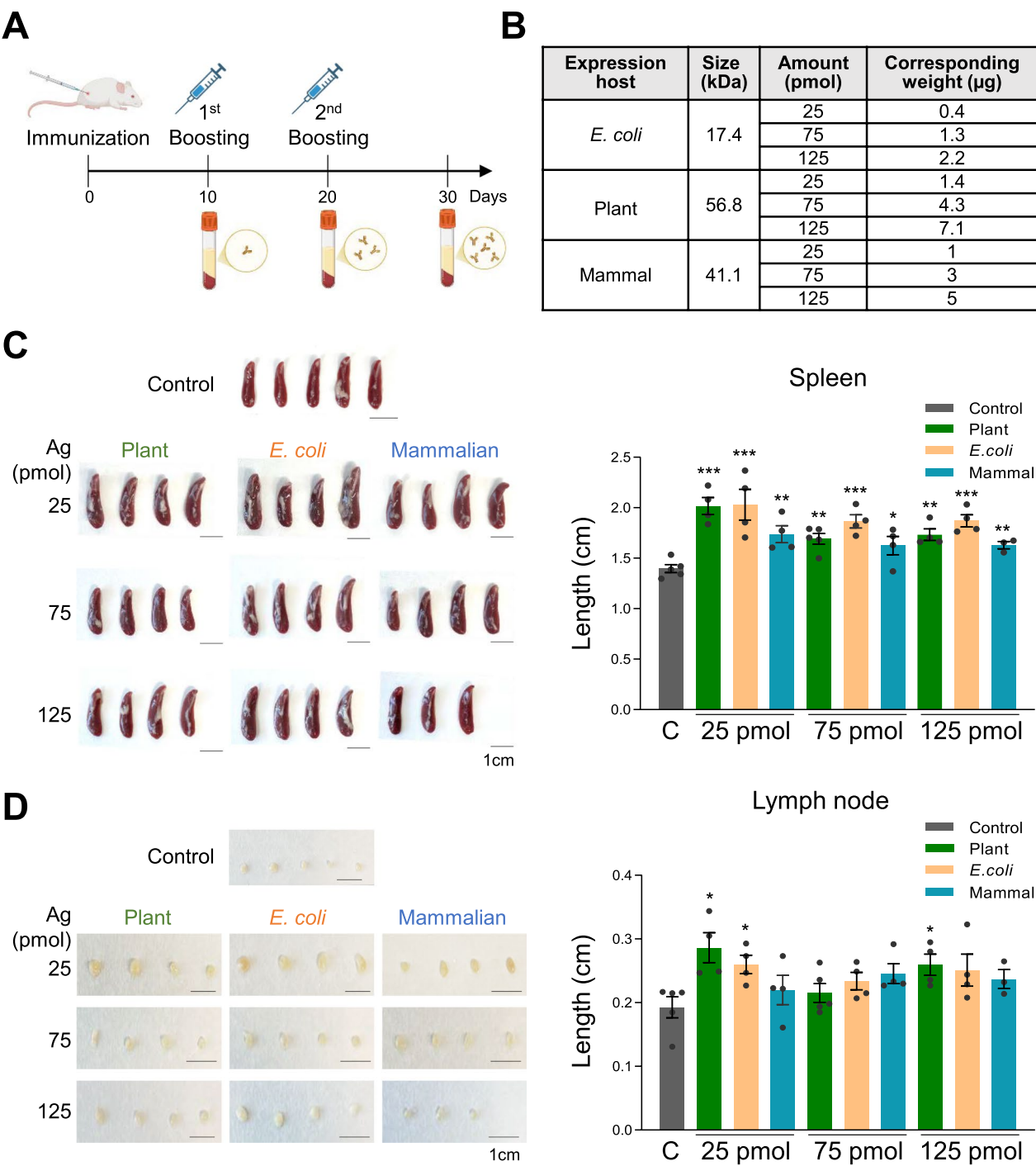


Fig. 4 Immunogenic evaluation of plant-derived PD-1 antigen in mice compared with bacterial and mammalian counterparts. **(A)** Experimental design of the immunization schedule. BALB/c mice were immunized three times with recombinant PD-1 proteins derived from *Nicotiana benthamiana*, *E. coli*, or mammalian expression systems on days 0, 10, and 20. Serum samples and lymphoid organs were collected on day 30 for subsequent analysis. **(B)** Antigen dose and physical property comparison among expression systems. The table summarizes the molecular weight, molar amount, and corresponding protein mass of PD-1 antigens produced in each system (*E. coli*, plant, and mammalian). Doses of 25, 75, and 125 pmol were prepared for immunization. **(C-D)** Comparison of spleen and lymph-node responses following immunization. Representative spleens (C) and lymph-nodes (D) from mice immunized with each antigen source are shown. The scale bar presents 1 cm. Spleen and lymph-node lengths were measured as indicators of antigen-specific immune activation. Data are presented as mean ± SD (n = 3 or 4); significance levels were determined by one-way ANOVA (*p < 0.05, **p < 0.01, ***p < 0.001)

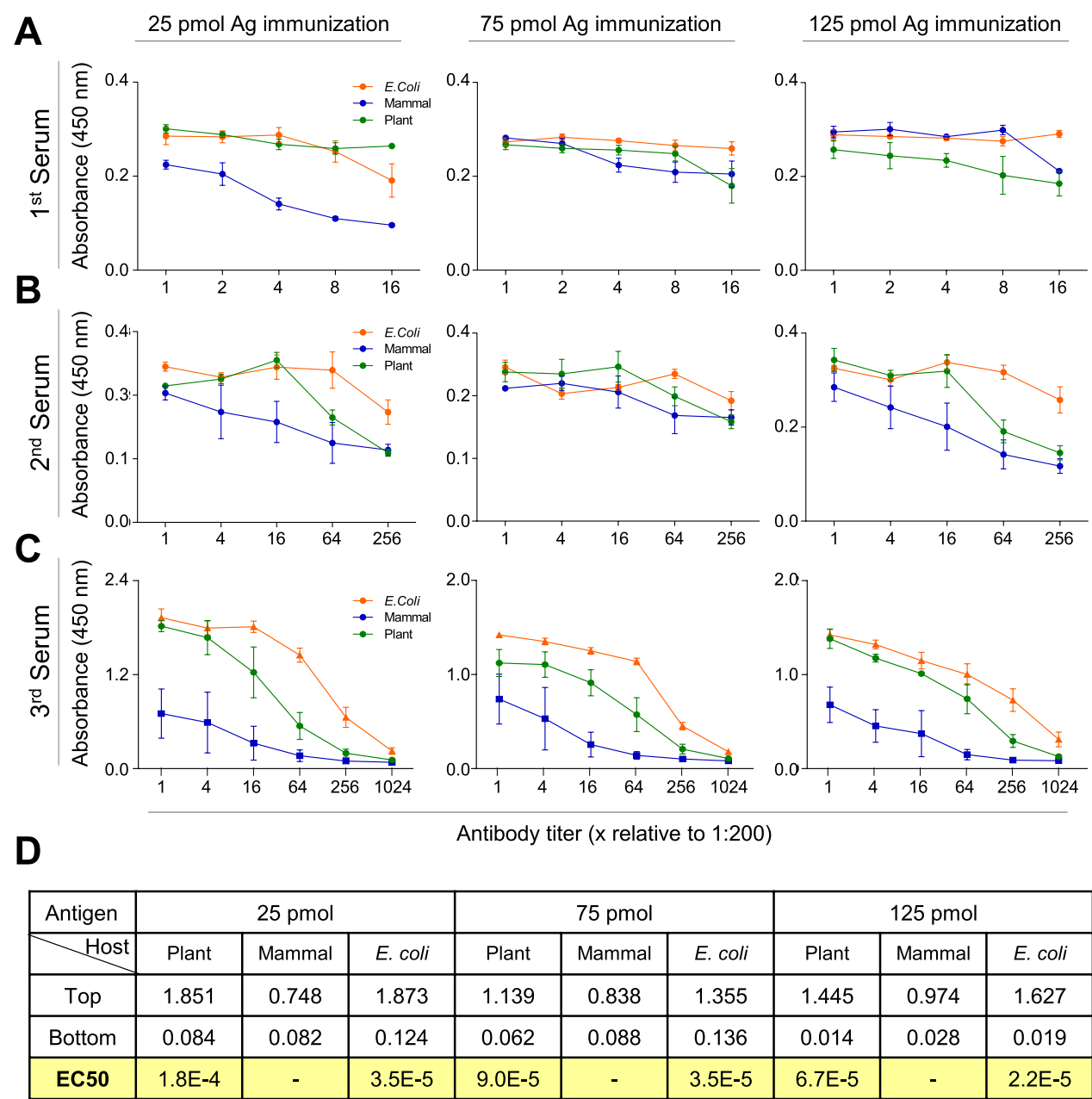


Fig. 5 Comparative antibody responses induced by PD-1 antigens produced in plant, bacterial, and mammalian systems. **(A–C)** Serum antibody titers following immunization with PD-1 antigens from different expression hosts. BALB/c mice were immunized twice with PD-1 proteins derived from *Nicotiana benthamiana* (Plant), *E. coli*, or mammalian cells at doses of 25 pmol, 75 pmol, and 125 pmol, respectively. Sera were collected after the 1st (A), 2nd (B), and 3rd (C) immunizations, and PD-1-specific IgG levels were determined by ELISA using *E. coli*-derived processed PD-1 (residues 25–167) as the coating antigen, with serial dilutions (x = 1—1024 relative to 1:200). Data represent mean ± SD (n = 3). Across all doses, *E. coli*-derived PD-1 generally induced the highest antibody titers, followed by mammalian and plant-derived PD-1. **(D)** Quantitative comparison of antibody-binding curves and EC₅₀ values. Top and bottom plateau values from nonlinear regression were used to calculate EC₅₀ titers for each antigen source. EC₅₀ values indicate that *E. coli*-derived PD-1 generated the strongest antibody binding (lowest EC₅₀), whereas plant-derived PD-1 exhibited moderate immunogenicity. Nevertheless, plant PD-1 consistently elicited measurable antibody responses, confirming its functional antigenicity despite expression under denaturing recovery conditions

the highest antibody binding potency. These results suggest that plant-produced PD-1 is capable of generating antibodies that effectively recognize human PD-1. To further assess the functional quality of the antibodies elicited by PD-1 antigens derived from plant, bacterial, and mammalian expression systems, a flow cytometry-based binding assay (FACS analysis) was performed using PD-1 expressing LR73 cells generated by transfection with a PD-1-GFP construct. PD-1-GFP expression in LR73 cells was confirmed by GFP fluorescence (Fig. 6c). Sera from

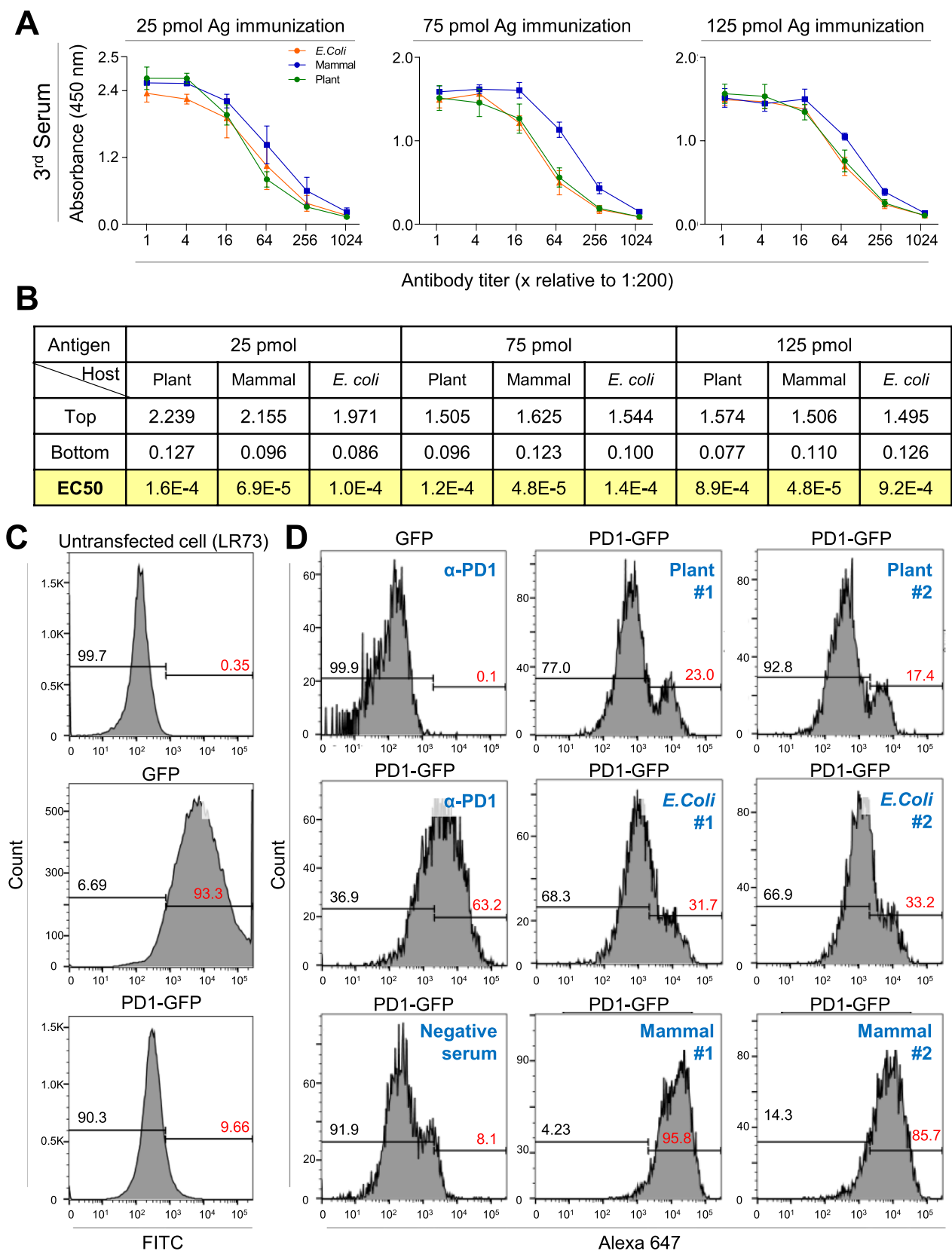


Fig. 6 (See legend on next page.)

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Fig. 6 Functional validation of antibody binding to plant-derived PD-1 antigen. **(A–B)** Serum antibody titers after the third immunization. Sera from mice immunized with PD-1 antigens derived from *Nicotiana benthamiana*, *E. coli*, or mammalian cells (25–125 pmol) were analyzed by ELISA using HEK293T cells-derived PD-1 (residues 1–167) as the coating antigen. Antibody titers showed similar overall trends among antigen sources, with *E. coli*-derived PD-1 producing slightly higher EC_{50} values (panel B). Top/bottom plateau and EC_{50} values were obtained by nonlinear regression fitting. **(C)** Validation of PD-1–GFP expression in LR73 cells. Flow-cytometric analysis confirmed strong GFP fluorescence and surface expression of PD-1–GFP in transfected LR73 cells, whereas untransfected controls showed negligible signal. Histograms indicate FITC (GFP) or Alexa 647 (anti-PD-1) fluorescence intensity. **(D)** Flow-cytometric analysis of antibody binding to PD-1–GFP–expressing cells. Sera from mice immunized with plant-, *E. coli*-, or mammalian-derived PD-1 were tested for binding to PD-1–GFP–expressing LR73 cells. Plant-derived antisera (Plant #1–2) bound PD-1–GFP–positive cells with fluorescence intensities of 17–23%, comparable to bacterial antisera (*E. coli* #1–2, 31–33%) and mammalian antisera (Mammal #1–2*, 85–96%). Negative serum and α -PD-1 commercial antibodies served as negative and positive controls, respectively. These data confirm that antibodies raised against plant-produced PD-1 specifically recognize the native PD-1 epitope on mammalian cell surfaces

mice immunized with plant-derived PD-1 bound to 23% and 17.4% of PD-1 expressing cells. Antibodies elicited by bacteria-derived PD-1 exhibited slightly higher binding, labeling 31.7% and 33.2% of cells. These results indicate that plant- and bacteria-derived PD-1 antigens generate functional antibodies capable of recognizing native, cell-surface PD-1. In contrast, sera from mice immunized with mammalian cell-derived PD-1 induces substantially higher levels of PD-1-specific antibodies with 95.8% and 85.7% (Fig. 6d). Collectively, these results demonstrate that plant-derived PD-1 can induce functional antibodies with the capacity to recognize native human PD-1, although at lower potency than antibodies elicited by mammalian cell-derived antigens. These findings support the potential of plant-produced PD-1 as an alternative immunogen for therapeutic antibody development, while highlighting the need for further optimization and evaluation in future studies.

Discussion

Plant-based recombinant protein systems have emerged as an alternative expression system, particularly for producing complex eukaryotic proteins such as antibodies, cytokines, and vaccine antigens [32]. In this study, we developed a plant-based expression platform for human PD-1, a key immune checkpoint receptor, and evaluated its expression characteristics, purification behavior, and immunogenic potential for therapeutic antibody development. A critical factor in the development of therapeutic monoclonal antibodies is the selection and preparation of the target antigen. Because antibodies recognize highly specific three-dimensional epitopes, the biochemical quality, conformation, and post-translational modifications of the antigen are key determinants for eliciting antibodies with high affinity and functional relevance [16]. For this reason, recombinant antigen production must carefully preserve native protein folding and epitope accessibility in order to generate antibodies that effectively bind the target in physiological contexts. In addition, when antigens are poorly immunogenic or highly conserved among species, strategies such as antigen engineering, peptide design, or conjugation to carrier proteins are often employed to enhance immune

recognition. Although sera from mice immunized with plant- or bacteria-derived PD-1 contained antibodies binding to PD-1 expressing cell, they were weak compared to sera from mice immunized with mammalian cell-derived PD-1 (Fig. 6d). The difference is that plant- or bacteria-derived PD-1 were purified under denaturation conditions. Success will require a method to purify the protein in its native state.

Recent advances in plant-based purification strategies may provide a path toward overcoming these limitations. Notably, natural peptidoglycan nanoparticles (NPNs) derived from Gram-positive lactic acid bacteria have emerged as a promising tool for the purification and delivery of plant-produced recombinant proteins [33]. A recent study demonstrated that optimized NPN processing—including trichloroacetic acid and trypsin treatment—can efficiently remove contaminating subcellular components while preserving the nanoparticle's strong protein-binding capacity. Importantly, when conjugated with plant-derived antigens, NPNs enabled purification under mild, non-denaturing conditions and supported robust humoral immune responses in vivo. These findings suggest that integrating NPN-based purification with our PD-1 expression platform could allow the recovery of properly folded, soluble PD-1 antigens from plants, thereby addressing the major limitation observed in our study: the loss of native conformation during denaturing purification. By enabling gentle protein capture and stabilization at low temperatures, NPN-mediated methods may help maintain epitope integrity and enhance immunogenicity compared with conventional denaturing workflows. Thus, applying nanoparticle-assisted purification technologies to plant molecular farming systems holds strong potential to overcome current barriers in the production of structurally sensitive targets such as human PD-1. Future work optimizing NPN-antigen conjugation and evaluating antibody responses to NPN-purified PD-1 will be essential for advancing plant-based platforms for therapeutic antibody development.

As autotrophic organisms, plants synthesize complex biomolecules using light, water and minimal nutrient inputs, reducing reliance on costly media and enabling energy-efficient production. This biological capacity

also supports the proper folding and post-translational modification of eukaryotic proteins within endogenous quality-control pathways, offering an inherent advantage over microbial systems that often require extensive engineering to achieve comparable molecular complexity. The scalability and environmental flexibility of controlled-environment plant cultivation further strengthen the utility of plant molecular farming [32]. Recombinant proteins can be produced in fully contained indoor systems, decoupling manufacturing from seasonal or geographical constraints and allowing consistent, contamination-resistant yields suitable for pharmaceutical applications. Such controlled-environment strategies also reduce the need for agricultural land and facilitate modular or urban bioproduction approaches. Notably, the self-sustaining growth of plants makes them uniquely suited for biomanufacturing in resource-limited or unconventional settings, including space-based environments. Recent work has demonstrated that plants can complete developmental and metabolic programs, including heterologous protein expression, under microgravity and controlled-atmosphere conditions [28]. These findings suggest that plant-based platforms could serve as lightweight, low-input bioreactors for long-duration missions where resupply capacity is limited.

Together, these characteristics underscore the broader potential of plant molecular farming as a versatile and sustainable production system. When combined with emerging non-denaturing purification strategies, such as NPN-assisted antigen recovery, plant-based platforms may provide a robust route to generating high-quality, conformationally intact antigens for therapeutic antibody development.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s13765-026-01083-4>.

Additional file 1: List of primers used for this study

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Author contributions

Designed the research, H.J.M., G.A., C.S.L., W.-Y.K. and M.G.K.; conducted the experiments, H.J.M., G.A. and S.A.S.; analyzed data or wrote the paper, H.J.M., G.A., S.A.S., C.S.L., W.-Y.K. and M.G.K.; supervision, W.-Y.K. and M.G.K.; project administration, W.-Y.K. and M.G.K.; funding acquisition, W.-Y.K. All authors have read and agreed to the published version of the manuscript.

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Data availability

All data generated or analyzed during this study are included in this published article.

Declarations

Competing interests

All authors declare that they have no conflicts of interest.

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