Monoclonal antibodies specific to human \(\Delta42PD1\): A novel immunoregulator potentially involved in HIV-1 and tumor pathogenesis

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Abbreviations: ART, antiretroviral therapy; ELISA, enzyme-linked immunosorbent assay; ESCC, esophageal squamous cell carcinoma; FBS, fetal bovine serum; h, hour(s); FSC, forward scatter; HIV-1, human immunodeficiency virus type 1; HRP, horseradish peroxidase; mAb, monoclonal antibody; MFI, mean fluorescence intensity; min, minute(s); OD\(_{450}\)nm, optical density at 450nm; PBMCs, peripheral blood mononuclear cells; PD1, programmed cell death 1; rpm, revolutions per minute; RT, room temperature; sec, second(s); s\(\Delta42PD1\), soluble \(\Delta42PD1\); sPD1, soluble PD1; SSC, side scatter

We recently reported the identification of \(\Delta42PD1\), a novel alternatively spliced isoform of human PD1 that induces the production of pro-inflammatory cytokines from human peripheral blood mononuclear cells and enhances HIV-specific CD8\(^+\) T cell immunity in mice when engineered in a fusion DNA vaccine. The detailed functional study of \(\Delta42PD1\), however, has been hampered due to the lack of a specific monoclonal antibody (mAb). In this study, we generated 2 high-affinity mAbs, clones CH34 (IgG2b) and CH101 (IgG1), from \(\Delta42PD1\)-immunized mice. They recognize distinct domains of \(\Delta42PD1\) as determined by a yeast surface-displaying assay and ELISA. Moreover, they recognize native \(\Delta42PD1\) specifically, but not PD1, on cell surfaces by both flow cytometry and immunohistochemical assays. \(\Delta42PD1\) appeared to be expressed constitutively on healthy human CD14\(^+\) monocytes, but its level of expression was down-regulated significantly during chronic HIV-1 infection. Since the level of \(\Delta42PD1\) expression on CD14\(^+\) monocytes was negatively correlated with the CD4 count of untreated patients in a cross-sectional study, \(\Delta42PD1\) may play a role in HIV-1 pathogenesis. Lastly, when examining \(\Delta42PD1\) expression in human esophageal squamous-cell carcinoma tissues, we found high-level expression of \(\Delta42PD1\) on a subset of tumor-infiltrating T cells. Our study, therefore, resulted in 2 \(\Delta42PD1\)-specific mAbs that can be used to further investigate \(\Delta42PD1\), a novel immune regulatory protein implicated in HIV-1 and tumor pathogenesis as well as other immune diseases.

Introduction

Programmed death 1 (PD1) is a member of the CD28 family, a type I transmembrane proteins composed of a single extracellular immunoglobulin variable-region-like domain, a transmembrane domain and an intracellular domain containing an immunoreceptor tyrosine-based inhibitory motif (ITIM), as well as an immunoreceptor tyrosine-based switch motif (ITSM).\(^1\) PD1 is transcriptionally induced in activated T cells, B cells, natural killer T (NKT) cells and monocytes. Signaling after PD1 interaction with its ligands PD-L1 (B7-H1; CD274) and PD-L2 (B7-DC; CD273) results in the phosphorylation of tyrosine-based motifs of its intracellular portion, and subsequently, the inhibition of lymphocyte activation and cytokine production.\(^2,4\) This PD1/PD-L pathway, therefore, delivers inhibitory signals that regulate the balance between T cell activation and tolerance.

The importance of the PD1/PD-L pathway has been demonstrated in various immune diseases. For example, PD1 plays a critical role in the maintenance of the symbiotic balance between mucosal bacterial communities and the host immune system through regulating secretory IgA diversification.\(^5\) Consistent with the role of PD1 in immunoregulation, \(pd1^{-/-}\) mice exhibit increased susceptibility to autoimmune diseases. In the C57BL/6 background, PD1-deficient mice develop lupus-like autoimmune

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diseases with deposition of IgG3 and C3 in the glomeruli; whereas in the BALB/c background, PD1 knockout mice display dilated cardiomyopathy due to production of high-titer autoantibodies against cardiac troponin. Anomalous expression of PD1 in humans has also been demonstrated to be associated with several autoimmune diseases, including systemic lupus erythematosus, myocarditis, encephalomyelitis, rheumatoid arthritis, multiple sclerosis and type I diabetes.

Although PD1 signaling is important for preventing excessive immune response, its over-expression can cause dysfunction and exhaustion of pathogen-specific T and B cells. The PD1/PD-L pathway has been reported to play a crucial role in inducing T and B cell exhaustion, anergy and apoptosis in chronic viral infections including lymphocytic choriomeningitis virus in mice, as well as HIV and hepatitis C virus infections in humans. These findings indicated that PD1 up-regulation might facilitate viral persistence in chronic viral infection. Blockade of the PD1/PD-L1 interaction, therefore, can partially reverse the exhaustion of virus-specific T cells and improve CD8+ T cell proliferation, cytokine production, and cytotoxicity. Manipulating the PD1/PD-L1 interaction may enhance virus-specific CD8+ T cell responses in the context of active vaccination.

We recently discovered in human peripheral blood mononuclear cells (PBMCs) a novel alternatively spliced human PD1 isoform, termed Δ42PD1, that contains a 42-nucleotide in-frame deletion located at exon 2 domain. Unlike PD1, Δ42PD1 does not interact with PD-L1 or PD-L2. The soluble extracellular domain of Δ42PD1 (sΔ42PD1) and cellular surface Δ42PD1 could induce interleukin (IL)-1β, IL-6 and tumor necrosis factor release from human PBMCs in vitro. Moreover, a mouse sΔ42PD1-based DNA vaccine amplifies the level of HIV-specific CD8+ T cell immunity in vivo. In the present study, we aimed to generate mAbs specific to Δ42PD1 that may facilitate the investigation of the biological relevance of this novel immunoregulatory protein in human diseases.

Results

Generation of mouse-derived mAb against human Δ42PD1

To elicit human Δ42PD1-specific antibodies, we used a DNA prime/protein boost regimen to immunize BALB/c mice (Fig. 1A). Serum samples at week 10 post-vaccination were analyzed for anti-sΔ42PD1 titers using sΔ42PD1His as coating protein in an indirect ELISA. We found that ELISA titers of anti-sΔ42PD1 antibodies were greater than 10,000 in sera of all 5 immunized mice (Fig. 1B). Before performing cell fusion assay for hybridoma generation, we sought to determine the binding bias of these serum samples. Using the indirect ELISA, polyclonal sera recognized both sΔ42PD1His and sPD1His proteins. However, the OD450nm value was at least 2-fold higher for sΔ42PD1His than PD1His for all samples, with the serum of mouse 4 (M4) displaying the strongest bias toward Δ42PD1 (Fig. 1C). Similarly, by flow cytometry analysis, all sera recognized Δ42PD1-expressing 293T cells more effectively than PD1-expressing 293T cells (n = 5, P = 0.0043), with serum of M4 possessing the highest mean fluorescence intensity (MFI) (Fig. 1D). Accordingly, M4 was immunized one more time at week 12 and sacrificed one week later to harvest splenocytes for fusion with SP2/0-Ag14 myeloma cells. Subsequently, we identified 3 positive hybridoma cell lines (clones CH34, CH36 and CH101). Clones CH34 and CH101 secreted mAbs highly reactive to Δ42PD1His by the indirect ELISA (Fig. 1E).

Two high-affinity Δ42PD1-specific mAbs

It is possible that Δ42PD1-elicited polyclonal antibodies could cross-react with PD1 because Δ42PD1 and PD1 shared 100% amino acid homology except for the deletion. This possibility was confirmed by the observation that Δ42PD1-elicited antiserum did recognize PD1 besides Δ42PD1 (Fig. 1C, D). To evaluate the specificity of Δ42PD1-elicited mAbs (clones CH34 and CH101), we analyzed their reactivity with Δ42PD1-and PD1-expressing 293T cells by flow cytometry and immunohistochemical assays. Both mAbs specifically recognized human Δ42PD1, but not PD1 (Fig. 2A, B). In Western blot analysis, however, both mAbs recognized denatured proteins of human Δ42PD1 and PD1, but not of the artificial mouse Δ42PD1 (msΔ42PD1fc) (Fig. 2C). It should be mentioned that human sΔ42PD1 shares approximately 64% amino acid sequence homology with its mouse homologue; notwithstanding that native mouse Δ42PD1 isoform has not been discovered yet. We also found that mAb clone CH36 reacted with both Δ42PD1 and PD1, although it showed higher affinity binding to the former by flow cytometry (data not shown). Since it did not meet our specificity requirement, CH36 was not further studied.

Both CH34 and CH101 could distinguish native human Δ42PD1 from PD1, arguing for their useful application in functional analysis of the novel PD1 isoform. We, therefore, further analyzed the isotype and avidity of these Δ42PD1-specific mAbs using a commercial mouse mAb isotyping kit and surface plasmon resonance, respectively. As shown in Fig. 2D, the isotype of clones CH34 and CH101 is IgG2b/Kappa and IgG1/Kappa, respectively. Both mAbs showed features of high-affinity to recombinant sΔ42PD1fc with Kd values of 1.15 x 10^-11 and 3.24 x 10^-10 for CH34 and CH101, respectively (Fig. 2E).

Epitope mapping via yeast surface display

To map the binding epitopes of mAbs CH34 and CH101, 6 fragments of human sΔ42PD1 were displayed on yeast cell surface. Each fragment contained 30 amino acids in length with 10 amino acids overlapping between 2 fragments (Fig. 3A). We found that mAb CH101 bound fragment-2 of sΔ42PD1 whereas CH34 did not recognize any of the 6 fragments by flow cytometry analysis (Fig. 3B). The crystal structure of the PD1/PD-L1 complex has been solved previously. Accordingly, the fragment-2 of sΔ42PD1 is likely exposed on the surface of PD1 molecule (Fig. 3C). ELISA further confirmed a linear peptide (Pet-1) in fragment-2 for CH101 binding (Fig. 3D). CH34 probably
recognizes a conformational structure that is not presented by these yeast-displayed epitopes. Flow cytometry analysis indicated that CH101, but not CH34, was able to compete the binding of pre-labeled Alexa Fluor 647-CH101 to 293T-Δ42PD1 cells (Fig. 3E), suggesting that the 2 mAbs are likely to have distinct binding domains. Since mAb CH101 did not recognize PD1 expressed on 293T cells, there is likely a major difference between the conformational structures of Δ42PD1 and PD1. This notion is also supported by the fact that PD1-specific mAbs (clones M1H4 and NAT105) did not recognize Δ42PD1 expressed on 293T cells (Fig. 2A and B). Further analysis of additional PD1-specific mAbs, including clones EH12.1, EH12.2H7 and J110, generated similar results (data not shown).

**Down-regulation of Δ42PD1 on monocytes during chronic HIV-1 infection**

Δ42PD1 was initially discovered by the detection of mRNA transcripts in PBMCs of healthy donors using reverse transcription PCR by our group. Therefore, we analyzed Δ42PD1 expression on normal human PBMCs by flow cytometry using home-made fluorescence-labeled Δ42PD1-specific mAb. As shown in Fig. 4A, lymphocyte and monocyte/macrophage populations in human PBMCs could be distinguished by their forward- and side-scatter profiles. We found that Δ42PD1 was preferentially expressed on monocyte/macrophage, but no significant expression was observed when total lymphocytes were examined (Fig. 4B). Our preliminary data showed that the stimulation of PBMC with phytohemagglutinin, pokeweed mitogen or CD3/CD28 antibodies for 2 d up-regulated PD1, but not Δ42PD1, on T cells in vitro (data not shown). Considering the critical role of monocytes/macrophages in HIV-1 infection, we then investigated Δ42PD1 expression on CD14+ monocytes from patients who either were not treated or were treated with antiretroviral therapy (No-ART and ART, respectively) and compared the result with that of healthy controls (HC). Based on the MFI, Δ42PD1 expression was significantly higher (~3-fold) on CD14+ monocytes in healthy subjects than in No-ART (P < 0.0001) and ART (P < 0.0001) subjects (Fig. 4C). No significant difference was observed between No-ART and ART (P = 0.64) subjects for Δ42PD1 expression on CD14+ monocytes (Fig. 4C). Interestingly, Δ42PD1 expression level was negatively correlated with CD4 count, but not with viral load, among No-ART subjects (Fig. 4D).

**Δ42PD1 expression on a subset of tumor-infiltrating T cells**

Since PD1 is also involved in tumor pathogenesis, we investigated Δ42PD1 expression in human tissue sections of esophageal squamous-cell carcinoma derived from 3 patients by immunohistochemistry. Interestingly, Δ42PD1+ cells were abundantly detected in stroma between tumor nests, the boundary between stroma and tumor nest, and even within tumor nest, in all 3 samples (Fig. 5). Dual immunofluorescence staining showed that Δ42PD1 was mainly expressed in a subset of CD3+ T cells (Fig. 5).
Discussion

In this study, we successfully generated 2 mouse mAbs that specifically recognize the native form of human Δ42PD1 when it is expressed on cell surfaces by both flow cytometry and immunohistochemical assays. These mAbs were elicited through a DNA prime/protein boost immunization regimen in BALB/c mice when human Δ42PD1 was used as an immunogen. Moreover, when delivered in the form of either DNA or fusion protein with rabbit IgG1 Fc, the native structure of human sΔ42PD1 was well presented to B cells for the induction of specific antibody responses. This notion was fully supported by our results that both polyclonal and monoclonal antibodies preferentially recognized human sΔ42PD1 but not PD1 when both were natively expressed on cell surface. Interestingly, although mAbs CH34 and CH101 both displayed high affinity binding to human sΔ42PD1, they seemed to recognize different epitopes of Δ42PD1. Since Western blot results indicated a cross-binding of mAb CH34 or CH101 to denatured PD1 protein, antibody reactive sequences are likely occluded in PD1 while exposed in Δ42PD1. Alternatively, the native epitope structure in Δ42PD1 differs from that in PD1. These findings warrant future investigation of these epitopes in the biological function of Δ42PD1.

The distinct immunogenicity profile of human Δ42PD1 may imply a functional difference between Δ42PD1 and PD1. The lack of cross-reactivity between Δ42PD1- and PD1-specific mAbs indicates clearly their distinct binding structures on Δ42PD1 and PD1. This finding is consistent to our findings that Δ42PD1 was not recognized by PD1-specific mAbs and could not interact with the native ligands of PD1, namely PD-L1 and PD-L2. Since human Δ42PD1, but not PD1, could induce the production of pro-inflammatory cytokines from PBMCs, it is reasonable to speculate that Δ42PD1 is not redundant, but rather a functional protein. To test this hypothesis, we measured the surface expression of Δ42PD1 on human peripheral lymphocytes and monocytes. For the first time, we discovered that Δ42PD1 was constitutively expressed primarily on the surface of CD14+ monocytes of healthy individuals by flow cytometry analysis. This finding is consistent with our previous observation that the level of Δ42PD1 transcripts was high in monocyte/macrophage and low in T and B cells. Since CD14+ monocytes among healthy individuals do not express PD1 (data not shown), these
findings prompted us to investigate the expression of Δ42PD1 in human diseases.

Human Δ42PD1 may play a role in HIV and tumor pathogenesis. A previous study had indicated that PD1 expression was upregulated on the surface of monocytes during HIV-1 infection, resulting in impaired CD4+ T cell activation. Here, we found that the Δ42PD1 expression on the surface of CD14+ monocytes was significantly downregulated during chronic HIV-1 infection. Moreover, this downregulation is unlikely to be reversible among patients who have received antiviral therapy with well-suppressed viral load (<500 copies/ml). Since there was an inverse correlation between Δ42PD1 expression on CD14+ monocytes and CD4 count among the study subjects, it is possible that Δ42PD1+/CD14+ monocytes play a role during HIV-1 infection. Although artificial soluble Δ42PD1 may induce the release of inflammatory cytokines from PBMCs in vitro, further studies should define the role of Δ42PD1 expression on CD14+ monocytes in modulating immune activation. Also, the effect of PD1/Δ42PD1 differential expression on CD14+ monocytes during HIV infection should be investigated. In addition, we also measured the Δ42PD1-expressing cells in tissues of human esophageal squamous cell carcinoma (ESCC). To our surprise, although Δ42PD1 is not highly expressed in lymphocytes of healthy individuals, a subset of ESCC-infiltrating CD3+ T lymphocytes exhibited elevated Δ42PD1 expression in all 3 patients tested. These Δ42PD1+/CD3+ T lymphocytes were mainly found in stroma areas surrounding the tumor nests. It is possible that the tumor microenvironment may either alter the T cell phenotype by up-regulating Δ42PD1 expression or recruit a distinct Δ42PD1+ T cell population from a different source (e.g., tissues). The underlying mechanism, however, remains to be determined by future experiments. In conclusion, the generation of human Δ42PD1-specific mAbs provides a useful tool for the investigation of the role of this novel immune regulatory protein in HIV-1 and tumor pathogenesis, as well as in other diseases.
Materials and Methods

Study subjects
The study was conducted with the approval of the Institutional Review Board of Shenzhen Third People’s Hospital. Blood samples were collected after written informed consent was obtained from each participant. A total of 28 HIV-1 infected patients, including 14 untreated patients (No-ART) and 14 patients on ART, were recruited at the Shenzhen Third People’s Hospital, Shenzhen, Guangdong province, China (Table 1). All patients were seronegative for hepatitis B and C. None showed signs of active tuberculosis. Human esophageal tumor specimens were obtained from 3 patients undergoing surgical resection for ESCC in accordance with the ethical standards of the Institutional Committee on human experimentation at the Queen Mary Hospital in Hong Kong. Clinical pathologists conducted histologic examination, and the diagnosis was made based on the microscopic features of the carcinoma cells.

Cell culture
SP2/0-Ag14 myeloma cells (Cat. No.CRL-1581, ATCC), 293T cells, and human PD1 or Δ42PD1 stably expressing 293T cell lines (293T-PD1, 293T-Δ42PD1) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Cat. No.11995, Gibco) supplemented with 10% fetal bovine serum (FBS) plus 1% pen/strep (Cat. No.15140, Gibco). All above-mentioned cells were maintained in a 37°C humidified 5% CO2 incubator. Suspension-adapted HEK293 cells (FreeStyle293-F, Cat. No.R79007, Life Technologies) were cultured in the serum-free FreeStyle293-F Expression Medium (Cat. No. 12338–018, Gibco) in 37°C incubator with a humidified atmosphere of 8% CO2 on an orbital shaker platform rotating at 135 rpm.

Figure 4. Flow cytometric analysis of Δ42PD1 expression on cells of healthy and HIV-1 infected subjects. (A) Gating of lymphocyte and monocyte/macrophage (Mo/Mφ) populations in human PBMCs by FSC and SSC, and representative dot plotting of Δ42PD1 expression by flow cytometric analysis. Iso Ctrl, isotype control. (B) Frequencies of Δ42PD1 positive cells among lymphocyte and Mo/Mφ populations of healthy donors (n = 15). (C) MFI of Δ42PD1 expression on total monocytes (CD14+ cells) in PBMCs of healthy donors (HC, n = 20) as compared with untreated HIV-1 patients (No-ART, n = 14) and antiretroviral drug treated HIV-1 patients (ART, n = 14) by flow cytometric analysis. (D) Correlation between Δ42PD1 MFI of CD14+ monocytes and CD4 count or viral load (copies/ml) among No-ART patients. ***p < 0.001.
Expression and purification of recombinant protein

Recombinant proteins sΔ42PD1Fc and sΔ42PD1His were expressed using Freestyle 293 Expression System (Cat. No. K9000–01, Life Technologies) and purified using Recombinant Protein G (rProtein G) Agarose (Cat. No. 15920–010, Life Technologies) for sΔ42PD1Fc and Dynabeads His-Tag Isolation & Pulldown (Cat. No. 10103D, Life Technologies) for sΔ42PD1His and sPD1His, respectively, following the manufactures’ instructions. Plasmids used for protein preparation were previously constructed. Concentration and purity of proteins were determined by BCA Protein Assay Kit (Cat. No. 23227, Pierce) and Coomassie Brilliant Blue-stained SDS-PAGE, respectively.

Immunization and cell fusion

All animal experiments received approval from the Committee on the Use of Live Animals in Teaching and Research, Laboratory Animal Unit, The University of Hong Kong, Hong Kong SAR, China. For immunization, mice were immunized with sΔ42pd1Fc plasmids, which encode the extracellular domain of human Δ42PD1 soluble Δ42PD1, sΔ42PD1) fused with rabbit IgG1 Fc region. Briefly, 100 μg sΔ42pd1Fc plasmid in 50 μl PBS was injected intramuscularly (i.m.) in the quadriceps of female BABL/c mouse (8–10 weeks of age) on week 0 and 3. PBS was injected intramuscularly (i.m.) in the quadriceps of female BABL/c mouse (8–10 weeks of age) on week 0 and 3. Immediately following injection, electroporation (EP) was performed at the injection site using a 2-needle array with a 0.5 cm gap. EP parameters were: 120 V/cm distance between the electrodes; 50-ms pulse length; 6 pulses, generated by a TERESA (Shanghai Teresa Healthcare) generator. After the DNA/EP priming, 20 μg sΔ42PD1Fc proteins emulsified in Freund’s complete adjuvant was immunized subcutaneously on week 6, followed by 20 μg immunogen in Freund’s incomplete adjuvant subcutaneously on week 9. Mouse sera were collected 7 d after the fourth immunization for ELISA and flow cytometry. At day 7 following the last boosting, 1.5 × 10⁸ spleen cells of the immunized mice were collected and fused with SP 2/0 myeloma cells at a ratio of 10:1 using polyethylene glycol solution (Cat. No. P7181, Sigma). Hybridoma producing mAb against human Δ42PD1 were generated as described by Kohler and Milstein.³⁴ Hybridoma cells were selected in HAT medium (DMEM supplemented with 20% FBS and 2% HAT) for 7 d and then switched to HT medium (DMEM supplemented with 20% FBS and 1% HT).

Hybridoma screening and antibody isotyping

Two weeks after cell fusion, culture supernatants were tested for specific antibody production by indirect ELISA. 100μl sa42PD1His (0.2 μg/ml) was coated in 96-well EIA/RIA plates (Cat. No. 3369, Corning) overnight at 4°C. The plates were then washed once with phosphate-buffered saline (PBS) containing 0.1% Tween-20 (PBS-T) and blocked with 200 μl of PBS containing 4% skim milk at 37°C for 1h. After three washes, hybridoma supernatants were added to the plates (100 μl/well) and incubated for 1h at 37°C. After 3 times washes, goat anti-Mouse IgG H&L (HRP) secondary antibody (100 μl/well, Cat. No. ab97040, Abcam) diluted 1:50,000 was added to plates. Plates were then incubated at 37°C for 1 h. After extensive washes, the enzymatic reaction was developed with 3,3',5,5'-tetramethylbenzidine (TMB) liquid substrate (Cat. No. T4444, Sigma) and stopped by adding 0.2 M H₂SO₄. The optical density was measured at 450 nm (OD₄₅₀) with a VICTOR™ 1420 Multilabel Counter (Perkin-Elmer). We performed the isotyping of the anti-Δ42PD1 mAbs, using the rapid ELISA mouse mAb isotyping kit (Cat. No. 37503, Pierce), according to the manufacturer’s instructions.

Table 1. Demographic characteristics of HIV-infected subjects

| Group             | No ART (n = 14) | ART (n = 14) | P*       |
|-------------------|----------------|-------------|----------|
| Mean age, years   | 39 ± 3.04      | 37 ± 2.13   | 0.59     |
| Sex (male/female) | 13/1           | 13/1        | 1        |
| Mean CD4 count    | 180.5 ± 32.54  | 32.69 ± 33.39 | 0.0042   |
| Mean viral load   | 824900 ± 403200 | <500³       | <0.0001  |
| Mean CD4%         | 11.20 ± 1.398  | 20.51 ± 2.382 | 0.0023   |
| Mean CD8%         | 44.06 ± 3.179  | 62.63 ± 2.744 | 0.0002   |
| Risk behavior     | 13/1           | 13/1        | 1        |

Some data are presented as mean ± SEM.

*P values were determined by Student t test or χ² test.

³Viral load is less than the detectable level (< 500 copies/mL).

NA, not applicable.

Figure 5. Analysis of Δ42PD1 expression in tissues of human esophageal squamous cell carcinoma by immunofluorescence histochemistry. Tissue samples from 3 patients (A–C) were stained for Δ42PD1 (green, 1), CD3 (red, 2) and nucleus blue (blue, 3) as color-coded. White dashed circle/line shows the distribution of tumor nests. Δ42PD1⁻/CD3⁻ T cells (white or yellow) in the large merged images were frequently observed in stroma between tumor nests (white arrow), tumor/stroma boundary (green arrow), and scattered within tumor nest (red arrow). 100X (A) or 200X (B and C) in magnification.
Flow cytometry analysis
For indirect staining, cells were initially incubated with mouse serum or hybridoma supernatant or purified mAbs followed by staining with fluorescein conjugated secondary antibody after washing with FACS buffer (PBS with 2% FBS and 0.1% NaN₃). For direct staining, cells were incubated with fluorescence-labeled mAbs. For intracellular staining, cells were fixed and permeabilized using Fixation/Permeabilization Solution Kit (Cat. No. 554714, BD Biosciences) according to the manufacturer’s instructions, and then stained using the above-mentioned method. All the stained tubes were incubated for 30 min at 4°C. After washing, cells were resuspended in 0.4 ml PBS and then subjected to acquisition with FACS Calibur or FACS Aria III Flow Cytometer (BD Biosciences), and data were analyzed with FlowJo software. Commercial antibodies used in flow cytometry include Alexa Fluor 647 Goat Anti-Mouse IgG (H+L) (Cat. No. A21235, Life Technologies), Goat anti-Human PD1 polyclonal antibody (Cat. No. AF1086, R&D Systems), Mouse anti-Human PD1 clone (clone CH101, Cat. No. 14–9969–82, ebioscience), Mouse IgG1 (clone P3.6.2.8.1, Cat. No. 16–4714–85, ebioscience), Mouse IgG2b (clone eBMG2b, Cat. No. 16–4732–85, ebioscience), Mouse Anti-Human PD1 antibody (clone NAT105, Cat. No. ab52587, Abcam), Alexa Fluor 568 Goat Anti-Mouse IgG1 (Cat. No. A-21124, Life Technologies); Alexa Fluor 488 Goat Anti-Mouse IgG1 (Cat. No. A-21121, Life Technologies); Alexa Fluor 488 Goat Anti-Mouse IgG2b (clone A-21141, Life Technologies). Antibody competitive assay was conducted by using equal amount of soluble mAbs CH101 and CH34 to block the binding of 0.25 μg of pre-labeled Alexa Fluor 467-CH101 to 293T-Δ42PD1 cells.

Immunocytochemistry assay
293T-PD1 and 293T-Δ42PD1 cells were harvested by vigorous pipetting, and then concentrated to 3 × 10⁶ cells/ml. 10 μl cell suspensions were dropped on clean glass slides followed by drying in a hood for 1 h. Samples were fixed in either cold methanol or 10% neutral buffered formalin for 10 min at RT and permeabilized with 0.5% Triton X-100 for 10 min. Samples were then stained with 20 μg/ml anti-human Δ42PD1 mAbs (clone CH34 and CH101) or anti-PD1 mAb (clone NAT105, Cat. No. ab52587, Abcam) for 1 h at RT, followed by staining with fluorescein conjugated secondary antibody (Alexa Fluor 568 Goat Anti-Mouse IgG1 (Invitrogen) for NAT105; Alexa Fluor 488 Goat Anti-Mouse IgG1 (Invitrogen) for CH101; Alexa Fluor 488 Goat Anti-Mouse IgG2b (Invitrogen) for CH34) for 1 h at RT after washing. Cells were incubated with 1:10,000 diluted Hoechst33258 (Cat. No. H3569, Life Technologies) for 10 min at RT. After washing, microscopic images were obtained under the Inverted Research Microscope ECLIPSÈ Ti (Nikon) with SPOT Advanced Software version 4.6 (Diagnostic Instruments).

Western blot analysis
Cells were harvested and lysed with RIPA Lysis and Extraction Buffer (Cat. No. 89900, Pierce) containing protease inhibitor cocktail (Cat. No. 78429, Pierce) 2 d post-transfection. Protein concentration was determined with a NanoDrop 8000 Spectrophotometer (Thermo Scientific). Approximately 50 μg of protein samples were boiled for 5 min, loaded onto 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to polyvinylidene difluoride (PVDF) membrane using a Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad). PageRuler Prestained Protein Ladder (Cat. No. 26616, Pierce) were used to determine molecular weight. After blocking with 5% skim milk for 1 h, membranes were incubated with specific primary antibodies for 1 h at RT with gentle agitation, followed by incubation with the appropriate IRDye secondary antibodies (1:10,000, LI-COR) for 1 h at RT with gentle agitation. Finally, the reactions were visualized with a LI-COR Odyssey infrared imager (LI-COR). Commercial antibodies used in current experiment include goat anti-mouse PD1 polyclonal antibody (Cat. No. AF1021, R&D Systems), α-Tubulin antibody (clone DM1A, Cat. No. sc-32293, Santa Cruz Biotechnology), IRDye 680RD Donkey anti-Goat IgG (Cat. No. 926–68074, LI-COR), IRDye 680RD Goat anti-Mouse IgG (Cat. No. 926–68072, LI-COR).

Surface plasmon resonance assay
Binding avidity analysis was performed with a Biacore×100 optical biosensor (GE Healthcare). Immobilization of recombinant sΔ42PD1Fc to CM5 sensor chip (Cat. No. BR100012, GE Healthcare) was performed following the standard amine coupling procedure. Carboxyl groups on the sensor chip surface were activated by injection of N-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) in Amine Coupling Kit (Cat. No. BR-1000-50, GE Healthcare). Recombinant sΔ42PD1Fc at a concentration of 30 μg/ml in 10 mM sodium acetate buffer (pH 5.0) was allowed to flow over the chip surface at a rate of 5 μl/min for 7 min, and the final response bound turned out to be 7379 RU. After unreacted protein was washed out, excess active ester groups on the sensor surface were capped by injection of 1 M ethanolamine (pH 8.5) at a flow rate of 5 μl/min for 7 min. As background to correct instrument and buffer artifacts, a reference was generated under the same conditions without immobilization of the recombinant protein. Binding experiments were performed at 25°C in HBS-EP buffer (Cat. No. BR.1006-69, GE Healthcare). Binding kinetics were analyzed by passing various concentrations of anti-human Δ42PD1 mAbs CH34 and CH101 over the chip surface for 3 min. Dissociation of bound analytes was monitored while the surface was washed with buffer for 4 min at a flow rate of 30 μl/min. Remaining analytes were removed in the surface regeneration step with injection of 10 mM glycine-HCl (pH 2.0) for 2 × 30 sec at a flow rate of 30 μl/min. The kinetic parameters were determined after subtraction of the blank cell from each response value, by collectively fitting the overlaid sensorgrams locally using Biacore × 100 Evaluation software (version 2.0.1) to the 1:1 Langmuir binding model.

Yeast surface display and epitope mapping
EBY100 yeast cells and the yeast expression plasmid pCTCON2 have been described previously.35,36 DNA sequences
encoding 6 fragments of human Δ42PD1 at corresponding position of L27-M56 for fragment-1 (F1), E47-G76 for fragment-2 (F2), A67-V96 for fragment-3 (F3), P87-P116 for fragment-4 (F4), Y107-E136 for fragment-5 (F5), E127-V156 for fragment-6 (F6) (Fig. 3A) were amplified by PCR using the Expand High Fidelity PCR System (Cat. No. 11732650001, Roche). The PCR products were then purified, digested and ligated to pCTCON2 vector. The ligation products were transformed into competent TG1 cells. After sequencing, recombinant plasmids with 100% homology were isolated and termed as pCTCON2–Δ42PD1-F1–6 accordingly. Competent EBV100 cells were then transformed with pCTCON2–Δ42PD1-F1–6 via EP 0.1 mm gene pulser cuvette (Cat. No. 165–2089, Bio-Rad) using Gene Pulser Xcell™ Total System (Cat. No. 165–2660, Bio-Rad) with EP parameters of 1.2 kv, 25 μF and resistance 200kΩ.

3.7 Surface expression of Δ42PD1 fragments was verified by analysis of surface expression of c-Myc tag with flow cytometry. For epitope mapping, after being cultured in SGCAA media for 30 h at 4°C, Δ42PD1 fragment-expressing yeast cells were analyzed by flow cytometry with a panel of antibodies including anti-Δ42PD1 mAbs clone CH34 and CH101, mouse anti-human PD1 (clone MIH4, Cat. No. 14–9969, eBioscience; clone EH12.1, Cat. No. 560795, BD PharMingen; clone EH12.2H7, Cat. No. 329911, BioLegend), goat-anti-human PD1 polyclonal antibody (Cat. No. AF1086, R&D Systems).

Further epitope mapping by ELISA
Three peptides of fragment-2 as indicated in Figure 3A were synthesized and subjected to ELISA for binding with CH101 and CH34, respectively (Fig. 3D). 1 μg of each peptide was coated into each well of ELISA plates. Serially diluted CH101 or CH34 were incubated for 30 min at 37°C, followed by detection with goat-anti-mouse IgG-HRP (Promega).

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Immunofluorescent histochemical staining
Immunofluorescence staining was performed on 5-μm paraffin sections of formalin-fixed tissue samples. Samples were deparaffinized and rehydrated. After blocking with normal goat serum for 30 min at room temperature, mouse anti-human Δ42PD1 antibody (clone CH101) was applied at 37°C for 1 h, followed by Alexa Fluor 488 conjugated goat anti-mouse IgG1 antibody (Cat. No. A-21121, Life Technologies) for 1 h at room temperature. The additional immunofluorescence staining was then performed by incubation of rabbit anti-CD3 antibody (Cat. A0452, DAKO) at room temperature for 1 h, followed by Alexa Fluor 568 conjugated goat anti rabbit IgG antibody (Cat. No. A-11036, Life Technologies) for 1 h at room temperature. Nuclei were counterstained with Hoechst33258 (Cat. No. H3569, Life Technologies).

Statistical analysis
We performed Student’s t-test and Spearman’s rank-correlation, using Prism 5.0 software (Prism). The error bars in figures represent ±1 standard deviation (SD) from the mean. P-values less than 0.05 were considered statistically significant.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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