Specific and high-affinity binding of tetramerized PD-L1 extracellular domain to PD-1-expressing cells: possible application to enhance T cell function

Seigo Terawaki1, Yoshimasa Tanaka2,3, Tomokazu Nagakura2, Tamon Hayashi4, Shiro Shibayama4, Kaori Muroi2, Taku Okazaki5, Bunzo Mikami6, David N. Garboczi7, Tasuku Honjo1 and Nagahiro Minato2

1Department of Immunology and Genomic Medicine, Graduate School of Medicine and 2Laboratory of Immunology and Cell Biology, Graduate School of Biostudies, Kyoto University, Yoshida-Konoe, Sakyo-Ku, Kyoto 606-8501, Japan
3Precursory Research for Embryonic Science and Technology, Japan Science and Technology Agency, 4-1-8 Honcho, Kawaguchi, Saitama 332-0012, Japan
4Tsukuba Research Institute, Ono Pharmaceutical Co., Ltd, Tsukuba, Ibaraki 300-4247, Japan
521st Century Center of Excellent Program, Graduate School of Medicine, Kyoto University, Yoshida-Konoe, Saky-Ku, Kyoto 606-8501, Japan
6Laboratory of Food Quality Design and Development, Graduate School of Agriculture, Kyoto University, Uji, Kyoto 611-0011, Japan
7Structural Biology Section, Laboratory of Immunogenetics, National Institute of Allergy and Infectious Diseases, 12441 Parklawn Drive, Rockville, MD 20852, USA

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Abstract

The negative co-stimulatory receptor, programmed cell death 1 (PD-1), is induced on activated T cells and delivers inhibitory signals upon engagement with its ligands PD-L1 and PD-L2, which are expressed on various somatic cells and certain cancers. Accumulating evidence suggests that interfering with the PD-1–PD-L1 interaction may result in the restoration of defective T cell functions in cancer and chronic viral infection. Herein, we established procedures to produce large amounts of renatured recombinant extracellular domain proteins of mouse PD-1 (mPD-1) and PD-L1. While monomeric mPD-1 and mouse PD-L1 (mPD-L1) only marginally interacted with the cells expressing their counterpart proteins, their tetramerization markedly enhanced the affinity with the $K_d$ of mPD-L1 tetramer being nearly 100-fold lower than that of the corresponding monomer. The affinity of mPD-L1 tetramer was even higher than a high-affinity anti-PD-1 mAb, and it efficiently inhibited the binding of mPD-L1/Fc-chimeric protein to mPD-1 cells. Functionally, mPD-L1 tetramer significantly enhanced the proliferative responses as well as the cytotoxic activity of T cells against specific target cells in vitro. The results suggest that oligomeric PD-L1 extracellular domains may provide a potential means to restore T cell functions in cancer and viral infection in humans.

Introduction

Stimulatory and inhibitory co-receptors play crucial roles in regulating TCR-mediated activation of T cells (1, 2). The stimulatory co-receptor CD28 is constitutively expressed on T cells as a covalent homodimer and essential for the activation of naive T cells by antigens. The cytoplasmic tail of CD28 contains a YMNM sequence motif, a phosphatidylinositol-3-kinase-binding site, which is responsible for the co-stimulatory functions (3–5). The inhibitory co-receptor, cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), disulfide-linked homodimer, is induced following T cell activation and delivers an inhibitory signal via a YVKM motif, a typical immunoreceptor tyrosine-based inhibitory motif (ITIM), in the cytoplasmic tail (6, 7). CD28 and CTLA-4 share their ligands, CD80 and CD86, on professional antigen-presenting cells (APC), and coordinately regulate the initiation and termination of naive T cell activation.

PD-1 is a member of the Ig superfamily (IgSF) containing a single V-set domain and is induced on activated T cells most
likely as a monomer (8, 9). Upon engagement with specific ligands, PD-1 delivers a negative signal to attenuate TCR-mediated T cell activation. The PD-1 cytoplasmic domain contains two tyrosine residues, with the membrane proximal one being in the context of an ITIM and the other an immunoreceptor tyrosine-based switch motif (ITSM). It was reported that tyrosine phosphorylation of PD-1 in the ITSM, rather than in the ITIM, was responsible for inhibitory signaling via recruitment of Src homology domain 2-containing tyrosine phosphatase-1 (SHP-1) and SHP-2 (10–12). Two ligands for the PD-1 receptor have been identified to date, PD-L1 and PD-L2, which are also IgSF members containing one V- and one C1-set Ig domain with weak overall homology to the B7 family proteins (13–16). Unlike B7 proteins, however, PD-L1 is constitutively expressed on various types of somatic cells in addition to professional APCs (17–21).

It was previously reported that PD-1+/− mice develop characteristic autoimmune diseases depending on the genetic backgrounds (22–24), suggesting that PD-1 played a role in maintaining self-tolerance. It was further suggested that PD-1–PD-L1 interaction might be involved in the escape of certain tumor cells from host immune attack. Thus, the expression of PD-1 on CD8+ effector T cells resulted in attenuated cytotoxic activity against specific tumor cells expressing PD-L1, and the administration of anti-PD-L1 mAb in vivo to block the PD-1–PD-L1 interaction caused significant inhibition of tumor growth in a mouse model (25–29). More recently, constitutive expression of PD-1 on CD8+ cells was associated with impaired effector functions in chronic lymphocytic choriomeningitis virus (LCMV) infection in mice (30) and in HIV infection in humans (31–33). These results suggest that effective blocking of the PD-1–PD-L interaction in vivo may provide a potential means to control human diseases such as malignancies and chronic viral infections.

In the present study, we developed strategies to prepare soluble recombinant extracellular domains of PD-1 and of PD-L1 capable of binding to PD-L1 and PD-1, respectively. We show that tetramerized PD-1 or PD-L1 binds to ligand or receptor with higher affinity than do monomers and provide evidence that the mouse PD-L1 (mPD-L1) tetramer may antagonize the negative signal mediated by PD-1 to enhance T cell functions.

Methods

PD-1 and PD-L1 tetramers

The DNA fragments encoding the V-set extracellular domain of PD-1 (residues L25 to T145) and the V-set and C1-set extracellular domains of PD-L1 (residues F19 to P229) in an Escherichia coli expression vector were PCR amplified with 5′ primer containing methionine (Met) and alanine (Ala) codons at the N-terminus and 3′ primer containing a BirA substrate peptide (BSP), GSLHHILDAQMWWNR (34), at the C-terminus. Each PCR product was digested with EcoRI and SalI and recloned into the vector. To achieve a high level of refolding yield, C83S and C113S mutations were introduced in PD-1 and PD-L1 cDNAs, respectively. The inclusion body proteins in E. coli were purified and renatured in a refolding buffer containing Tris–HCl, pH 8.0, arginine hydrochloride, reduced glutathione, oxidized glutathione, Na2EDTA and phenylmethylsulfonylfluoride at 4°C overnight. The samples were then applied to a DE52 anion-exchange column connected to a Q Sepharose HP column and eluted with a 0–400 mM NaCl linear gradient for PD-1 and 0–700 mM for PD-L1 in 20 mM Tris–HCl buffer, pH 8.0, followed by purification on Superdex 200 column chromatography. The refolded and purified recombinant monomers were biotinylated by using BirA at 25°C, and excess biotin was removed by gel filtration. The biotinylated proteins were tetramerized by incubating with streptavidin (Wako Pure Chemical Industries Ltd, Chuo-Ku, Osaka, Japan) or R-PE-conjugated streptavidin (Molecular Probes, Inc., Eugene, OR, USA) (35) followed by the purification on Superdex 200 gel filtration. Endotoxin levels in the final preparations were <1 EU µg−1 as judged by the Limulus amebocyte lysate (LAL) test (Cambrex, Walkersville, MD, USA).

Circular dichroism spectral analysis

Biotinylation substrate-tagged mouse PD-1 (mPD-1) and mPD-L1 protein samples were dialyzed against PBS and adjusted to final concentrations of 63.9 and 38.7 µM, respectively. UV circular dichroism (CD) spectra of the preparations were measured in a quartz cuvette of 0.01 cm path length using a spectropolarimeter (Japan Spectroscopic, Tokyo, Japan). CD spectra were recorded from 260 to 190 nm at a digital resolution of 0.1 nm with scan speed of 10 nm min−1. The scans were single averaged and the data sets were concatenated and curve fitted by ninth order polynomial regression.

Cell lines and cell cultures

A cDNA-encoding mPD-L1 or mouse PD-L2 (mPD-L2) was digested with EcoRI or Smal and Xbal and incorporated into a pApuroXS or a pEF-BOS-neo plasmid kindly provided by Shigekazu Nagata, Osaka University, Suita, Osaka, Japan. A murine P815 mastocytoma line was transfected with mPD-L1 and mPD-L2 cDNAs in a pApuroXS and a pEF-BOS vector, respectively, by electroporation. The established transfectants were maintained in complete RPMI 1640 medium containing 3 µg ml−1 of puromycin for P815/mPD-L1 or 1 mg ml−1 of G418 for P815/mPD-L2. A murine IIA1.6 B cell line was similarly transfected with the mPD-L1 cDNA and was maintained in a medium containing 3 µg ml−1 of puromycin. Primary spleen cells from normal or mPD-1−/− B6 littermate mice were cultured in the complete Iscove’s modified DMEM containing 10% FCS in the presence of 0.3 µg ml−1 of plate-coated anti-CD3 mAb (2C11), 50 µg ml−1 of LPS or 15 µg ml−1 of goat anti-mouse IgM antibody and the bone marrow (BM) cells with 50 µg ml−1 of LPS for 3 days.

Antibodies and flow cytometric analysis

Cell lines were directly stained with R-PE–streptavidin-conjugated recombinant tetramers or with biotin-conjugated recombinant monomers followed by R-PE-conjugated streptavidin and analyzed by using FACS Calibur (Becton Dickinson, San Diego, CA, USA). Control staining was done with R-PE-conjugated streptavidin alone. For blocking experiments, IIA1.6/mPD-1 cells were incubated with 10 µg ml−1 of the mPD-L1/Fc chimera (R&D Systems, Inc., Minneapolis, MN, USA).
USA) in the absence or presence of various concentrations of mPD-1 tetramer, followed by Alexa647-conjugated goat anti-human IgG (Fc) antibody (Rockland, Gilbertsville, PA, USA). For primary cells, two-color staining was performed. Spleen cells cultured with anti-CD3 and LPS were incubated with allophycocyanin (APC)-conjugated anti-Thy1.2 and APC-conjugated anti-CD11b (Becton Dickinson), respectively, followed by R-PE-conjugated mPD-L1 tetramer or R-PE-conjugated anti-mPD-1 mAb (J43, eBioscience, San Diego, CA, USA) together with anti-FcyRIII (2.4G2). BM cells cultured with LPS were incubated with APC-conjugated anti-CD11b (Becton Dickinson) followed by R-PE-conjugated mPD-L1 tetramer or R-PE-conjugated anti-mPD-1 mAb (J43) together with anti-FcyRIII (2.4G2). R-PE-conjugated streptavidin alone served as a control for the secondary staining. Other antibodies used included R-PE-anti-mPD-L1 (MIH5), R-PE-anti-mPD-L2 (TY25), R-PE-IgG and R-PE-anti-mPD-1 (J43, eBioscience).

Reverse transcription–PCR
Splenic T cells, B cells and BM monocytes from PD-1++ and PD-1−− B6 mice were enriched by staining with anti-Thy1.2, anti-B220 and anti-CD11b, respectively, followed by positive sorting by using autoMACS (Miltenyi Biotec, Gladbach, Germany). The sorted cells were cultured with Concanavalin A (ConA) (2 μg ml−1) or immobilized anti-CD3 (0.3 μg ml−1) for the T cell-enriched population, LPS (50 μg ml−1) or anti-IgM (15 μg ml−1) for the B cell-enriched population and LPS (50 μg ml−1) for monocyte-enriched population. After 3 days, the cells were harvested and resorted for Thy1.2++ CD3+ cells, B220+ CD19+ cells and CD11b+ CD14+ cells from the respective cultures by using FACS Aria (Becton Dickinson, CA, USA). The two-step isolation procedures yielded highly pure activated T, B and monocyte populations. Total RNAs were extracted from the sorted cells with TRIreagent (Sigma–Aldrich, Saint Louis, MO, USA) according to the manufacturer’s protocols and subjected to reverse transcription (RT) with oligo-dT primer using Superscript III cDNA synthesis system (Invitrogen, Carlsbad, CA, USA). The primer sets used were as follows: glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (5′-GAACGGATTTGCCTATTG-3′ and 5′-GTGATGACCTTCTGGCTC-3′), mPD-1 (5′-GCAGAGCTCGTGTTAACAGAGA-3′ and 5′-TCATAGGCCACACTAGGGA-3′) and cyclophilin (5′-TGGAGAGCACCAAGACAGA-3′ and 5′-TGCCGGAGTGCACATGAT-3′). cDNA input of each sample was normalized to GAPDH as a reference gene. Real-time PCR was performed by using LightCycler® 480 (Roche, Basel, Switzerland).

Surface plasmon resonance analysis
Surface plasmon resonance (SPR)-binding assays were carried out at 25°C using a Biacore 2000 (Amersham Biosciences Corp., Piscataway, NJ, USA). CM5 sensor chips were activated by a 7 min injection of a 1:1 mixture of N-hydroxysuccinimide and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride at a flow rate of 10 ml min−1. The mPD-1/Fc-chimeric protein was prepared at a concentration of 20 μg ml−1 in 10 μM acetate buffer, pH 4.0, and immobilized to the sensor chips by amine linkage, with the typical immobilization levels being 1000–3000 response units. Blocking sensor chips, 70 μl of 1 M ethanolamine–HCl buffer, pH 8.5, was injected and the chips were further washed with 10 μl of 10 mM glycine–HCl buffer, pH 1.5. For analyzing the binding affinity of PD-L1 and PD-L2 for the mPD-1/Fc chimera, 1 mg ml−1 of each refolded protein in HBS-EP buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% v/v surfactant p20, Biacore AB, Uppsala, Sweden) (Biacore AB, Uppsala, Sweden) was injected at a flow rate of 10 μl min−1 over the sensor chips. For determining the equilibrium affinity binding, serial dilutions of recombinant proteins starting from 100 μg ml−1 or anti-mPD-1 antibody (J43) starting from 200 μg ml−1 were injected at a flow rate of 30 μl min−1 over sensor chips coupled with the mPD-1/Fc-chimeric protein. The data sets were analyzed using a model for 1:1 binding with drifting baseline (BIAevaluation Software, version 4.1, Biacore AB).

T cell proliferation assay
Thy1.2+ T cells were isolated from splenocytes of 2C TCR transgenic (Tg) mice (22, 36) by using autoMACS. 2C T cells, 1 × 105, were co-cultured with 5 × 105 of mitomycin C (MMC)-treated BALB/c splenocytes in the absence or presence of 10 μg ml−1 of anti-mPD-1 mAb (J43), 10 μg ml−1 anti-mPD-L1 (1-111A) or various concentrations of mPD-L1 tetramers in round-bottom 96-well plates in triplicate at 37°C for 3 days. The cultures were pulsed with 2 μCi of [3H]-thymidine for the last 12 h and the thymidine incorporation was determined by a solid γ-counter (PerkinElmer Inc., Wellesley, MA, USA).

Cytotoxicity assay
Spleen and lymph nod cells from 2C TCR Tg mice were co-cultured with MMC-treated splenocytes from BALB/c mice twice for 2 weeks. One week after the second stimulation, the viable cells were harvested and incubated with 1 × 104 of 51Cr-sodium chromate-labeled P815/mPD-L1 in the absence or presence of 5 μg ml−1 of J43, 1-111A or mPD-L1 tetramer at the varying effector to target ratios at 37°C and the specific 51Cr release was measured at 4 h.

Results
Production of mPD-1 and mPD-L1 tetramers
In order to optimize bacterial expression and refolding yields of mPD-1 and mPD-L1 ectodomains, we first tested several constructs for each protein. The highest refolding efficiencies and yields were obtained from constructs encoding residues L25-T145 for mPD-1 and residues F19-P229 for mPD-L1 (Fig. 1A). After the addition of translation initiation codons (Met Ala) at the N-terminus and BSP at the C-terminus, the recombinant proteins were expressed in E. coli as inclusion bodies, solubilized, renatured in an arginine-based refolding buffer and purified through consecutive chromatographies as described in Methods. On anion-exchange chromatography, refolded protein species were separated from misfolded soluble aggregates (Fig. 1B). Both proteins emerged as single peaks from a size exclusion column with elution times corresponding to their monomeric forms (Fig. 1B). By CD analyses, the purified proteins exhibited CD spectra that were characteristic of β-sheet secondary
mPD-1 and mPD-L1 tetramers specifically bind to cells expressing PD-L and PD-1, respectively

We first assessed the binding of mPD-1 and mPD-L1 tetramers to cells expressing their counterpart proteins by flow cytometric analysis. As target cells for mPD-1, P815 cells transfected with mPD-L1 (P815/PD-L1) or mPD-L2 (P815/mPD-L2) were used and for mPD-L1, IIA1.6 cells transfected with mPD-1 (IIA1.6/mPD-1) were employed. mPD-1 monomers did not bind to P815/mPD-L1 and bound marginally to P815/mPD-L2 only at 30 µg ml⁻¹ (Fig. 2A). In contrast, mPD-1 tetramers showed strong binding to both P815/mPD-L1 and P815/mPD-L2 cells in a dose-dependent manner, with significant fluorescence being detected even at a concentration of 3 µg ml⁻¹ (Fig. 2A). It was noted that parental P815 cells were weakly stained by anti-PD-L1 mAb, while mPD-1 tetramer did not bind to them at all (Fig. 2A). Similarly, mPD-L1 monomers showed no detectable binding to IIA1.6/mPD-1 cells or to mock-transfected IIA1.6 cells, whereas mPD-L1 tetramers bound strongly to IIA1.6/mPD-1

Fig. 1. Preparation of mPD-1 and mPD-L1 proteins. (A) Schematic illustrations of mPD-1 and mPD-L1 full-length proteins and ectodomains. cDNA fragments encoding ectodomains were ligated with the sequence coding BSP via a glycine-serine (GS) spacer. Cysteines were mutated to serines (C83S, C113S) to improve renaturing efficiency and a Met and an Ala were added at the N-termini for efficient translation and its removal, respectively. (B) Recombinant mPD-1 and mPD-L1 produced in Escherichia coli as inclusion bodies were renatured as described in Methods. The renatured proteins were purified with an anion-exchange column (arrowheads in upper panel) followed by preparative size exclusion chromatography (lower panel). (C) Analysis of the purified mPD-1 and mPD-L1 by CD.
The results indicated that binding of mPD-1 and mPD-L1 to their corresponding partner proteins was markedly enhanced by tetramerization. The mPD-1 monomer was more labile in solution than was mPD-L1 (data not shown), similar to the instability of the CD28 recombinant ectodomain protein observed in a recent report (37). We therefore focused our further analysis on the mPD-L1 monomer and tetramer.

We next compared the binding capacities of the mPD-L1 tetramer with those of an anti-mPD-1 mAb, J43, using primary cells from normal and PD-1^−/−/− mice. Normal splenic T and B cells activated by anti-CD3 (or ConA) and anti-IgM, respectively, could be stained by both anti-PD-1 mAb and mPD-L1 tetramers at comparable levels (Fig. 3A). Curiously, however, LPS-stimulated B cells were stained only marginally and LPS-stimulated BM monocytes (CD11b+) were not bound at all by mPD-L1 tetramers, while anti-PD-1 mAb showed weak yet significant binding to both of them even in the presence of excess anti-FcR mAb (Fig. 3A). To elucidate the minor discrepancy, we then examined the cells from PD-1^−/−/− mice. As expected, mPD-L1 tetramers did not bind at all to any types of activated cell populations (Fig. 3A). In contrast, anti-PD-1 mAb showed significant binding to all cell populations even in the presence of anti-FcR mAb. In particular, it bound to LPS-activated B cells and monocytes at the level comparable to those from wild-type littermates (Fig. 3A), strongly suggesting that the interaction of the mAb with these cells was not specific for PD-1. To further confirm this, we examined the expression of PD-1 transcripts in these populations by RT-PCR. As shown in Fig. 3(B), LPS-stimulated B cells expressed a much lower level of PD-1 transcripts (~5%) than did anti-IgM-activated B cells, and there was no detectable PD-1 transcripts in LPS-stimulated BM monocytes. Thus, we conclude that the mPD-L1 tetramer binds to cell-surface PD-1 in a highly specific manner.

**SPR analyses of the binding of mPD-L1 tetramer to PD-1 molecules**

We then examined the binding affinities of mPD-L1 monomer and tetramer molecules to mPD-1/Fc-chimeric molecules in comparison with anti-PD-1 mAb by SPR analyses. While the mPD-L1 monomer specifically bound to the mPD-1/Fc chimera in a dose-dependent manner, the dissociation was quite rapid and the $K_d$ value was calculated to be $8.6 \times 10^{-6}$ M (Fig. 4A). The dissociation rate of the mPD-L1 tetramer was >100 times lower than that of the corresponding monomer, with the $K_d$ value being calculated as $5.9 \times 10^{-8}$ M (Fig. 4B). The $K_d$ value was even smaller than that of an anti-PD-1 mAb (J43), $1.7 \times 10^{-7}$ M (Fig. 4C). Thus, it was revealed that the tetramerization of mPD-L1 resulted in a marked increase in the binding affinity to the specific ligand molecules.

**Enhanced T cell responses by mPD-L1 tetramer in vitro**

To investigate the functional effects of the mPD-L1 tetramer, we first examined whether or not it could competitively interfere with the binding of mPD-L1/Fc-chimeric protein to the
surface of IIA1.6/mPD-1 cells. While the mPD-L1 monomer hardly affected the binding of mPD-L1/Fc to the cells as detected by Alexa647–anti-Fc antibody, mPD-L1 tetramer could competitively interfere with it in a dose-dependent manner (Fig. 5A). In a reciprocal combination, mPD-L1/Fc also interfered with the binding of R-PE-labeled mPD-L1 tetramer to the cells, but the efficiency was apparently much lower (Fig. 5A). We then investigated the effects of mPD-L1 tetramer on T cell functions using 2C TCR Tg B6 mice (22, 36). T cells from the Tg mice were co-cultured with MMC-treated BALB/c spleen cells in the absence or presence of anti-PD-1 mAb (J43, 10 µg ml⁻¹), anti-PD-L1 mAb (1-111A, 10 µg ml⁻¹) or mPD-L1 tetramer (3–30 µg ml⁻¹) for 3 days and proliferative responses were assessed. It was confirmed that the 2C T cells expressed significant level of PD-1 on day 3 after stimulation (Fig. 5B), while freshly isolated T cells hardly expressed PD-1 (data not shown). As also shown in Fig. 5(C), inclusion of mPD-L1 tetramer resulted in enhanced proliferative response in a dose-dependent manner. The extent at 10 µg ml⁻¹ was nearly comparable to those of J43 and 1-111A mAbs, both of which were shown to have the ability to block PD-1–PD-L1 interaction (25, 28). To confirm that the enhancing effect was indeed mediated by the interference with PD-1 on activated T cells, we repeated the same experiments using T cells from 2C PD-1−/− littermate mice. As expected, 2C PD-1−/− T cells showed enhanced proliferative response as compared with 2C PD-1+/+ T cells. However, inclusion of mPD-L1 tetramer did not affect the proliferative response at any concentration or did J43 and 1-111A mAbs (Fig. 5C). 2C PD-1−/− T cells showed a largely comparable proliferative response to 2C PD-1+/+ T cells in the presence of optimal doses of mPD-L1 tetramer or blocking mAbs. Finally, to investigate the function of effector T cells, 2C Tg spleen cells were stimulated twice in vitro with MMC-treated BALB/c spleen cells for 2 weeks. The recovered viable cells were mostly CD8⁺ T cells (data not shown), and they were incubated with ⁵¹Cr-labeled P815/mPD-L1 cells at varying effector/target ratios in the absence or presence of mPD-L1 tetramer, J43 or 1-111A mAb at 5 µg ml⁻¹ each for 4 h followed by specific ⁵¹Cr-release assay. The mPD-L1 tetramer
PD-1 might be responsible for aberrant immune responses, or immune exhaustion, during chronic virus infection such as LCMV in mice (30) and HIV in humans (31–33). In a mouse model, it was demonstrated that administration of anti-PD-L1 mAb could significantly improve eradication of tumor cells or viruses in vivo (25–27, 29, 30). These results raised the possibility that effective blockade of PD-1–PD-L interaction in vivo might provide a potential means to reverse impaired immune responses to tumors and chronic viral infection in humans.

In the present study, we set out to prepare soluble recombinant ectodomain of PD-1 and PD-L1 that would specifically interfere with the PD-1–PD-L interaction. Although both mPD-1 and mPD-L1 proteins could be poorly expressed in ordinary E. coli expression systems, a high level of protein expression could be achieved by modifications of cDNAs including synonymous codon changes and addition of a strong ribosomal-binding site (43, 44). Even after extensive renaturing, however, recombinant proteins in monomeric form barely bound to cells over-expressing the partner proteins as judged by flow cytometry. We then examined the effects of molecular polymerization of recombinant mPD-1 and mPD-L1 proteins on their binding activity. A specific biotinylation site was added at the C-termini of constructs and recombinant proteins were tetramerized by streptavidin. Present results clearly indicated that both tetramers now could strongly and specifically bind to cells expressing their counterpart proteins. The $K_d$ of mPD-L1 tetramer for PD-1/Fc protein was $5.9 \times 10^{-8}$ M, which was >100 times lower than that of mPD-L1 monomer ($K_d$, $8.6 \times 10^{-6}$ M) and largely comparable to that of a high-affinity anti-PD-1 mAb (J43) ($K_d$, $1.7 \times 10^{-7}$ M). Consistently, mPD-L1 tetramer could effectively interfere with the binding of mPD-L1/Fc protein to IIA1.6/mPD-1 cells, while the corresponding monomer failed to elicit a significant effect. Unlike CD28 and CTLA-4, mPD-L1 tetramer also bound strongly to normal T and B cells stimulated with anti-CD3 and anti-IgM antibodies, respectively, while it did not bind to those from PD-1–/– mice at all, confirming that the binding was highly specific for cell-surface PD-1. Interestingly, mPD-L1 tetramer showed only a marginal binding to LPS-stimulated B cells and virtually no binding to LPS-stimulated BM monocytes, and this was consistent with the expression of very low and no PD-1 transcripts in respective cell types. It may be worthy of note that BCR and LPS signaling shows distinct effects on PD-1 expression in B cells. On the contrary, anti-PD-1 mAb (J43) showed a significant residual binding to the activated T and B cells from PD-1–/– mice even in the presence of anti-FcγRII/III mAb. In particular, the mAb showed almost comparable binding to LPS-activated BM monocytes from both

Discussion

The negative receptor PD-1 plays important roles in various aspects of immune responses. PD-1-deficient mice develop various autoimmune diseases depending on the genetic background and PD-1 deficiency accelerated the disease phenotypes of other genetically autoimmune-prone mice (22, 38, 39), strongly suggesting that PD-1 is crucial in maintaining self-tolerance. Also, PD-1 might contribute to compromised T cell immune responses against tumor cells expressing PD-1 ligands, facilitating immune escape of tumor cells (25, 40–42). More recently, it was reported that...
PD-1\(^{+/+}\) and PD-1\(^{-/-}\) mice in the presence of anti-FcγRII/III mAb. Similar unexpected binding was also observed in an anti-PD-L1 mAb (1-111A) to P815 cells. The apparent ‘non-specific’ binding may be due to FcR other than FcγRII/III on the cell surface or undefined cross-reactivity. In either case, present results indicate that mPD-L1 tetramer serves as a highly specific reagent to identify the cells expressing PD-1.

Although a previous report implied that monocytes might also express PD-1 (45), the current studies indicated that PD-1 expression is essentially restricted to T and B cells activated via antigen receptors.

Functionally, the mPD-L1 tetramer was shown to enhance the proliferation of 2C Tg T cells in response to specific H-2L\(^{d}\)\(^{+}\) stimulator cells. The effects were specifically targeted for PD-1\(^{+}\) T cells, because no effect was observed on 2C PD-1\(^{-/-}\) T cells. The enhancing effects were largely comparable to those of anti-PD-1 or anti-PD-L1 mAb. While antibodies are highly stable in culture, the stability of mPD-L1 tetramer in culture remains to be examined at the moment, and it may be possible that improvement of the molecular stability may further enhance the functional effects of the mPD-L1 tetramer. Nonetheless, the results strongly suggested that mPD-L1 tetramer could functionally interfere with the interaction of PD-1\(^{+}\) T cells and target cells expressing the ligands, exerting significant antagonistic effect.

Accumulating evidence strongly suggests that functional interference of PD-1–PD-L1 interaction by specific antibodies in vivo could provide a means to restore the compromised immune responses in cancer and chronic viral infections (25, 27–30). Suitably polymerized recombinant PD-L1 and possibly PD-1, which are more convenient and possibly more specific than corresponding antibodies, may provide an alternative and effective means to control cancer and infections in humans.

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Fig. 5. Enhanced T cell responses by mPD-L1 tetramer in vitro. (A) IIA1.6/mPD-1 cells were first incubated with unlabeled mPD-L1 monomer or tetramer (none, red line; 10 \(\mu\)g/ml–1, orange line; 30 \(\mu\)g/ml–1, green line and 100 \(\mu\)g/ml–1, blue line), washed and then with mPD-L1/Fc chimera (10 \(\mu\)g/ml–1) followed by Alexa647-conjugated anti-Fc antibody and analyzed with FACS (left and middle panels). Or reciprocally, IIA1.6/mPD-1 cells were incubated with unlabeled mPD-L1/Fc chimera (none, red line; 10 \(\mu\)g/ml–1, orange line; 30 \(\mu\)g/ml–1, green line and 100 \(\mu\)g/ml–1, blue line), washed and then with 10 \(\mu\)g/ml–1 R-PE–mPD-L1 tetramer (right panel). Dotted lines indicate negative control staining. (B) Spleen T cells from 2C TCR Tg mice were cultured with MMC-treated BALB/c splenocytes for 72 h. The cultured cells were three-color stained with anti-2C TCR (1B2), anti-CD8 and anti-PD-1 mAbs and the expression of PD-1 in 2C TCR\(^{+}\)CD8\(^{+}\) population was analyzed. Dotted line indicates negative control staining. (C) Splenic T cells from PD-1\(^{+/+}\) or PD-1\(^{-/-}\) 2C TCR Tg littermate mice (1 \(\times\) 10\(^{6}\)) were cultured with BALB/c splenocytes (5 \(\times\) 10\(^{5}\)) in the absence or presence of mPD-L1 tetramer (3, 10 and 30 \(\mu\)g/ml–1), anti-PD-1 mAb (J43, 10 \(\mu\)g/ml–1) or anti-PD-L1 mAb (1-111A, 10 \(\mu\)g/ml–1) for 3 days and the [\(^{3}H\)]-thymidine incorporation during the last 12 h was determined. The means and standard error of triplicate cultures are shown. The same experiments were repeated five times with similar results. **P < 0.01 and ***P < 0.005 by Student’s t-test. (D) The spleen cells from 2C TCR Tg mice were stimulated with MMC-treated BALB/c splenocytes for 2 weeks in the culture. The cells were harvested and incubated with \(^{51}\)Cr-labeled P815/mPD-L1 cells at varying effector/target ratios in the absence (open circle) or presence of 5 \(\mu\)g/ml–1 mPD-L1 tetramer (solid square), 1-111A mAb (solid triangle) or J43 mAb (solid circle) for 4 h. The means of specific \(^{51}\)Cr release and standard error in triplicate cultures are indicated.
References


