Glycosylation-independent binding of monoclonal antibody toripalimab to FG loop of PD-1 for tumor immune checkpoint therapy

Hongchuan Liu#, Lijing Guo,b,c#, Jing Zhang#, Yuehua Zhou, Jinwei Zhou, Jian Yao*, Hai Wu, Sheng Yao*, Bo Chen*, Yan Chai, Jianxun Qi*, George F. Gao*, Shuguang Tan, Hui Feng*, and Jinghua Yanb,c,d*

#Department of Antibody Discovery and Engineering, Shanghai Junshi Biosciences Co., Ltd, Shanghai, China; *Institute of Physical Science and Information Technology, Anhui University, Hefei, China; bCAS Key Laboratory of Pathogenic Microbiology and Immunology, Institute of Microbiology, Chinese Academy of Sciences, Beijing China; cCAS Key Laboratory of Microbial Physiological and Metabolic Engineering, Institute of Microbiology, Chinese Academy of Sciences, Beijing, China

ABSTRACT
Monoclonal antibody (mAb)-based blockade of programmed cell death 1 (PD-1) or its ligand to enable antitumor T-cell immunity has been successful in treating multiple tumors. However, the structural basis of the binding mechanisms of the mAbs and PD-1 and the effects of glycosylation of PD-1 on mAb interaction are not well understood. Here, we report the complex structure of PD-1 with toripalimab, a mAb that is approved by China National Medical Products Administration as a second-line treatment for melanoma and is under multiple Phase 1-Phase 3 clinical trials in both China and the US. Our analysis reveals that toripalimab mainly binds to the FG loop of PD-1 with an unconventionally long complementarity-determining region 3 loop of the heavy chain, which is distinct from the known binding epitopes of anti-PD-1 mAbs with structural evidences. The glycan modifications of PD-1 could be observed in three potential N-linked glycosylation sites, while no substantial influences were detected to the binding of toripalimab. These findings benefit our understanding of the binding mechanisms of toripalimab to PD-1 and shed light for future development of biologics targeting PD-1. Atomic coordinates have been deposited in the Protein Data Bank under accession code 6JBT.

Introduction
Monoclonal antibody (mAb)-based immune checkpoint therapy (ICT), which involves blocking immune checkpoint receptor-ligand interactions to restimulate antitumor T-cell immunity for tumor immunotherapy, has gain particular interest since the approval of cytotoxic T-lymphocyte-associated protein 4 (CTLA-4)-targeting ipilimumab in 2011. As a critical inhibitory molecule in modulation of T-cell reactivity, programmed cell death 1 (PD-1) plays pivotal roles in immune suppression within the tumor microenvironment. The blockade of the interaction between PD-1 and its ligand, PD-L1, to interrupt the inhibitory signaling in T cells could release the preexisting antitumor T-cell activity to kill tumor cells. To date, seven immune checkpoint-blocking mAbs have been approved by US Food and Drug Administration (FDA), i.e. CTLA-4-targeting ipilimumab (2011, Bristol Myers-Squibb); PD-1-targeting nivolumab (2014, Bristol Myers-Squibb), pembrolizumab (2014, Merck & Co., Inc.) and cemiplimab (2018, Sanofi and Regeneron); and PD-L1-targeting atezolizumab (2017, Genentech), durvalumab (2017, AstraZeneca) and avelumab (2017, Merck KGaA and Pfizer). Clinical studies reveal that these mAbs not only induce durable tumor suppression in multiple tumors as monotherapy, but also play important roles in improving antitumor efficacy in combination with chemotherapy, targeted therapy, and other immunotherapies, e.g. chimeric antigenic receptor engineered T cells (CAR-T) or oncolytic virus.

The structural basis of the binding of PD-1/PD-L1-blocking mAbs to their targets has been reported, which facilitates our understanding of the blocking mechanisms and differential binding characteristics of these mAbs. The binding of nivolumab is mainly located on an unexpected N-terminal loop of PD-1, which is out of the immunoglobulin (Ig)-like domain that contributes binding to PD-L1. On the other side, pembrolizumab binds to the flexible CD loop of PD-1, which is also not involved in the interaction with PD-L1. Although these two PD-1-targeting mAbs bind to different regions on PD-1 to block the PD-1/PD-L1 interaction, they compete with each other with stereospecific hindrance when binding to PD-1. PD-L1-targeting mAbs bind to PD-L1 with distinct orientations, and mainly target the loops of PD-L1 to interrupt PD-1/PD-L1 interaction. Nanobodies targeting PD-L1 that block the binding of PD-1/PD-L1 have also been reported. However, whether these mAbs are representative of all the binding modes of PD-1/PD-L1 blocking mAbs is unknown. Nevertheless, there are more mAbs targeting PD-1/PD-L1 in clinical trials and investigations of the binding mechanisms of these mAbs would provide better understanding of mAb-based PD-1/PD-L1 blockade for tumor ICT.

Glycosylation modifications of PD-1 or PD-L1 and the effects of these glycosylations to the interaction with therapeutic mAbs,
which may be correlated with the clinical responsiveness of these mAbs, are concerns in this field.\textsuperscript{15,21} Both PD-1 and PD-L1 have glycosylation modifications; in particular, PD-1 is highly glycosylated.\textsuperscript{15} Li et al. recently reported that glycan modifications of PD-L1 influence the interaction with PD-1, and subsequent immunosuppression in triple-negative breast cancer.\textsuperscript{22} In addition to the expression in T cells, PD-1 is also reported to exist in tumor cells, which commonly have dysregulated glycosylation modifications.\textsuperscript{23} It is reported that the binding of the two FDA-approved mAbs, pembrolizumab and nivolumab, with PD-1 is independent of PD-1 glycosylation.\textsuperscript{24,25} However, the glycan modifications of PD-1 and the effects of binding to other PD-1 targeting mAbs are less understood.

Toripalimab, a humanized IgG4 mAb targeting PD-1, was approved by the China National Medical Products Administration in 2018 as a second-line treatment for melanoma, and it is also undergoing multiple Phase 1-Phase 3 clinical trials in numerous indications, including nasopharyngeal carcinoma, and nonsmall cell lung cancer. Here, we determined the complex structure of toripalimab and PD-1 to elucidate the structural basis of the interaction between toripalimab and PD-1. The dominant binding to the FG loop of PD-1 with a long complementarity-determining region 3 (CDR3) loop of heavy chain (H chain) is substantially different compared to that of nivolumab and pembrolizumab. Glycosylation modifications of PD-1 could be observed in three potential N-linked glycosylation sites, and the binding to toripalimab was investigated. These findings improve our understanding of the binding mechanisms of toripalimab, and broaden our knowledge about mAb-based tumor ICT.

Results

Tumor suppression of the PD-1 targeting toripalimab

Toripalimab could block the interaction between PD-1 and its ligands. The ability of toripalimab to compete with PD-L1 or PD-L2 for PD-1 binding was assessed in enzyme-linked immunosorbent assay (ELISA) and flow cytometry-based competition experiments (Figure 1(a,b)). In ELISA-based assays, recombinant human PD-1 (hPD-1) extracellular domain (ECD) protein was immobilized to microtiter plates. The results showed that toripalimab could efficiently block the binding of ligands PD-L1 and PD-L2 to PD-1 (Figure 1(a)). Toripalimab was also confirmed to block interactions between PD-1 and PD-L1 and PD-L2 in flow cytometry-based competition assays, which involved 293T cells stably transfected with human PD-1 (Figure 1(b)). Half-maximal inhibitory concentrations (IC\textsubscript{50}) of toripalimab in ELISA-based PD-1/PD-L1 inhibition assays were determined to be 0.8 nM for PD-L1 and 1.3 nM for PD-L2, while the IC\textsubscript{50} of toripalimab was determined to be 1.3 nM for PD-L1 and 3.7 nM for PD-L2 in flow cytometry-based assays. The results indicated that toripalimab could block the interaction of PD-1 with its ligands, PD-L1 and PD-L2, and showed a higher blocking efficacy to PD-1/PD-L1 than PD-1/PD-L2.

The in vivo tumor suppression efficacy of toripalimab was examined in hPD-1 knock-in mice of C57BL/6 background (C57/hPD-1) by inoculation of the syngeneic tumor cell line MC38. The C57/hPD-1 mice were subcutaneously inoculated with $1 \times 10^6$ MC38 cells and the size of the tumor was monitored after injection of the toripalimab or negative control IgG4 (antikeyhole limpet hemocyanin (KLH) IgG4) (Figure 1(c)). The results showed that inhibition of tumor growth was observed in a dose-dependent manner with substantial antitumor efficacy in 1, 3, and 10 mg/kg treatment groups with toripalimab (Figure 1(d)). Compared with the negative control IgG4-treated group, the tumor sizes in the toripalimab-treated groups decreased significantly at the end of the observation period (day 23), with $P$ values being less than 0.05 in the 1 and 3 mg/kg groups, and $P < 0.01$ in the 10 mg/kg group. The low dose group (0.3 mg/kg) showed no significant change in tumor size compared to control Ig (P > 0.05). The EC\textsubscript{50} dose for toripalimab in this MC38 tumor model likely falls between 0.3 and 1 mg/kg. Therefore, the PD-1 targeting toripalimab exhibits substantial tumor suppressive efficacy in a dose-dependent manner.

FG loop of PD-1 dominates the binding to toripalimab

To elucidate the binding characteristics of toripalimab to PD-1 and the blocking mechanisms of toripalimab to PD-1/PD-L1 interaction, the complex structure of toripalimab and PD-1 was determined at a resolution of 2.6 Å after screening of crystals of toripalimab-antigen-binding fragment (Fab)/PD-1 complex proteins (Table S1 and Figure 2(a)). The toripalimab binds to PD-1 with a total buried surface of 2011 Å\textsuperscript{2}, while H chain and light (L) chain contributes comparable buried surfaces to PD-1, with a buried surface of 961 Å\textsuperscript{2} and 1, 049 Å\textsuperscript{2}, respectively. Overall, all three CDRs of the heavy chain (HCDRs) of toripalimab are involved in the interaction with PD-1, while CDR1 and CDR3 of its light chain (LCDR1 and LCDR3) are engaged in recognition to PD-1 (Figure 2(b)). The binding of toripalimab to PD-1 is mainly located on the FG loop of PD-1, which is mainly contributed by HCDR3 and LCDR1 of toripalimab, with multiple hydrogen bond interactions. Toripalimab possesses a long HCDR3 loop with 18 amino acids, which forms multiple contacts with the FG loop of PD-1. Specifically, the amino acids of HCDR3 (E99, T102, Y108, W110, and Y111) contributed major hydrogen bond interactions with amino acids from FG loop of PD-1 (P130, K131, A132, and I134) (Figure 2(b)). The H31 of LCDR1 of toripalimab also forms hydrogen bond interactions with P130 of the FG loop. Additionally, amino acids from HCDR1, HCDR2, and LCDR1 contact with FG loop of PD-1 with multiple van der Waals’ forces (Table 1). Taken together, the binding of toripalimab to PD-1 is mainly contributed by the long HCDR3 loop of toripalimab, while FG loop of PD-1 contributed most of the interactions with toripalimab.

Blocking mechanisms of toripalimab to PD-1/PD-L1 interaction

To explore the blocking mechanisms of toripalimab to the interaction of PD-1/PD-L1, the structure of toripalimab/PD-1 complex was superimposed with PD-1/PD-L1 complex (PDB code: 4ZQK). Overall, the binding of toripalimab to PD-1 exhibited stereospecific hindrance to that of PD-L1. Specifically, the H chain of toripalimab provides major conflicts with PD-L1, while L chain is away from the binding interface of PD-1/PD-L1 (Figure 3(a)). Further analysis of the binding
surface of toripalimab on PD-1, in comparison with that of PD-L1, revealed that the overlapped binding surface mainly locates on FG loop (P130, K131, A132, and I134), which is also the major target for toripalimab binding (Figure 3(a)). Taken together, these findings suggest that the binding of toripalimab to PD-1 would abrogate the binding of PD-L1, which is mainly induced by H chain-derived stereo clash.

Glycosylation-independent binding of toripalimab to PD-1

Previous studies revealed that PD-1 is highly glycosylated. Therefore, the influences of PD-1 glycosylation to the binding of toripalimab were further evaluated. PD-1 protein has four potential N-linked glycosylation sites, N49, N58, N74, and N116. The structural analysis of PD-1 showed that glycosylation modifications could be observed in three of these N-glycosylation sites, N49, N58, and N116 (Figure 4). Similar glycosylation modifications were found on N49 and N58, which consist of two N-acetylglucosamines (NAG) and one fucose (FUC), while only one NAG was visible on N116. Of note, N58 is located near the interface of toripalimab and PD-1. Glycosylation modifications might also affect the overall structure of PD-1, and further affect the binding to toripalimab. Therefore, the binding affinity of toripalimab to PD-1 was evaluated with PD-1 proteins obtained from 293T expressing system, which enables full glycosylation.

Figure 1. Antitumor efficacy of toripalimab in a MC38 bearing mouse model. (a,b) Blocking of the binding of PD-1 to PD-L1 or PD-L2 using protein-based ELISA assay (a) or cell-based flow cytometry assay (b). (c) Flow chart of the animal study. MC38 was inoculated into human PD-1 knock-in mice of the C57BL/6 background. Toripalimab was administered via intraperitoneal (i.p.) injection every 3 or 4 d from day 7 (D7) after MC38 tumor inoculation. The size of the tumor was monitored every 3 or 4 d after injection of toripalimab or control mAb. Saline and control mAb were enrolled as negative control. (d) Mice bearing subcutaneous MC38 palpable tumors for 7 d were treated i.p. with four doses of toripalimab, 0.3, 1, 3, and 10 mg/kg, or saline or control IgG. The data with each dot show the average tumor volume of the group while the SE was presented as longitudinal bars. (e–j) Individual follow-up of tumor sizes is presented for each experimental group with each line showing the changes of the tumor size of each mouse.
E. coli

The complex structure of toripalimab and PD-1. (a) Gel filtration elution profiles of PD-1 (red), toripalimab-Fab (black), and the PD-1/toripalimab-Fab complex (green) were analyzed by size-exclusion chromatography as indicated. The SDS-PAGE analyses are shown in reducing (+DTT) or nonreducing (-DTT) conditions, one for PD-1, two for toripalimab-Fab, and three for toripalimab-Fab/PD-1 complex. (b) The complex structure of toripalimab and PD-1. The V fragment of toripalimab is shown as cartoon (heavy chain (VH), wheat; light chain (VL), lemon), and PD-1 is shown as surface representation (light blue). The CDR1, CDR2, and CDR3 loops of the heavy chain (HCDR1, HCDR2, and HCDR3) are colored in light pink, marine, and magenta, respectively. The FG loop is invisible in the PD-1/pembrolizumab complex with PD-L1, nivolumab or pembrolizumab was superimposed with that from the toripalimab/PD-1 complex (Figure 5(a)). We found that these mAbs bind mainly on the loops of PD-1. The pembrolizumab mainly binds to the C'D loop, while the binding of nivolumab mainly involves the N-terminal loop. In the meanwhile, the binding of toripalimab to PD-1 mainly locates on the FG loop, as also described above (Figures 2(b) and 5(a)). The C'D loop and N terminal loop of PD-1 could only be visible upon binding with the mAbs, indicating the flexibility of these loops. Of note, the FG loop exhibits varied conformations upon binding to different partners (Figure 5(b)). The FG loop is invisible in the PD-1/pembrolizumab complex, which indicates the flexibility of this loop. A substantial shift of 8.19 Å was observed with the FG loop when PD-1 binds to nivolumab or toripalimab. The binding of nivolumab is mainly contributed by the N-terminal loop, though multiple contacts were also observed with the FG loop. This finding suggests that the FG loop of PD-1 adopts different conformations upon binding to different mAbs.

Structures of the nivolumab/PD-1 and pembrolizumab/PD-1 complexes were superimposed with toripalimab/PD-1 complex, with the structure of PD-1 fixed to investigate the binding mode of different mAbs. Comparative analysis revealed that the binding orientation of toripalimab to PD-1 is similar to that of nivolumab, while pembrolizumab adopts a distinct binding orientation (Figure 5(c)). Though similar binding orientation is

modification of PD-1, or refolded from inclusion bodies expressed in E. coli, which is deficient in any glycosylation modifications.

The binding profiles of toripalimab with PD-1 proteins obtained from 293T or E. coli expression system were analyzed using a surface plasmon resonance (SPR) assay with toripalimab immobilized on the chip. The results revealed that the binding affinity ($K_D$) of toripalimab with PD-1 proteins from E. coli ($K_D = 0.324$ nM) showed no substantial difference with that from 293T cells ($K_D = 0.238$ nM) (Figure 4(b)). This finding suggests that the binding of toripalimab to PD-1 is independent of any glycosylation modifications.

Comparative binding characteristics of PD-1 targeting mAbs

The complex structures of two US-FDA approved anti-PD-1 mAbs, nivolumab and pembrolizumab, have been reported, which enabled us to investigate the binding characteristics of these mAbs, which have promising efficacy as tumor immunotherapy.\textsuperscript{15,16}

The PD-1 extracted from the complex with PD-L1, nivolumab or pembrolizumab was superimposed with that from the toripalimab/PD-1 complex (Figure 5(a)).

Table 1. Residues contributed interaction between toripalimab and PD-1.

| Residues | Contacts\textsuperscript{a} | Total |
|----------|------------------|-------|
| PD-1     |                  |       |
| H chain  |                  |       |
| E33      | S127, L128, A152 | 1,15,6|
| W47      | A129             | 2     |
| V50      | L128, A129       | 3,1   |
| F52      | L126, L128       | 3,2   |
| T55      | V64, N66, L126  | 2,3,3 |
| G57      | V64, L128       | 1,6   |
| T58      | L128             | 4     |
| A59      | S62, L128, A129 | 4,4,1 |
| E99      | K131, A132       | 9 (1) |
| G100     | A132             | 2     |
| I101     | A132, Q133, I134 | 3,4,5 |
| T102     | A132, Q133, I134 | 5 (1) |
| T103     | I134             | 3     |
| Y108     | K131             | 5 (1) |
| Y109     | K131             | 3     |
| W110     | K131             | 7 (1) |
| Y111     | P130, K131       | 3, 15 (1) |
| L chain  |                  | 91    |
| H31      | W32, P130, K131 | 3, 10 (1) |
| S32      | W32, T59         | 9, 2  |
| N33      | W32              | 18    |
| Y37      | K131             | 18    |
| G96      | P130, K131       | 4, 1  |
| S97      | P130             | 5     |
| H98      | P130             | 7     |
| Y99      | A129, P130       | 4, 7  |
| L101     | P130             | 1     |

\textsuperscript{a}Numbers represent the number of atom-to-atom contacts between toripalimab and PD-1 residues, which were analyzed by the Contact program in CCP4 suite (the distance cutoff is 4.5 Å).

\textsuperscript{b}Numbers in the parentheses represent the number of hydrogen bonds between toripalimab and PD-1 residues which were analyzed by the Contact program in CCP4 suite (the distance cutoff is 3.5 Å).
observed between toripalimab and nivolumab, the major binding regions on PD-1 are substantially different between these two mAbs, as also described above. These findings suggest that therapeutic mAbs targeting PD-1 adopt distinct binding modes to interrupt the interaction of PD-1 and PD-L1.

Discussion

In this study, we report the antitumor efficacy of toripalimab in a mouse model and the structural basis of the interaction between toripalimab and PD-1. Toripalimab has been reported to be...
Figure 5. Comparative binding of PD-1 targeting mAbs. (a) Superimposition of the PD-1 from complex structures of PD-1/PD-L1 (cyan) (PDB code: 4ZQK), PD-1/pembrolizumab (light pink) (PDB code: 5JXE), PD-1/nivolumab (lemon) (PDB code: 5WT9), and PD-1/toripalimab (light blue) (PDB code: 6JBT). The loops contributed major binding to the mAbs were highlighted in dashed circles. (b) Comparison of the FG loop of the PD-1s from the complex structures. The FG loop of PD-1 shifted 8.19 Å upon the binding to nivolumab or toripalimab. (c) Superimposition of the complex structures of PD-1/pembrolizumab, PD-1/nivolumab and PD-1/toripalimab with the mAbs shown as ribbon in red, blue, and lemon, respectively. The PD-1 from PD-1/toripalimab complex is shown as surface in light blue with the FG loop highlighted in cyan.
T cells. All these findings broaden our understanding of the binding mechanisms of toripalimab, and may facilitate its clinical applications.

PD-1 binds to PD-L1 or PD-L2 with the front β-sheet faces consisting of CC’FG strands. In our study, we found that the binding of toripalimab to PD-1 is mainly located on the FG loop of PD-1, while amino acids from FG loop of PD-1 (L128, P130, K131, and A132) also contributed multiple interactions with PD-L1. The toripalimab contains an unconventional, long HCDR3 loop consisting of 18 amino acids, which provided major hydrogen bond interactions with the FG loop of PD-1. It could be deduced that this HCDR3 loop would be responsible for the high binding affinity of toripalimab to PD-1, as observed in the SPR assay. Of note, the FG loop of PD-1 is highly flexible, such that it adopts different conformations to bind to different counterparts, e.g. nivolumab and toripalimab. The binding of toripalimab to PD-1 has induced substantial stereo clash with the binding of PD-L1, which is mainly mediated through the H chain of toripalimab. Therefore, toripalimab’s blockade of PD-1/PD-L1 interactions is due to both the overlapping binding regions of toripalimab and PD-L1 on PD-1, and stereo hindrance of the H chain of toripalimab to PD-L1. Meanwhile, nivolumab and pembrolizumab mainly bind to the N-terminal loop and the CD loop of PD-1, indicating that the flexible loops of PD-1 are more prone to be targeted by therapeutic mAbs targeting PD-1.

Disordered glycosylation modifications of the proteins in tumor cells have played critical roles in the development and progression of multiple tumors. PD-1 protein is highly glycosylated, and all of the four potential N-linked glycosylation sites are suggested to be glycosylated. Intrinsic expression of PD-1 in tumor cells has been reported to play critical roles in promotion of tumor growth, and mAbs targeting tumor cell-intrinsic PD-1 would induce substantially decreased progression of the tumors. Therefore, PD-1-specific mAbs not only restimulate the exhausted T cells to evoke antitumor immunity, but also suppress tumor growth by directly targeting tumor cell-intrinsic PD-1. The disordered glycan transferase expression in tumor cells raises the antitumor mechanisms, especially when targeting PD-1 in tumor cells, which are usually accompanied with deficiency in glycosylation modifications. All these findings broaden our understanding of the binding mechanisms of toripalimab, and may facilitate its clinical applications.

Materials and methods

Plasmid construction and protein purification

The DNA encoding the ectodomain of human PD-1 (residues M1-Q167, including signal peptide) with six histidines at the N terminus of the sequence was cloned into pCAGGS vector (Addgene) with EcoRI and BglII restriction sites as previously described. Plasmid pCAGGS-PD-1 was transiently transfected into 293T cells for protein expression; the cells were cultured with 5% CO2 at 37°C. The supernatant was collected after 72 h, and the protein was purified by sequentially His-Trap HP column (GE Healthcare) and Superdex™ 200 10/300 GL (GE Healthcare) in a buffer containing 20 mM Tris and 150 mM NaCl (pH 8.0).

The DNA encoding the ectodomain of human PD-1 (residues L25-R147) was cloned into the pET-21a vector (Novagen) with NdeI and XhoI restriction sites and transformed into Escherichia coli strain BL21 (DE3) for protein expression. The inclusion bodies of recombinant proteins were purified and then refolded as previously described. Soluble proteins were further purified by a Superdex™ 200 10/300 GL (GE Healthcare) with a running buffer of 20 mM Tris (pH 8.0) and 150 mM NaCl.

Full-length toripalimab proteins were obtained from Shanghai Junshi Biosciences. The purified mAbs were digested with the Human IgG Fab and F(ab)2 Preparation Kits (Thermo Scientific) according to the manufacturer’s instructions. The protein fragments were purified by HiTrap Protein A FF (GE Healthcare), and then exchanged to the buffer of 20 mM Tris (pH 8.0), 150 mM NaCl.

Blocking of PD-1 and its ligands with ELISA and flow cytometry

The ability of toripalimab to compete with PD-L1 or PD-L2 for PD-1 binding was assessed in ELISA and flow cytometry-based
competition experiments. In ELISA-based assays, recombinant human PD-1 ECD fusion protein was immobilized to microtiter plates, washed, and blocked using 1% bovine serum albumin (BSA). Biotin-labeled PD-L1 and PD-L2 Fc fusion (R&D Systems) were added to the plate, together with a titration of toripalimab antibody. The bound PD-L1 and PD-L2 Fc fusion proteins were detected by incubation with horseradish peroxidase-labeled streptavidin secondary antibody (Jackson ImmunoResearch, catalog no. 016-030-084). Half-maximal inhibitory concentrations (IC_{50}) of toripalimab were determined using a log(inhibitor) vs. response—variable slope curve fit (GraphPad Prism).

In flow cytometry-based competition assay, biotin-labeled PD-L1 and PD-L2 Fc fusion were incubated with 293T cells stably transfected with human PD-1 (293T-hPD1). Cell surface-bound PD-L1 and PD-L2 Fc fusion protein was subsequently detected by incubation with PE-labeled Streptavidin secondary antibody (eBioscience, catalog no. 12-4317-87). Half-maximal inhibitory concentrations (IC_{50}) of toripalimab were determined using a log (inhibitor) vs. response—variable slope curve fit (GraphPad Prism).

In vivo antitumor activity in a syngeneic tumor model

A genetically modified mouse strain with human PD-1 knock-down was employed in our study. For the MC38 syngeneic model, mice were subcutaneously inoculated with 1 × 10^6 MC38 cells in 100 µL phosphate-buffered saline on day 0. On day 6, the inoculated mice were randomized into six groups (tumor volume averages 20–70 mm^3) and treated with either control Ig (anti-KLH, Junshi Biosciences, lot no. 20160929), saline, or toripalimab at 0.3, 1, 3, and 10 mg/kg via intraperitoneal injection twice a week. The tumor growth was monitored twice a week and the volume of the tumors was calculated by the formula: ½ length × width^2.

Complex preparation and crystallization

The mammalian cell-expressed PD-1 protein and toripalimab-Fab were mixed at a molar ratio of 1:1. The mixture was incubated on ice for 30 min and further purified by Superdex™ 200 10/300 GL (GE Healthcare). A total of 10 mg/mL of toripalimab/PD-1 proteins were used for crystal screening by vapor-diffusion sitting-drop method at 4°C. Diffractable crystals were obtained in a condition consisting of 0.09 M halogens consisting of NaF, NaBr, and NaI additives, 0.1 M Tris·Base (pH 8.5), 37.5% (v/v) MPD-P1K-P3350 consisting of MPD (racemic), PEG 1K and PEG 3350 (Morphus* MD1-46 kit, Molecular Dimensions). Subsequent model building and refinement were performed using coot and phenix to refine the results, respectively. The stereocchemical qualities of the final model were assessed with MolProbity. Data collection and refinement statistics are summarized in Table S1. All structural figures were generated using Pymol (http://www.pymol.org).

SPR analysis

The SPR analysis was performed at room temperature using a BLAcore T100 system with CM5 chips (GE Healthcare). For all the analyses, an HBS-EP buffer consisting of 10 mM HEPES (pH 7.4), 150 mM NaCl and 0.005% (v/v) Tween-20 was used as running buffer, and all proteins were exchanged to the same buffer in advance via gel filtration. The blank channel of the chip was used as the negative control. To detect the toripalimab binding to different forms of PD-1 proteins, toripalimab protein was immobilized on the chip by antihuman IgG at about 70 response units. Gradient concentrations of PD-1 (0.975, 1.95, 3.9, 7.81, and 15.625 mM) were then flowed over the chip surface. After each cycle, the sensor surface was regenerated with 3M MgCl2. The binding kinetics were all analyzed with the software of BIA evaluation” Version using a 1:1 Langmuir binding model.

Data deposition

Atomic coordinates have been deposited in the Protein Data Bank (PDB, http://www.rcsb.org/pdb) under accession code 6JBT.

Abbreviations

CAR-T chimeric antigenic receptor engineered T cells
CDR complementarity-determining region
CTLA-4 cytotoxic T-lymphocyte-associated protein 4
ELISA enzyme-linked immunosorbent assay
ECD extracellular domain
Fab antigen-binding fragment
Fv variable fragment domain
H chain heavy chain
ICT immune checkpoint therapy
IC_{50} Half-maximal inhibitory concentrations
Ig immunoglobulin
KLH keyhole limpet hemocyanin
mAb monoclonal antibody
NAG N-acetylglucosamines
NAG N-acetylglucosamines
NAG N-acetylgalactosamines
FUC fucose
PD-1 programmed cell death 1
SPR surface plasmon resonance.

Data collection and structure determination

To collect the diffraction data, all crystals were flash-cooled in liquid nitrogen after incubating in reservoir solution containing 20% (v/v) glycerol. The diffraction data were collected at Shanghai Synchrotron Radiation Facility (SSRF) BL17U, and all data were processed with HKL2000. The complex structure was solved by molecular replacement method using phase with the reported PD-1 structure (PDB: 5WT9) and Fab structure (PDB: 5WT9) as the search models. The stereochemical qualities of the final model were assessed with MolProbity. Data collection and refinement statistics are summarized in Table S1. All structural figures were generated using Pymol (http://www.pymol.org).

Acknowledgments

This study was supported by National Major Science & Technology Major Project (2015ZX09102017, 2017ZX09302009). Strategic Priority Research Program of Chinese Academy of Sciences (CAS) (XDA12020358, XDB29040201). G.F.G. and J.Y. are supported by the NSFC Innovative Research Group (grant no. 81621091). We thank the staff of BL17U and BL19U beamline at the Shanghai Synchrotron Radiation Facility for assistance with data collection. We also thank Yuanjuan Chen, Bingxue Zhou, and Zhenwei Yang from the Institute of Biophysics, CAS, for their technical support in the SPR assay.
Research Program of Chinese Academy of Sciences (CAS) [XDA12020358, XDB29040201].

This study was supported by National Science & Technology Major Project [2015ZX09102017, 2017ZX09302009], Strategic Priority Research Program of Chinese Academy of Sciences (CAS) [XDA12020358, XDB29040201].

The authors declare that they have no conflict of interests.

Conflict of interests

Involvement of PD-L1 on tumor cells by targeting glycosylated PD-L1. Cancer Cell. 2015;28:84–97. doi: 10.1016/j.ccr.2015.06.006.

Distinct PD-L1 binding characteristics of therapeutic monoclonal antibody durvalumab. Protein Cell. 2018;9:135–39. doi: 10.1007/s13238-017-0412-8.

Tan S, Zhang CW and Gao GF. Seeing is believing: anti-PD-1/PD-L1 monoclonal antibodies in action for checkpoint blockade tumor immunotherapy. Signal Transduct Target Ther. 2016;1:16029. doi: 10.1038/sigs.2016.29.

Zhang F, Wei H, Wang X, Bai Y, Wang P, Wu J, Jiang X, Wang Y, Cai H, Xu T, et al. Structural basis of a novel PD-L1 nanobody for immune checkpoint blockade. Cell Discov. 2017;3:17004. doi: 10.1038/celldisc.2017.4.

Tan S, Chen D, Liu K, He M, Song H, Shi Y, Liu J, Zhang CW, Qi J, Yan J, et al. Crystal clear: visualizing the intervention mechanism of the PD-1/PD-L1 interaction by two cancer therapeu-tic monoclonal antibodies. Protein Cell. 2016;7:866–77. doi: 10.1038/s13238-016-0337-7.

Pinho SS, Reis CA. Glycosylation in cancer: mechanisms and clinical implications. Nat Rev Cancer. 2015;15:540–50. doi: 10.1038/nrc3989.

Li CW, Lim SO, Chung EM, Kim YS, Park AH, Yoo J, Cha JH, Xia W, Chan LC, Kim T, et al. Eradication of triple-negative breast cancer cells by targeting glycosylated PD-L1. Cancer Cell. 2018;33:187–201. doi: 10.1016/j.ccell.2018.01.009.

Klefel S, Posch C, Barthel SR, Mueller H, Schlapbach C, Guenova E, Elco CP, Lee N, Janeva VR, Zhan Q, et al. Melanoma cell-intrinsic pd-1 receptor functions promote tumor growth. Cell. 2015;162:1242–56. doi: 10.1016/j.cell.2015.08.052.

Fu J, Wang F, Dong LH, Zhang J, Deng CL, Wang XL, Xie XY, Zhang J, Deng RX, Zhang LB, et al. Preclinical evaluation of the efficacy, pharmacokinetics and immunogenicity of Toripalimab, a programmed cell death protein-1 (PD-1) monoclonal antibody. Acta Pharmacol Sin. 2017;38:710–18. doi: 10.1038/aps.2016.161.

Lin DY, Tanaka Y, Iwasaki M, Gittis AG, Su HP, Mikami B, Okazaki T, Honjo T, Minato N and Garboczi DN. The PD-1/PD-L1 complex resembles the antigen-binding Fv domains of antibodies and T cell receptors. Proc Natl Acad Sci U S A. 2008;105:3011–16. doi: 10.1073/pnas.0712278105.

Lazar-Molnar E, Yan Q, Cao E, Ramagopalan U, Nathenson SG and Almo SC. Crystal structure of the complex between programmed death-1 (PD-1) and its ligand PD-L2. Proc Natl Acad Sci U S A. 2008;105:10483–88. doi: 10.1073/pnas.0804453105.

Xu C, Ng DT. Glycosylation-directed quality control of protein folding. Nat Rev Mol Cell Biol. 2015;16:742–52. doi: 10.1038/nrm4073.

Otwonoski Z, Minor W. Processing of X-ray diffraction data collected in oscillation mode. Methods Enzymol. 1997;276:307–26.

Read RJ. Pushing the boundaries of molecular replacement with maximum likelihood. Acta Crystallogr D Biol Crystallogr. 2001;57:1373–82.

Collaborative computational project N. The CCP4 suite: programs for protein crystallography. Acta Crystallogr D Biol Crystallogr. 1994;50:760–63. doi: 10.1107/S0907444994003112.

Emsley P, Cowtan K. Coot: model-building tools for molecular graphics. Acta Crystallogr D Biol Crystallogr. 2004;60:2126–32. doi: 10.1107/S0907444904019158.
32. Adams PD, Afonine PV, Bunkóczi G, Chen VB, Davis IW, Echols N, Headd JJ, Hung LW, Kapral GJ, Grosse-Kunstleve RW, et al. PHENIX: a comprehensive Python-based system for macromolecular structure solution. Acta Crystallogr D Biol Crystallogr. 2010;66:213–21. doi:10.1107/S0907444909052925.

33. Chen VB, Arendall WB 3rd, Headd JJ, Keedy DA, Immormino RM, Kapral GJ, Murray LW, Richardson JS, Richardson DC. MolProbity: all-atom structure validation for macromolecular crystallography. Acta Crystallogr D Biol Crystallogr. 2010;66:12–21. doi:10.1107/S0907444909042073.