The FG Loop of PD-1 Serves as a “Hotspot” for Therapeutic Monoclonal Antibodies in Tumor Immune Checkpoint Therapy

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HIGHLIGHTS
GY-5 and GY-14 show efficient anti-tumor efficacy in NCG mouse model
Both GY-5 and GY-14 bind to the FG loop of PD-1
Glycosylation is observed in PD-1, but not involved in binding to GY-5 and GY-14
The loops of PD-1 may serve as “hotspot” for development of PD-1-targeting biologics

DATA AND SOFTWARE
AVAILABILITY
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The FG Loop of PD-1 Serves as a “Hotspot” for Therapeutic Monoclonal Antibodies in Tumor Immune Checkpoint Therapy

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SUMMARY

Programmed cell death 1 (PD-1)/PD-1 ligand-1 (PD-L1)-blocking monoclonal antibodies (mAbs) have taken center stage for tumor immune checkpoint therapy. Identification of the “hotspots” on PD-1 for mAbs will help to develop next-generation oral deliverable agents with long-lasting efficacy. Here, we identified two PD-1-targeting mAbs, GY-5 and GY-14, with PD-1/PD-L1-blocking efficacy. Complex structural information revealed that both mAbs mainly bind to the FG loop of PD-1, which also contributes multiple interactions with PD-L1. The FG loop adopts substantially varied conformations upon binding to different mAbs, providing a novel targetable region for the development of PD-1-specific biologics and small chemical molecules. Glycosylation modifications of PD-1 could be observed in three of the four potential N-linked glycosylation sites. However, the binding of GY-5 and GY-14 to PD-1 was not affected by glycosylation. These findings broaden our understanding of the mechanism of anti-PD-1 mAbs and provide insight into the development of agents targeting PD-1.

INTRODUCTION

Immune checkpoint therapy (ICT) that targets co-inhibitory or co-stimulatory molecules to modulate antitumor T cell reactivity has achieved clinical success since the approval of the cytotoxic T-lymphocyte-associated protein 4 (CTLA-4)-targeting drug ipilimumab by the US Food and Drug Administration (FDA) in 2011 (Callahan et al., 2016; Tan and Gao, 2015). Programmed cell death 1 (PD-1), a member of the CD28-B7 superfamily, is an important co-inhibitory molecule in the modulation of T cell activity (Ishida et al., 1992). PD-1 ligand 1 (PD-L1) and then PD-1 ligand 2 (PD-L2) were subsequently identified as the ligands of PD-1 (Freeman et al., 2000;Latchman et al., 2001; Nishimura et al., 1999). Interruption of the PD-1/PD-L1 interaction with a monoclonal antibody (mAb) to re-stimulate tumor-specific T cell reactivity has been proved to be a promising strategy for treating multiple tumors in clinical applications (Motzer et al., 2015; Robert et al., 2015; Topalian et al., 2012).

The clinical success of anti-PD-1 or anti-PD-L1 mAbs in tumor therapy has initiated an era of anti-tumor drug development to modulate tumor-specific immune responses by targeting immune checkpoint molecules, either co-stimulatory (e.g., 4-1BB) or co-inhibitory (e.g., CTLA-4, PD-1) molecules, to treat tumors (He et al., 2017; Tan and Gao, 2015; Tan et al., 2016). Six mAbs targeting PD-1 or PD-L1 have been approved by the US FDA since 2014 (Tan et al., 2016). However, clinical responsiveness and benefits from these therapies are still limited owing to the disadvantage of the monotherapies in highly heterogeneous tumors. Therefore a combination of mAbs targeting varied immune checkpoints or other immune therapeutic strategies, e.g., oncolytic virus and chimeric antigen receptor-engineered T cells, are under evaluation at both the basic research and clinical levels to improve the clinical responsiveness and benefits of the tumor ICT. Looking for mAb replacement like small molecules for convenient drug delivery is another focal point for the field.

The interaction of PD-1 and PD-L1 plays pivotal roles in immune suppression within the tumor microenvironment (Tan and Gao, 2015; Tan et al., 2016). Upregulated PD-L1 expression on tumor cells is correlated with tumor progression and, hence, is a valuable indication for unfavorable prognosis (Iwai et al., 2002; Gridelli et al., 2017). On the other hand, higher PD-L1 levels in tumor tissue indicate better responsiveness.
to PD-1/PD-L1 blockade treatment, to a certain extent, in ovarian, kidney, pancreatic, and gastric cancers (Powles et al., 2017; Massard et al., 2016; Apolo et al., 2017; Balar et al., 2016). Inducible PD-1 expression on T lymphocytes (tumor-infiltrating lymphocytes in particular) can lead to the tolerance of tumor-specific T cells to tumors (Tumeh et al., 2014). Moreover, PD-1 has also been found to be expressed in tumor cells, and tumor-cell-intrinsic PD-1 can promote tumorogenesis by modulating downstream mammalian target of rapamycin signaling (Kleffel et al., 2015). Forced expression of PD-1 or PD-L1 on T cells or tumor cells
Figure 2. Blocking and Binding Characteristics of Humanized GY-5 and GY-14

(A) The blocking of the binding of PD-1-mFc to PD-L1s expressed on the surface of 293T cells by humanized GY-5 and GY-14 mAbs. The PD-L1-expressing HEK293T cells were stained with PBS and used as negative controls, whereas the staining with PD-1-mFc proteins was used as a positive control. The blocking efficacies of GY-5 and GY-14 were analyzed by

B

GY-5

chimeric GY-5

KD = 1.22 nM

humanized GY-5

KD = 9.62 nM

C

GY-14

chimeric GY-14

KD = 1.54 nM

humanized GY-14

KD = 2.21 nM

D

nivolumab

KD = 1.53 nM



The blocking of the binding of PD-1-mFc to PD-L1s expressed on the surface of 293T cells by humanized GY-5 and GY-14 mAbs. The PD-L1-expressing HEK293T cells were stained with PBS and used as negative controls, whereas the staining with PD-1-mFc proteins was used as a positive control. The blocking efficacies of GY-5 and GY-14 were analyzed by...
underlies the rationale that blockade of the PD-1 signaling would restore tumor-specific T cell function to eliminate tumor cells (Curiel et al., 2003; Hirano et al., 2005).

Recently, the complex structures of FDA-approved mAbs targeting PD-1 or PD-L1 were determined, providing critical information for our understanding of mAb-based PD-1/PD-L1 blockage for ICT (Tan et al., 2018; Liu et al., 2017; Tan et al., 2016, 2017; Lee et al., 2016; Na et al., 2017). The binding and blocking mechanisms of the two PD-1-targeting mAbs, nivolumab (Opdivo, Bristol-Myers Squibb) and pembrolizumab (Keytruda, Merck) are reported to be quite different (Tan et al., 2016). Nivolumab binds to the N-terminal loop of PD-1, which is outside the Ig-like domain, and competes for the binding of PD-L1 with its light chain (L chain) (Lee et al., 2016; Tan et al., 2017). On the other side, pembrolizumab binds to the C'D loop of PD-1, involving mainly the heavy chain (H chain) of the mAb, and competes with the PD-1/PD-L1 interaction with both its H and L chains (Na et al., 2017). Although the binding regions of nivolumab and pembrolizumab on PD-1 are different, the binding of nivolumab to PD-1 would abrogate the additional binding of pembrolizumab, indicating the competitive binding profiles of these two mAbs (Lee et al., 2016; Tan et al., 2017; Na et al., 2017). However, whether there are “hotspots” for mAb-based anti-PD-1 checkpoint blockage therapy or additional novel “hotspot” regions within PD-1 for therapeutic mAb development remains unknown.

Here, we report the screening of therapeutic mAbs targeting PD-1 and the structural basis of two of these mAbs for PD-1/PD-L1 blockage. We found that the FG loop of PD-1 was targeted by both mAbs, indicating that the PD-1 FG loop may serve as a novel “hotspot” for mAb-based PD-1 ICT. Moreover, the dependency of glycosylation modifications of PD-1 to the binding of these two mAbs was also investigated. Our findings will aid in the future development of biologics or small chemical molecules by targeting PD-1.

RESULTS

Tumor Suppression Efficacy and Humanization of PD-1-Targeting mAbs

To investigate the hotspots on PD-1 for mAbs, B6/C57 mice were vaccinated with human PD-1 protein expressed by HEK293T cells. Thirty-one cell hybrid clones that yield PD-1-specific mAbs were obtained after hybridization. Among these mAbs, 23 were found to interrupt the interaction between PD-1 and PD-L1 through a flow cytometry-based assay in which PD-L1 was expressed on 293T cells (Figure S1). Evaluation of the efficiency of enhancing T cell reactivity with enzyme-linked immunospot assays revealed that 11 of the mAbs elevated the T cell responses against influenza A virus M1 peptide pool (Figure S2). Sequences of eight mAb clones were obtained, and the representative GY-5 and GY-14 mAbs were selected for further investigations for their tumor suppressive efficacy and blocking mechanistic studies (Table S1).

Tumor-bearing mouse models were used to investigate anti-tumor activity of chimeric mAbs of GY-5 and GY-14 of human IgG4 subtype. The anti-tumor efficacies of GY-5 and GY-14 were evaluated in the human non-small-cell lung cancer cell line HCC-827-bearing NOD-SCID-IL2rg−/− (NCG) mouse model with pre-established human immunity by inoculation with 1 × 10⁶ peripheral blood mononuclear cells from healthy donors (Figure 1A). An Ebola virus GP-protein-specific mAb, 13C6 (Audet et al., 2014), was used as a negative control. The mAbs were injected intraperitoneally, twice a week for six doses, and tumor volumes were monitored twice a week. The results revealed that both GY-5 and GY-14 showed significant tumor suppression 2 weeks after their first dose compared with 13C6 (Student’s t test, p < 0.05 or <0.01) (Figure 1).

GY-5 and GY-14 were subsequently humanized via the “CDR grafting” method, and the PD-1/PD-L1 blocking efficiency and binding affinity of the humanized mAbs were evaluated (Figures S3 and S4). Humanized GY-5 and GY-14 could efficiently block the binding of PD-1 to PD-L1s expressed on 293T cells (Figure 2A).
The binding affinities (K_D) of both chimeric and humanized GY-5 or GY-14 for PD-1 were then analyzed using surface plasmon resonance (SPR) (Figures 2B–2D). We found that the binding affinity of humanized GY-5 and GY-14 (K_D = 9.62 and 2.21 nM, respectively) for PD-1 was similar to that of the chimeric GY-5 and GY-14 mAbs (K_D = 1.22 and 1.54 nM, respectively) (Table S2). Therefore the humanized GY-5 and GY-14 could serve as promising PD-1-targeting therapeutics for tumor ICT.

**Structural Basis of GY-5 and GY-14 Binding to PD-1 for PD-1/PD-L1 Blockage**

The complex structures of GY-5/PD-1 and GY-14/PD-1 were determined at a resolution of 2.6 and 1.4 Å, respectively, which enabled us to analyze the binding and blocking mechanisms of these two mAbs (Table S3). Overall, GY-5 and GY-14 bind to PD-1 with similar binding orientations, and both mAbs mainly bind to the FG loop of PD-1 (Figures 3A and 3B). The binding of GY-5 mainly involves the CDR2 and CDR3
of its H chain, and CDR1 and CDR3 of its L chain, with a buried surface of 1,730.1 Å² (Figure 3 A and Table S4). On the other side, GY-14 utilizes all six CDRs of both the H and L chains to contact PD-1, with a buried surface of 1,602.6 Å² (Figure 3 B and Table S5). The previously reported complex structures of PD-1/PD-L1, nivolumab/PD-1, and pembrolizumab/PD-1 enabled us to comprehensively compare the binding surface of these mAbs and the ligand (Figure 3 C). Nivolumab mainly binds to the N-terminal loop of PD-1, with partial contacts with the FG loop, whereas pembrolizumab mainly binds to the C'D loop. Both GY-5 and GY-14 mainly bind to the FG loop of PD-1, with the binding surfaces of GY-5 and GY-14 being more proximal to that of nivolumab.

Detailed analysis shows that the FG loop of PD-1 contributes major hydrogen bond interactions with GY-5 and GY-14 (Figure 4). Specifically, amino acids of the FG loop (P130, K131, Q133, and I134) formed multiple hydrogen bond interactions with HCDR3 (E97, D100, Y103, and Y104), LCDR1 (H31 and D33), and LCDR3 (Y101) of GY-5 (Figure 4 A). Similarly, amino acids of the FG loop (L128, P130, K131, A132, Q133, and I134) formed multiple hydrogen bond interactions with HCDR1 (E33), HCDR3 (E99, M101, N102, T103, W105, and Y106), LCDR2 (H31), and LCDR3 (Y101) of GY-14 (Figure 4 B).

We next analyzed the blocking mechanisms of GY-5 and GY-14 to the PD-1/PD-L1 interaction by superimposition of the structure of the previously reported PD-1/PD-L1 complex (PDB code: 4ZQK) with the GY-5/PD-1 complex or GY-14/PD-1 complex, individually (Figure 5). These analyses revealed that the binding of GY-5 and GY-14 induced stereospecific hindrance involving both their H chains to interrupt the binding of PD-L1 to PD-1 (Figures 5 A and 5B). The amino acids of the FG loop (L128-Q132) display competitive binding to PD-L1 by both GY-5 and GY-14 (Figures 5C and 5D). However, the competitive binding surfaces of GY-5 and GY-14 are substantially different from each other. These findings suggest that GY-5 and GY-14 bind to PD-1 with similar binding and blocking modes, which is distinct from that of nivolumab and pembrolizumab. In addition, the overwhelming binding affinity of GY-5 or GY-14 (K_D = 1.22 and 1.54 nM, respectively) for PD-1 over PD-L1 (K_D = 0.7–8.3 μM) also ensures the binding priority of the mAbs (Tan et al., 2017). Taken together, the blockade binding mechanism of GY-5 and GY-14 lies in both the overwhelming binding affinity and H-chain-induced stereo-hindrance to the binding of PD-L1 to PD-1.

Glycosylation-Independent Binding of GY-5 and GY-14
PD-1 has four potential N-linked glycosylation sites (N49, N58, N74, and N116) in its IgV domain. We previously reported the N-linked glycosylation modifications at N58, which consist of two N-acetylglucosamines (NAG) and one fucose, for which the protein was prepared from mammalian cells (Tan et al., 2017). In the present structure of PD-1 from the GY-5/PD-1 complex, which was expressed in insect cells, N-linked glycan modifications were visible in three of the four potential N-linked glycosylation sites: N49, N58, N116, and N74.
and N116 (Figure 6A). The glycosylation modification at N58 is similar to that observed previously, whereas only a NAG was visible at N49 and N116 (Figures 6 B–6D). Considering the flexibility of the glycan chains, N49 and N116 may have more complicated glycan modifications, which is also possible for N74.

The glycosylation may play a role in the folding and function of PD-1 and may further affect the binding of these mAbs (Pinho and Reis, 2015). Therefore we analyzed the binding of GY-5 and GY-14 to PD-1 proteins obtained from the insect cell expression system, which enables partial glycosylation of PD-1 compared with the HEK293T cell expression system, or PD-1 proteins refolded from inclusion bodies expressed in E. coli cells as previously described (Li et al., 2005; Tan et al., 2017), which have no glycosylation modifications at all. The binding characteristics of these two mAbs were further investigated with SPR analysis. Similar to the glycosylation-independent binding of nivolumab to PD-1, the binding affinity of GY-5 and GY-14 to PD-1 proteins obtained from insect cells (K_D = 1.64 nM and 0.52 nM, respectively) or E. coli (K_D = 3.52 and 0.34 nM, respectively) showed no substantial differences from those of PD-1 proteins from 293T cells (Figures 2 B, 2C, and 6A and, Table S2). These results indicate that the binding of both GY-5 and GY-14 to PD-1 is independent of PD-1 glycosylation.

The FG Loop of PD-1 Serves as a Novel “Hotspot” for PD-1-Targeting mAbs

To investigate the conformational variations of PD-1 upon binding to different mAbs, the PD-1 structures extracted from the GY-5/PD-1 and GY-14/PD-1 complexes and the other two structurally known nivolumab/PD-1 and pembrolizumab/PD-1 complexes were superimposed. The fold motif of the extracellular PD-1 consists of two β-sheets with multiple strands, together with multiple connecting loops (Figure 7A). Superimposition of the structure of PD-1 from the GY-5/PD-1 complex and GY-14/PD-1 complex yields a root-mean-square deviation of 0.509 Å for 85 Cz pairs, demonstrating the conformational conservation of the
DISCUSSION

In the present study, we report two PD-1-specific mAbs, GY-5 and GY-14, with potent tumor suppressive efficacy. PD-1 contains a front β-sheet face comprising the CC′FG strands and a back β-sheet face comprising the AA′BDE strands. The binding of PD-1 to PD-L1 involves the front β-sheet faces of both molecules, with additional contributions of the FG loop. Structural analysis revealed that both GY-5 and GY-14 mainly bind to the FG loop of PD-1. Together with the previously reported complex structures of nivolumab/PD-1 and pembrolizumab/PD-1, the binding of the four mAbs targeting PD-1 exhibits “loop-dominated” binding characteristics, which is different from PD-L1 binding. The highly flexible loops of PD-1 adopt different conformations when binding to these mAbs. The binding of nivolumab mainly involves the N-terminal loop, whereas pembrolizumab mainly binds to the C′D loop. Both the N-terminal and the C′D loops of PD-1 are away from the binding interface of PD-1/PD-L1. In contrast, both GY-5 and GY-14 mainly bind to the FG loop of PD-1, which shows varied conformations upon binding to different mAbs or its ligand, PD-L1. In contrast to the loops targeted by nivolumab or pembrolizumab, which are away from the PD-1/PD-L1 binding interface, the FG loop of PD-1 plays critical roles in the interaction with PD-L1 (Lin et al., 2008; Lazar-Molnar et al., 2008). Therefore the blocking of the PD-1/PD-L1 interaction by GY-5 and GY-14 relies on occupancy of the FG loop of PD-1 with overwhelming binding affinity compared with the PD-1/PD-L1 interaction. Although the N-terminal loop of PD-1 exhibits partial contacts with GY-5, the overall binding affinity of GY-5 to PD-1 is not affected, as determined through SPR assays.
with an N-terminally loop-truncated PD-1. The FG loop-dominated binding of GY-5 and GY-14 indicates a completely different binding mode from that of nivolumab and pembrolizumab, suggesting that GY-5 and GY-14 are potential therapeutic mAbs in addition to the two commercially available mAbs. Taken together, the FGl loop of PD-1 may serve as an important region for the development of PD-1-targeting biologics or small chemical molecules.

Li et al. recently reported that glycosylation of PD-L1 in tumor cells is essential for interaction with PD-1, and an mAb targeting glycosylated PD-L1 would promote PD-L1 internalization and degradation (Li et al., 2018). A wide range of alterations in the glycoproteins on tumor cells can occur, which may correlate with the development and progression of multiple tumors (Pinho and Reis, 2015). Disordered glycosylation modification of the proteins in tumor cells was usually correlated with dysregulated protein folding, trafficking, and protein-protein interactions (Xu and Ng, 2015). In addition to the expression in T cells, PD-1 is also expressed in tumor cells and macrophages, and the expression in these cells may also correlate with the treatment efficacy of PD-1/PD-L1-blocking mAb-based tumor ICT (Kleffel et al., 2015; Gordon et al., 2017; Huang et al., 2009). Therefore investigations of PD-1 glycosylation and the glycan dependency of the interaction of PD-1 with these mAbs would be valuable for our understanding of immune checkpoint blockade therapy (Tan et al., 2017).

PD-1 has four potential N-linked glycosylation sites, and mutational analysis indicates glycosylation modifications at each of these sites (Tan et al., 2017). In the present study, glycosylation modifications were structurally visible at three of the four glycosylation sites: N49, N58, and N116. Considering the flexibility of the glycans, the possibility of glycosylation at N74 cannot be excluded, and more complicated glycan structures may exist at the other three N-linked glycosylation sites. Structural analysis revealed that all these glycosylation sites are located away from PD-1/PD-L1-binding face, suggesting that the glycosylation modifications have no direct influence on the PD-1/PD-L1 interaction. Previous studies reveal that the binding of nivolumab is independent of PD-1 glycosylation, as determined through the analysis of binding affinity to glycosylation-site-mutated PD-1s or PD-1 proteins obtained from different expression systems that

Figure 7. Comparative Binding of PD-1-Targeting mAbs
(A) The location of the loops on PD-1, with the N-terminal loop colored in red, BC loop in green, and FG loop in blue. The invisible C’D loop is depicted as dashed lines in purple.
(B) Superimposition of apo-PD-1 (gray) and the PD-1s extracted from the complex structures of PD-1/PD-L1 (orange) (PDB code: 4ZQK), PD-1/nivolumab (yellow) (PDB code: 5WT9), PD-1/pembrolizumab (light pink) (PDB code: 5JXE), GY-5/PD-1 (green), and GY-14/PD-1 (cyan). The loops that contributed major binding to the mAbs are highlighted in dashed circles.
(C) Comparison of the FG loop of the PD-1s from the complex structures. The FG loop of PD-1 shifted 10.3 Å upon the binding to nivolumab or GY-5.
enable different levels of glycosylation of PD-1 (Tan et al., 2017). Although pembrolizumab binds to
different regions compared with nivolumab, the structural analysis reveals that the N-linked glycosylation
sites are also located away from the pembrolizumab/PD-1 interface (Na et al., 2017). The structural analysis
and binding assays of GY-5 and GY-14 to PD-1 proteins obtained from different expression systems
demonstrated that the binding of GY-5 and GY-14 is also independent of PD-1 glycosylation. Therefore
the FG loop-targeting mAbs may be promising therapeutics independent of dysregulated glycosylation
modifications of PD-1 in both immune and tumor cells.

Taken together, we identified two PD-1/PD-L1 blocking mAbs targeting PD-1 with tumor suppressive effi-
cacy. These two mAbs mainly bind to the FG loop of PD-1, which is distinct from the other structurally clear
anti-PD-1 mAbs. Glycosylation modifications could be observed at three N-linked glycosylation sites, but
the glycosylation modifications were not involved in the binding of these two mAbs to PD-1. These findings
have broadened our understanding of mAb-based ICT and will aid in the future development of therapeu-
tics by targeting PD-1.

Limitations of the Study
We do not know the functional anti-tumor advantage of the two mAbs identified in the present study over
commercially available PD-1-targeting mAbs. Moreover, there are more PD-1-specific mAbs under clinical
investigations and the binding regions of these mAbs on PD-1 may be different from the currently known
binding epitopes. Especially, the N58 glycosylation is near the PD-1/PD-L1-binding interface, and future
studies should take considerations whether this glycosylation would affect the binding of the mAb.

METHODS
All methods can be found in the accompanying Transparent Methods supplemental file.

DATA AND SOFTWARE AVAILABILITY
The accession number for the atomic coordinates of GY-5/PD-1 complex and GY-14/PD-1 complex re-
ported in this paper is PDB: 6J15 and 6J14.

SUPPLEMENTAL INFORMATION
Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2019.03.017.

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AUTHOR CONTRIBUTIONS
G.F.G., J.Y., and S.T. designed and supervised the study. D.C., H.Z, H.W., S.T., R.S., and Z.T. conducted the
experiments. Y.C. and J.Q. collected the datasets and solved the structures. S.T., D.C., W.H., J.Z., H.C.,
S.G., M.X., J.Y., and G.F.G. analyzed the data and wrote the manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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Ishiida, Y., Agata, Y., Shibahara, K., and Honjo, T. (1992). Induced expression of PD-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell death. EMBO J. 11, 3887–3895.

Iwai, Y., Ishida, M., Tanaka, Y., Okazaki, T., Honjo, T., and Minato, N. (2002). Involvement of PD-L1 on tumor cells in the escape from host immune system and tumor immunity by PD-L1 blockade. Proc. Natl. Acad. Sci. U S A 99, 12293–12297.

Kleffel, S., Posch, C., Barthel, S.R., Mueller, H., Schlappbach, C., Guenova, E., Elco, C.P., Lee, N., Juneja, V.R., Zhan, Q., et al. (2015). Melanoma cell-intrinsic PD-1 receptor functions promote tumor growth. Cell 162, 1242–1256.

Latchman, Y., Wood, C.R., Chernova, T., Chaudhary, D., Borde, M., Chernova, I., Iwai, Y., Long, A.J., Brown, J.A., Nunes, R., et al. (2001). PD-L2 is a second ligand for PD-1 and inhibits T cell activation. Nat. Immunol. 2, 261–268.

Lazar-Molnar, E., Yan, Q., Cao, E., Ramagopal, U., Nathenson, S.G., and Almo, S.C. (2008). Crystal structure of the complex between programmed death-1 (PD-1) and its ligand PD-L2. Proc. Natl. Acad. Sci. U S A 105, 10483–10488.

Lee, J.Y., Lee, H.T., Shin, W., Chae, J., Choi, J., Kim, S.H., Lim, H., Won Heo, T., Park, K.Y., Lee, Y.J., et al. (2016). Structural basis of checkpoint blockade by anti-PD-1 monoclonal antibodies in cancer immunotherapy. Nat. Commun. 7, 13354.

Li, C.W., Lin, S.O., Chung, E.M., Kim, Y.S., Park, T.H., and Garboczi, D.N. (2008). The PD-1/PD-L1 interaction by two antibody avelumab for tumor therapy. Cell Res. 28, 151–153.

Lin, D.Y., Tanaka, Y., Iwasaki, M., Gittis, A.G., Su, H.P., Mikami, B., Okazaki, T., Honjo, T., Minato, N., and Garboczi, D.N. (2008). The PD-1/PD-L1 complex resembles the antigen-binding Fv fragments. J. Immunol. 174, 195–204.

Lin, D.Y., Tanaka, Y., Iwasaki, M., Gittis, A.G., Su, H.P., Mikami, B., Okazaki, T., Honjo, T., Minato, N., and Garboczi, D.N. (2008). The PD-1/PD-L1 complex complexes the antigen-binding Fv domains of antibodies and T cell receptors. Proc. Natl. Acad. Sci. U S A 105, 3011–3016.

Liu, K., Tan, S., Choi, Y., Chen, D., Song, H., Zhang, C.W., Shi, Y., Liu, J., Zhang, C.W., Qi, J., and Yan, J., et al. (2016). Crystal clear: visualizing the intervention mechanism of the PD-1/PD-L1 interaction by two cancer therapeutic monoclonal antibodies. Protein Cell 7, 866–877.

Liu, S., Chen, D., Liu, K., He, M., Song, H., Shi, Y., Liu, J., Zhang, C.W., Qi, J., and Yan, J., et al. (2016). Crystal clear: visualizing the intervention mechanism of the PD-1/PD-L1 interaction by two cancer therapeutic monoclonal antibodies. Protein Cell 7, 866–877.

Tang, Y., Wu, D., Li, X., Kong, Y., Xin, Z., Liu, Y., and Gu, S., et al. (2017). Structural basis of anti-PD-1 monoclonal antibody avelumab for tumor therapy. Cell 162, 1242–1256.
Supplemental Information

The FG Loop of PD-1 Serves as a “Hotspot” for Therapeutic Monoclonal Antibodies in Tumor Immune Checkpoint Therapy

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Figure S1. Screening of PD-1/PD-L1 blocking MAbs targeting PD-1, related to Figure 1. The blocking of the binding of PD-1-mFc to PD-L1s expressed on the
surface of 293T cells by MAbs targeting PD-1. The PD-L1 expressing HEK 293T cells were stained with PBS as mock, while the staining with PD-1-mFc proteins was involved as positive control. The blocking efficacy of the MAbs was analyzed by staining of a protein complex of MAbs and PD-1-mFc at a molar ratio of 2:1 and a final concentration of 10 μg/mL. The absence of the PD-1-mFc staining positive subpopulation indicates the blockade of PD-1/PD-L1 interaction. The data presented here is representative of three independent experiments with similar results.

Figure S2. T cell activating efficacy of the MAbs, related to Figure 1. IFN-γ secreting cells were detected with PBMCs from three healthy donors, donor 1, donor 2 and donor 3, stimulated with a peptide pool of M1 of 2009 pN1N1 virus. The activating efficacy of PD-1 specific MAbs to M1 specific T cell responses were evaluated with simultaneous stimulation of the PBMCs with the MAbs and M1 peptide pool. PBMCs incubated with medium alone were enrolled as mock. PBMCs stimulated with M1 peptide pool alone or together with Ebola virus GP protein specific 13C6 MAb were served as negative control. The SE was represented on each MAb bar. *: p< 0.05. The data presented here is representative of three independent experiments with similar results.
Figure S3. Humanization strategy of GY-5, related to Figure 2. Sequence alignments highlighting the humanization strategy of GY-5 by retaining all the CDRs and substituting the remaining amino acids with the equivalent residues of the human immunoglobulins. The human antibody of IGHV1-67*01, which exhibits the highest sequence identity to GY-5 in heavy chain, was selected as the humanization backbone, while IGKV2-30*02 was selected as the humanization backbone for the light chain.

Figure S4. Humanization strategy of GY-14, related to Figure 2. Sequence alignments highlighting the humanization strategy of GY-14 by retaining all the CDRs
and substituting the remaining amino acids with the equivalent residues of the human immunoglobulins. The human antibody of IGHV1-2*02, which exhibits the highest sequence identity to GY-5 in heavy chain, was selected as the humanization backbone, while IGKV2-29*02 was selected as the humanization backbone for the light chain.

**Figure S5.** SPR assay-based characterization of the binding of GY-5 to N-terminal truncated PD-1, related to Figure 6. The assay was accomplished using a single-cycle BIAcore® T100 system. The equilibrium dissociation constant (KD) of the binding are labeled accordingly. The fits of the binding curves are shown in red. The data presented here are representative of two independent experiments with similar results.
Table S1. Sequence of the MAbs with T cell activating efficacy, related to Figure 1

| MAb   | L-V       | L-J | LCDR3       | H-V allele | H-J | HCDR3            |
|-------|-----------|-----|-------------|------------|-----|------------------|
| GY-1  | KV1-117*01| 2*01| QGSHVPYT    | HV1-15*01  | 1*01| TREGMNTDWDYDV    |
| GY-2  | KV1-117*01| 2*01| QGSHVPYT    | HV1-67*01  | 2*01| AREEWDVYYDYW     |
| GY-5  | KV1-117*01| 2*01| QGSHVPYT    | HV1-67*01  | 2*01| SREEWDVYYDYW     |
| GY-6  | KV3-12*01 | 5*01| QHSWELPLT   | HV1-S81*02 | 2*01| TRRDYRYDGGDY     |
| GY-11 | KV3-12*01 | 5*01| QHSWELPLT   | HV1-S81*02 | 2*01| TRRDYRYDGGYD    |
| GY-14 | KV1-117*01| 2*01| QGSHVPYT    | HV1-15*01  | 1*01| TREGMNTDWDYDV    |
| GY-17 | KV1-117*01| 2*01| QGSHVPYT    | HV1-67*01  | 2*01| SREEWDVYYDYW     |
| GY-25 | KV3-12*01 | 5*01| QHSWELPLT   | HV1-S81*02 | 2*01| TRRDYRYDGGDY     |
| Antibody          | PD-1 protein expressing cell | $K_a (M \cdot s)^1$ | $K_d (s)^2$ | $K_D (nM)$ |
|-------------------|-----------------------------|--------------------|--------------|-------------|
| Chimeric GY-5     | 293T cell                   | $2.13 \times 10^5$ | $2.59 \times 10^{-4}$ | 1.22        |
| Chimeric GY-14    | 293T cell                   | $3.42 \times 10^5$ | $5.27 \times 10^{-4}$ | 1.54        |
| Humanized GY-5    | 293T cell                   | $1.13 \times 10^5$ | $10.9 \times 10^{-4}$ | 9.62        |
| Humanized GY-14   | 293T cell                   | $2.38 \times 10^5$ | $5.26 \times 10^{-4}$ | 2.21        |
| nivolumab         | 293T cell                   | $8.08 \times 10^5$ | $12.36 \times 10^{-4}$ | 1.53        |
| Chimeric GY-5     | Insect cell                 | $4.17 \times 10^5$ | $6.84 \times 10^{-4}$ | 1.64        |
| Chimeric GY-5     | *E. coli*                   | $5.41 \times 10^5$ | $19.06 \times 10^{-4}$ | 3.52        |
| Chimeric GY-14    | Insect cell                 | $4.81 \times 10^5$ | $2.51 \times 10^{-4}$ | 0.52        |
| Chimeric GY-14    | *E. coli*                   | $6.62 \times 10^5$ | $2.26 \times 10^{-4}$ | 0.34        |

$^1$ $K_a$, association rate constant.  
$^2$ $K_d$, dissociation rate constant.
Table S3. Crystallographic data collection and refinement statistics, related to Figure 3.

|                      | GY-5/PD-1     | GY-14/PD-1    |
|----------------------|---------------|--------------|
| **Data collection**  |               |              |
| Space group          | P 1 2 1 1     | P 1 2 1 1    |
| Wavelength (Å)       | 0.979         | 0.978        |
| Unit cell dimensions |               |              |
| \(a, b, c\) (Å)      | 61.59, 64.35, 151.48 | 43.42, 75.68, 55.87 |
| \(\alpha, \beta, \gamma\) (°) | 90.0, 98.04, 90.0 | 90.0, 104.3, 90.0 |
| Resolution (Å)       | 50.0-2.60 (2.69-2.60) | 50.0-1.40 (1.40-1.45) |
| No. reflections      | 33831         | 66956        |
| \(R_{merge}\)       | 0.109 (0.859) | 0.039 (0.222) |
| \(I / \sigma\)      | 3.17 (2.61)   | 7.69 (1.40)  |
| Completeness (%)     | 92.2          | 97.07        |
| Redundancy           | 6.2 (6.5)     | 7.0 (7.2)    |
| **Refinement**       |               |              |
| Resolution (Å)       | 41.6-2.6      | 44.0-1.4     |
| \(R_{work} / R_{free}\) | 0.212/0.276  | 0.188/0.215  |
| No. atoms            |               |              |
| Protein              | 8342          | 2958         |
| Ligands              | 0             | 0            |
| Water                | 0             | 392          |
| \(B\)-factors       |               |              |
| Protein              | 41.2          | 13.0         |
| Ligands              |               |              |
| Water                |               |              |
| R.m.s. deviations    |               |              |
| Bond lengths (Å)     | 0.010         | 0.007        |
| Bond angles (°)      | 1.248         | 0.933        |
| Ramachandran plot    |               |              |
| Favored (%)          | 95.83         | 97.12        |
| Allowed (%)          | 4.17          | 2.56         |
| Outliers (%)         | 0.00          | 0.32         |

*Values in parentheses are for highest-resolution shell.*
Table S4. Interaction between GY-5 and PD-1, related to Figure 4

| GY-5 | PD-1                  | Contacts |
|------|-----------------------|----------|
| H chain |                     |          |
| A31  | L128, A132            | 1\(^1,1\) |
| V48  | L128, A129            | 9,1      |
| I49  | L128                  | 5        |
| S50  | L128                  | 6        |
| Y52  | V64, I126             | 1,7      |
| N53  | V64                   | 1        |
| I55  | S62, F63, L128        | 5,2,6    |
| T56  | S62, L128             | 4(1)\(^2,3\) |
| N57  | S60, S62, L128, A129  | 2,7(1),1,4 |
| E97  | K131, A129            | 13(2),7  |
| W99  | I126, S127, L128, A132,Q133,I134 | 4,6,6,13,8,4 |
| D100 | A132,Q133,I134        | 3(1),5,16(1) |
| V101 | K131                  | 5        |
| F102 | K131                  | 3        |
| Y103 | K131                  | 6(1)     |
| Y104 | P130, K131            | 1,17(1)  |
| L chain |                     |          |
| Y101 | L128, A129            | 1,3,7,4(1) |
| V30  | I30                   | 3        |
| H31  | I30, P130, K131, Q133 | 2,10(1),5,6 |
| S32  | I30, W32, T59         | 3,8,4    |
| D33  | I30, W32, Q133, K135  | 1,7,4(1),1 |
| G34  | I30                   | 4        |
| Y37  | K131, Q133            | 15,1     |
| G96  | P130, K131            | 1,1      |
| S97  | P130                  | 4        |
| H98  | P130                  | 2        |
| V99  | A129, P130            | 3,6      |

\(^1\) Numbers represent the number of atom-to-atom contacts between the antibody residues and the hPD-1 residues, which were analyzed by the Contact program in CCP4 suite (the distance cutoff is 4.5 Å).

\(^2\) Numbers in the parentheses represent the number of hydrogen bonds between the antibody residues and the hPD-1 residues, which were analyzed by the Contact program in CCP4 suite (the distance cutoff is 3.5 Å).
Table S5. Interaction between GY-14 and PD-1, related to Figure 4

|        | GY-14  | PD-1     | Contacts |
|--------|--------|----------|----------|
| H chain| T29    | Q75      | 10\(^1\) |
|        | Y32    | K78, D85, Q88, P89 | 6(1) \(^2\), 3, 12, 12 |
|        | Y34    | S87, P89 | 6(1), 3  |
|        | G49    | P89      | 4        |
|        | I50    | P89      | 8        |
|        | N51    | P89      | 8        |
|        | S53    | Q75, D77 | 4(1), 6(1) |
|        | N54    | G90      | 1        |
|        | G56    | P89, G90 | 6, 6     |
|        | T57    | P89, G90 | 3, 6     |
|        | N58    | S87, Q88, P89 | 4, 4, 14 |
|        | R98    | K78, D85, S87, Q88 | 1, 9(2), 8(1), 1 |
|        | Y100   | T76, D77, K78, D85 | 11 (1), 2, 12(1), 1 |
|        | R101   | V64, N66, Y68, K78, I126, I134 | 1,7(1),4,1,4,2 |
|        | Y102   | V64, I126, L128 | 1,3,5 |
|        | D103   | S87      | 2        |

| L chain| H31    | R86      | 2        |
|        | S32    | P83      | 5        |
|        | D33    | E61, S62, F63, F82, P83 | 7,14(1),15,1,3 |
|        | G34    | S62      | 6(1)     |
|        | N35    | P83      | 2        |
|        | Y37    | P83, E84, R86 | 2,1,10 |
|        | Y54    | L128     | 2        |
|        | K55    | L128     | 1        |
|        | N58    | L128, A129 | 5        |
|        | G96    | R86      | 3        |
|        | S97    | R86      | 12(1)    |
|        | Y101   | S87      | 17(2)    |

\(^1\) Numbers represent the number of atom-to-atom contacts between the antibody residues and the hPD-1 residues, which were analyzed by the Contact program in CCP4 suite (the distance cutoff is 4.5Å).

\(^2\) Numbers in the parentheses represent the number of hydrogen bonds between the antibody residues and the hPD-1 residues, which were analyzed by the Contact program in CCP4 suite (the distance cutoff is 3.5 Å).
Transparent Methods

Ethics statement.

The study was approved by the Research Ethics Committee of Institute of Microbiology, Chinese Academy of Sciences. All of the subjects provided written informed consent for the studies performed on their samples and publication of their cases. Animals used in this study (6-to-8-week-old female NCG mice) were bought from Model Animal Research Center of Nanjing University. The study was conducted in accordance with the principles of the Declaration of Helsinki and the standards of good clinical practice (as defined by the International Conference on Harmonization), and Chinese regulatory requirements, as stipulated by the Chinese Food and Drug Administration.

Protein expression and purification

Three expression systems, i.e., E.coli, insect cell, HEK 293T cell, were used to express PD-1 proteins for crystal screening or binding affinity analysis as previously described (Tan et al., 2017). Two constructs with PD-1 fragment of L25-R147 or N33-R147 (UniProt: Q15116) were cloned into pET21a vector (Invitrogen). The protein was expressed in E.coli as inclusion bodies and refolded in vitro as described previously (Liu et al., 2017; He et al., 2017; Tan et al., 2018; Li et al., 2015). The construct encoding PD-1 fragment (residue N33-R147) was cloned into pFastbac1 vector (Invitrogen) with an N-terminal gp67 signal peptide and a C-terminal hexa-His tag. Transfection and virus amplification were performed according to the Bac-to-Bac baculovirus expression system manual (Invitrogen). The Hi5 cells (Invitrogen) were
infected by the recombinant baculovirus to produce soluble PD-1. Then the supernatant containing soluble PD-1 was purified by sequentially His-Trap HP column (GE Healthcare) and Superdex® 200 10/300 GL (GE Healthcare) in a 20 mM Tris and 150 mM NaCl (pH 8.0) buffer. The DNA encoding the ectodomain of PD-1 (residues M1-Q147, including signal peptide, UniProt: Q15116) with either a C-terminal hexa-His tag (plasmid PD-1-pCAGGS) or mouse IgG-Fc Fragment at the C terminus (plasmid PD-1-mFc-pCAGGS) were cloned into pCAGGS vector (Addgene) with EcoRI and BglII restriction sites. Plasmid PD-1-pCAGGS or PD-1-mFc-pCAGGS was transiently transfected into HEK 293 T cells (ATCC) for protein expression. The PD-1-mFc protein was purified using a Protein G affinity column (GE Healthcare), while PD-1-his protein was purified by His-Trap HP column (GE Healthcare). PD-1-mFc protein or PD-1-his protein was subsequently purified by gel filtration on a Superdex 200 10/300 GL (GE Healthcare) in a buffer containing 20 mM Tris (pH 8.0) and 150 mM NaCl.

The mouse ascites fluid with GY-5 was diluted with an equal volume of 20 mM Na3PO4 (pH 7.0) buffer and filtered through a 0.22 μm filter (Millipore). The antibodies were purified on a Protein G affinity column. GY-5 Fab fragments were obtained respectively by papain (Pierce) digestion according to the manufacturer’s instruction and subsequently purified by gel filtration on a Superdex 200 10/300 GL (GE Healthcare) in a buffer containing 20 mM Tris and 150 mM NaCl (pH 8.0). GY-14-single-chain variable fragment (scFv) was constructed as VL-(GGGGS)₄-VH which expressed and refolded as described previously (Liu et al., 2017; He et al., 2017; Tan et al., 2018). Recombinant GY-14-scFv were further purified by Superdex 200 10/300 GL
The H chain and L chain of chimeric GY-5 and GY-14 constructed with human IgG4 subtype were cloned into pCAGGS plasmids with EcoRI and BglII restriction sites. The H chain and L chain plasmids of chimeric GY-5 and GY-14 were transiently co-transfected into HEK 293 T cells (ATCC) for protein expression. The protein was purified using a Protein G affinity column (GE Healthcare) and subsequently purified by gel filtration on a Superdex 200 10/300 GL (GE Healthcare) in a buffer containing 20 mM Tris (pH 8.0) and 150 mM NaCl.

**Mouse model for anti-PD-1 antibody treatment.**

Highly immunodeficient NCG mice (Model Animal Research Center of Nanjing University) were injected with human PBMC i.v. 3 days prior to engraftment of HCC-827 NSCLC cells. Then the mice were inoculated with NSCLC cells into one of the flanks of each mouse (5×10⁶ cells/mouse) subcutaneously. Three days after engraftment, GY-5, GY-14 or 13C6 (anti-Ebola virus antibody) antibody was injected i.p. at 10 mg/kg twice a week. Growth rates were determined by measuring three dimensions (length, width and depth) of tumors with calipers twice a week. After 4 weeks, mice were sacrificed with a CO₂ chamber. Volumes of tumors were calculated according to the formula: Volume = length × width × height × 0.5236. Animal care was carried out in accordance with the guidelines of Animal Care and Use Committee of Institute of Microbiology, Chinese Academy of Sciences.
Flow cytometry of PD-1/PD-L1 blockade assay

The full-length PD-L1 was cloned into pEGFP-N1 vector (Clontech). HEK 293 T cells were transiently transfected with this recombinant plasmid for 24 hours and resuspended in PBS at $1 \times 10^7$ cells/mL. PD-1-mFc protein at a concentration of 2 μg/mL and GY-5 or GY-14 antibody at a concentration of 20 μg/mL were pre-incubated respectively at room temperature for 30 minutes. Then these mixtures were used to incubate with the 293T cells expressing PD-L1 fused EGFP protein at room temperature for further 30 min. After washing with PBS for three times, the cells were stained with secondary APC-anti-human IgG (Clone G18-145, BD Pharmingen) for another 30 min and analyzed using flow cytometry (BD FACSCalibur Flow Cytometer).

Enzyme-linked immunospot (ELISpot) assay

Peripheral blood mononuclear cells (PBMCs) from three healthy donors were isolated by use of the Ficoll-Paque gradient technique according to the manufacturer’s instruction (Hao Yang Biological Manufacture). PBMCs isolated were incubated respectively with M1 peptide pool (Liu et al., 2016) (10 μg/mL) after culturing in the 24-well-plate for 24 hours. Recombinant interleukin 2 (IL-2) at a concentration of 50 UI/mL was added to the culture medium on day 3. The next day, half of the medium from the plate was replaced with fresh RPMI-1640 media (Gibco, Thermo Fisher Scientific). On day 7, the PBMCs were seeded on the ELISpot plate (BD) with M1 peptide pool (10 μg/mL) and PD-1 specific MAbs (10 μg/mL). PBMCs incubated with medium alone were enrolled as mock while PBMCs stimulated with M1 peptide pool
alone or together with Ebola virus GP protein specific 13C6 MAb were served as negative control. Secretion of IFN-γ from activated T cells was examined by the ELISpot assay kit (Human IFN-γ Set, BD) following the manufacturer’s instructions. The reaction was finally stopped using demineralized water and spots were analyzed with an ELISpot reader (ImmunoSpot® S6 analyzers, Cellular Technology).

**Surface plasmon resonance (SPR)**

SPR measurements were done at room temperature using a BIAcore® T100 system with CM5 chips (GE Healthcare). For all measurements, the buffer consisting of 150 mM NaCl, 10 mM HEPES, pH 7.4 and 0.005% (v/v) Tween-20 was used as running buffer, and all proteins were exchanged into this buffer in advance through gel filtration. The blank channel of the chip served as the negative control. To detect the anti-PD-1 antibodies binding to PD-1 proteins, full-length GY-5 or GY-14 was immobilized on the chip at a concentration of 1 mg/mL by anti-human IgG at B70 response units. Gradient concentrations of PD-1 (0 nM, 3.125 nM, 6.25 nM, 12.5 nM, 25 nM and 50 nM) 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 4.1 using a 1:1 Langmuir-binding model.

**Crystal screening and structure determination**

PD-1 protein expressed in insect cells and GY-5-Fab, or PD-1 proteins refolded from inclusion bodies from *E. coli* cells and GY-14-scFv were mixed respectively at a molar
ratio of 1:1. The mixed samples were incubated for 30 min on ice and then purified by gel-filtration. Pooled proteins (10 mg/mL) were used for crystal screening by vapor-diffusion sitting-drop method at 4 °C. Diffracting crystals of PD-1/GY-5 were obtained in conditions consisting of 0.1 M citrate (pH 5.0), 20% w/v polyethylene glycol 6,000 and 0.2 M ammonium acetate, while crystals of PD-1/GY-14 were obtained in conditions consisting of 0.06 M MgCl$_2$ and CaCl$_2$, 0.1 M imidazole-MES (pH 6.5), 18% v/v ethylene glycol and polyethylene glycol 8,000, respectively. After incubation in a reservoir solution containing 20% (v/v) glycerol crystals were flash-cooled in liquid nitrogen. The diffraction data were collected at Shanghai Synchrotron Radiation Facility BL17U, and all data were processed with HKL2000 (Otwinowski and Minor, 1997). The complex structure was solved by molecular replacement method using Phaser (Read et al., 2001) from the CCP4 programme suite (Collaborative computational project N, 1994), with the reported PD-1 structure (PDB: 3RRQ) and Fab structure (PDB: 3EYQ) as the search models. COOT (Emsley and Cowtan, 2004) and PHENIX (Adams et al., 2010) were used for subsequent model building and refinement. The stereochemical qualities of the final model were assessed with MolProbity (Chen et al., 2010). All structure figures were prepared with Pymol (http://www.pymol.org).

Data and software availability

The accession number for the atomic coordinates of GY-5/PD-1 complex and GY-14/PD-1 complex reported in this paper is PDB: 6J15 and 6J14.
Supplemental References

Adams, P. D., Afonine, P.V., Bunkóczi, G., Chen, V.B., Davis, I.W., Echols, N., Headd, J.J., Hung, L.W., Kapral, G.J., Grosse-Kunstleve, R.W., et al. (2010). PHENIX: a comprehensive Python-based system for macromolecular structure solution. Acta Crystallogr. D Biol. Crystallogr. 66, 213-221.

Chen, V.B., Arendall, W.B.3rd., Headd, J.J., Keedy, D.A., Immormino, R.M., Kapral, G.J., Murray, L.W., Richardson, J.S., Richardson, D.C., et al. (2010). MolProbity: all-atom structure validation for macromolecular crystallography. Acta Crystallogr. D Biol. Crystallogr. 66, 12-21.

Collaborative computational project N. (1994). The CCP4 suite: programs for protein crystallography. Acta Crystallogr. D Biol. Crystallogr. 50, 760-763.

Emsley, P. and Cowtan, K. (2004). Coot: model-building tools for molecular graphics. Acta Crystallogr. D Biol. Crystallogr. 60, 2126-2132.

He, M., Chai, Y., Qi, J., Zhang, C.W., Tong, Z., Shi, Y., Yan, J., Tan, S., Gao, G.F. (2017). Remarkably similar CTLA-4 binding properties of therapeutic ipilimumab and tremelimumab antibodies. Oncotarget 8, 67129-67139.

Li, H., Zhou, M., Han, J., Zhu, X., Dong, T., Gao, G.F., Tien, P. (2005). Generation of murine CTL by a hepatitis B virus-specific peptide and evaluation of the adjuvant effect of heat shock protein glycoprotein 96 and its terminal fragments. J. Immunol. 174, 195-204.

Liu, K., Tan, S., Chai, Y., Chen, D., Song, H., Zhang, C.W., Shi, Y., Liu, J., Tan, W., Lyu, J., et al. (2017). Structural basis of anti-PD-L1 monoclonal antibody avelumab for tumor therapy. Cell Res. 27, 151-153.

Otwinowski, Z. and Minor, W. (1997). Processing of X-ray diffraction data collected in oscillation mode. Methods Enzymol. 276, 307-326.

Read, R. J. (2001). Pushing the boundaries of molecular replacement with maximum likelihood. Acta Crystallogr. D Biol. Crystallogr. 57, 1373-1382.

Tan, S., Zhang, H., Chai, Y., Song, H., Tong, Z., Wang, Q., Qi, J., Wong, G., Zhu, X., Liu, W.J., et al. (2017). An unexpected N-terminal loop in PD-1 dominates binding by nivolumab. Nat. Commun. 8, 14369.

Tan, S., Liu, K., Chai, Y., Zhang, C.W., Gao, S., Gao, G.F., Qi, J., et al. (2018). Distinct PD-L1 binding characteristics of therapeutic monoclonal antibody durvalumab. Protein Cell 9, 135-139.