Cancer cells express tumour-specific antigens derived via genetic and epigenetic alterations, which may be targeted by T-cell-mediated immune responses. However, cancer cells can avoid immune surveillance by suppressing immunity through activation of specific inhibitory signalling pathways, referred to as immune checkpoints. In recent years, the blockade of checkpoint molecules such as PD-1, PD-L1 and CTLA-4, with monoclonal antibodies has enabled the development of breakthrough therapies in oncology, and four therapeutic antibodies targeting these checkpoint molecules have been approved by the FDA for the treatment of several types of cancer. Here, we report the crystal structures of checkpoint molecules in complex with the Fab fragments of therapeutic antibodies, including PD-1/pembrolizumab, PD-1/nivolumab, PD-L1/BMS-936559 and CTLA-4/tremelimumab. These complex structures elucidate the precise epitopes of the antibodies and the molecular mechanisms underlying checkpoint blockade, providing useful information for the improvement of monoclonal antibodies capable of attenuating checkpoint signalling for the treatment of cancer.
As the immune system plays an important role in controlling cancer, utilizing the immune system to eliminate cancer holds great potential. Although various immunotherapeutic approaches have been shown to enhance the immune system’s ability to modulate cancer, therapeutic antibodies that target regulatory pathways in T-cells to enhance antitumor immune responses, have attracted significant recent attention. T-cell-mediated immune responses are triggered through the recognition of antigenic peptide/HLA complexes on the surface of antigen presenting cells (APCs) by T-cell receptors and are tightly regulated by antigen-independent co-receptor signals, either costimulatory or coinhibitory, providing the optimal balance between immune responses to antigens and maintenance of self-tolerance under normal physiological conditions. Costimulatory signals are required to enhance and sustain the function of T-cells, the most important of which is provided by the interaction of CD28, a co-receptor on T-cells, with its ligands B7-1 and B7-2 on APCs. Programmed death-1 (PD-1) is also an antigen-independent co-receptor and plays a pivotal role in modulating immune responses. Although both CD28- and PD-1 are coinhibitory receptors, each plays a non-redundant role in the negative regulation of immune responses. While engagement of CTLA-4 by B7 ligands attenuates the early activation of naive and memory T-cells, PD-1 modulates the function of T-cells later in peripheral tissues via interaction with PD-L1 and PD-L2.

As cancer cells harbour genetic and epigenetic modifications, tumour-specific antigens are presented on the cancer cell surface and can be recognized by T-cells, therefore causing immune responses. However, cancer cells can also evade immunological recognition and destruction through the activation of coinhibitory signalling by overproduction of immune checkpoint proteins such as PD-1 and CTLA-4 on immune effector cells and PD-L1 on cancer cells. Furthermore, expression of PD-L1 on cancer cells can directly lead to the death of antigen-specific effector T-cells expressing PD-1. In an inflamed tumour microenvironment, engagement of PD-1 or CTLA-4 can self-limit the antitumor immune responses and permit cancer cells to proliferate unrestrained. Advances in the understanding of the molecular mechanisms underlying the ability of cancer cells to suppress immune surveillance have devised strategies to overcome cancer-induced immune tolerance, thereby protecting the host from tumour progression. Blockade of the ligand-receptor interaction of these immune checkpoint molecules can directly enhance the function of T-cells, which represents a critical paradigm shift whereby checkpoint blockade aims at disallowing the activity of T-cells compared with the previous immuno-ontocology concept, whereby cancer vaccines and cytokine therapies aimed at de novo activation of immune responses.

Monoclonal antibodies blocking immune checkpoints have demonstrated unprecedented therapeutic benefits in clinical trials and provided a major breakthrough in oncology. While targeted therapies elicit transient clinical responses because of acquisition of cancer drug resistance usually occurring within months after an initial response, the clinical responses of checkpoint blockade therapies are often durable, with some patients free from cancer progression for many years. The US Food and Drug Administration (FDA) has approved monoclonal antibodies that block CTLA-4 (ipilimumab), PD-1 (pembrolizumab and nivolumab), and PD-L1 (atezolizumab) for the treatment of melanoma, non-small-cell lung cancer, renal cell carcinoma, Hodgkin lymphoma and bladder cancer. As a number of clinical trials for antibodies targeting immune checkpoints are ongoing, additional approvals are expected for other antibodies and expanded indications in the near future.

There have been a number of structural studies for immune checkpoint molecules. The structures of the extracellular portion of CTLA-4 revealed an unusual mode of CTLA-4 dimerization that places the B7 binding sites distal to the dimerization interface. The crystal structures of CTLA-4 in complex with its B7 ligands showed the formation of a unique alternating network of bivalent CTLA-4 and B7-1/2 dimers, providing a structural basis for formation of unusually stable signalling complexes to regulate T-cell responsiveness within the immunological synapse. The structures of murine PD-1 in complex with human PD-L1, murine PD-1 in complex with murine PD-L2, and human PD-1 in complex with human PD-L1 have established the structural foundations of the interaction of PD-1 with its ligands. These structural studies for checkpoint molecules and their binding complexes have provided invaluable information for understanding the molecular mechanism of coinhibitory signals of T-cells through the communication of checkpoint molecules.

As the exact epitope and mechanism are crucial elements for antibody drugs, we report the crystal structures of checkpoint molecules in complex with the Fab fragments of therapeutic antibodies approved by the FDA or currently undergoing clinical trials, and reveal the binding modes of the complexes and the conformational changes induced by the antibody binding, thereby providing a structural basis of checkpoint blockade by monoclonal antibodies for the treatment of cancer.

**Results**

**Crystal structures of PD-1 in complex with anti-PD-1 drugs.** Pembrolizumab (trade name KEYTRUDA) is a humanized IgG4 antibody that blocks PD-1. The FDA approved pembrolizumab for the treatment of advanced melanoma in September 2014 and metastatic non-small-cell lung cancer (NSCLC) in October 2015. The PD-1/pembrolizumab Fab fragment complex was crystallized and its structure was determined and refined to a resolution of 2.0 Å. The pembrolizumab epitope of PD-1 consists of numerous discontinuous segments. The formation of hydrogen bonds between PD-1 and pembrolizumab involves side chain atoms of PD1N66, PD1T76, PD1K78, PD1S87 and PD1K131 and main chain atoms of PD1F63, PD1E84, PD1S87, PD1G90 and PD1A132, whereas a salt bridge is formed between PD1D85 and heavyR99. In addition, the main chain atom of PD1K78 and the side chain atoms of PD1S87 and PD1D85 participate in water-mediated interactions with heavyN52 and heavyR99. The residues of PD-1 involved in van der Waals contact with pembrolizumab are PD1S62, PD1V64, PD1Y68, PD1Q75, PD1D77, PD1A81, PD1F82, PD1P83, PD1R86, PD1Q88, PD1P89, PD1G90, PD1I126, PD1L128, PD1A129, and PD1I134. The paratope of pembrolizumab consists of heavyT30, heavyY33, and heavyY35 of HCDR1; heavyN52, heavyS54, heavyN55, heavyG57, heavyT58, and heavyN59 of HCDR2; heavyR99, heavyY101, heavyR102, heavyF103, heavyD104, heavyM105, and heavyD108 of HCDR3; lightT31, lightS32, lightY34, and lightY36 of LCDR1; lightY53, lightL54, lightY57, and lightE59 of LCDR2; and lightS95, lightR96, lightD97, lightL98 and lightL100 of LCDR3.

Nivolumab (trade name OPDIVO) is a fully human IgG4 that blocks PD-1. The FDA approved nivolumab for the treatment of melanoma in December 2014, NSCLC in March 2015, renal cell carcinoma in November 2015, and Hodgkin...
and lightS91 of LCDR3 (Fig. 2b). Binding as they also interact with PD-L1 in distinct conformations (residues 127–134) of PD-1, which are incompatible with PD-L1 changes in the BC loop (residues 57–63) and the FG loop binding site (Fig. 2c). In addition, the binding of PD-1 with epitopes of the two antibodies directly occupy part of the PD-L1 residues involved in van der Waals interactions with BMS-936559, including PDL1A51, PDL1A53, PDL1K57, and heavyK59 of HCDR2; heavyK99, heavyS104, heavyG105, and heavyS106, heavyP107 and heavyF108 of HCDR3; lightT32 of LCRD1; and lightS92, lightN93, and lightW94 of LCRD3. The total buried surface area of PD-L1/BMS-936559 is 1,349 Å², which is much smaller than that of PD-1/PD-L1 (1,970 Å²) (ref. 30). However, the epitope of BMS-936559 occupies a large part of the PD-1 binding site (Fig. 5b), in addition, high avidity, resulting from IgG bivalency, is expected due to the high expression level of PD-L1 in many cancer types36, leading to efficient blockade of the PD-1/PD-L1 interaction.

**Crystal structure of CTLA-4 in complex with tremelimumab Fab.** Tremelimumab is a fully human IgG2 antibody that blocks CTLA-4. In 2015, tremelimumab was granted Fast Track Designation and Orphan Drug Designation by the FDA as a potential treatment for malignant mesothelioma. In addition, tremelimumab is being studied in combination with an anti-PD-L1, durvalumab, in multiple tumour types37. The CTLA-4/tremelimumab Fab complex and free tremelimumab Fab were crystallized and their structures were determined and refined to a resolution of 2.0 Å and 2.3 Å, respectively (Fig. 6). The
tremelimumab epitope of CTLA-4 consists of several discontinuous segments (Fig. 7a). The side chain atoms of CTLA4K1 and CTLA4K95 and main chain atoms of CTLA4M3, CTLA4Q41, CTLA4M99, CTLA4Y104, CTLA4L106, and CTLA4I108 participate in the formation of hydrogen bonds between CTLA-4 and tremelimumab, whereas CTLA4E97 participates in salt bridge formation. A large number of residues, including CTLA4A2, CTLA4E33, CTLA4R35, CTLA4S44, CTLA4Q45, CTLA4V46, CTLA4E48, CTLA4L91, CTLA4I93, CTLA4M99, CTLA4P102, CTLA4P103, CTLA4Y104, CTLA4Y105, CTLA4L106, CTLA4I108, and CTLA4N110, contribute to van der Waals contact with tremelimumab. The tremelimumab residues involved in the interaction with CTLA-4
and b (grey) and PD-1/nivolumab (yellow), yielding r.m.s. deviation of 1.38 Å. In position of PD-1 molecules extracted from the complexes of PD-1/PD-L1 (grey) and PD-1/pembrolizumab (green), yielding r.m.s. deviation of 1.26 Å. (extracted from the complexes of PD-1/PD-L1 (grey, PDB code 4zqk) and PD-lizumab is also represented. The Cpale cyan, light chain: pale orange). Surface of the Fv region of pembrolizumab is presented of PD-1 (green) in complex with pembrolizumab Fab (heavy chain: (cis-trans-cis unusual loop of CTLA-4 in complex with tremelimumab adopt an residues of the MYPPPYY (residues 99–105) sequence of the FG loops of the PD-1 molecule in PD-1/PD-L1 is coloured orange. This N-terminal region in PD-1/PD-L1 and PD-1/pembrolizumab and the C&D loop in PD-1/PD-L1 are not shown in the structures due to the lack of interaction. (c) Interaction of the C&D loop with pembrolizumab with ribbon representation of PD-1 (green) in complex with pembrolizumab Fab (heavy chain: pale cyan, light chain: pale orange). Surface of the Fv region of pembrolizumab is also represented. The C&D loop of PD-1 is coloured red.

Figure 3 | Conformational changes in the loops of PD-1 induced by the binding of anti-PD-1 antibodies. (a) Superposition of PD-1 molecules extracted from the complexes of PD-1/PD-L1 (grey, PDB code 4zqk) and PD-1/pembrolizumab (green), yielding r.m.s. deviation of 1.26 Å. (b) Superposition of PD-1 molecules extracted from the complexes of PD-1/PD-L1 (grey) and PD-1/nivolumab (yellow), yielding r.m.s. deviation of 1.38 Å. In a and b, the BC, C&D, and FG loops of the PD-1 molecule in PD-1/anti-PD-1 are coloured blue, red and violet, respectively. In b, the N-terminal region (residue 27–33) of the PD-1 molecule in PD-1/nivolumab is coloured orange. This N-terminal region in PD-1/PD-L1 and PD-1/pembrolizumab and the C&D loop in PD-1/PD-L1 are not shown in the structures due to the lack of interaction. (c) Interaction of the C&D loop with pembrolizumab with ribbon representation of PD-1 (green) in complex with pembrolizumab Fab (heavy chain: pale cyan, light chain: pale orange). Surface of the Fv region of pembrolizumab is also represented. The C&D loop of PD-1 is coloured red.

are heavyW52, heavyY53, heavyN57 and heavyY59 of HCDR2; heavyR101, heavyG102, heavyY103, heavyT104, heavyL105, heavyY106, heavyY107, heavyY108 and heavyY110 of HCDR3; lightQ27, lightS28, lightN30 and lightY32 of LCDR1; and lightY91, lightY92, lightY93 and lightY94 of LCDR3. Superposition of the CTLA-4 molecules extracted from CTLA-4/tremelimumab and CTLA-4/B7-1 yielded a root mean square deviation of 1.08 Å for all of the Cα atoms, indicating no significant deviation in the two structures except for several residues at the antibody–antigen interface, which are not involved in the interaction with B7 ligands (Supplementary Fig. 3). As observed in the structure of free CTLA-4 and that of CTLA-4 in complex with B7-1/2, the three consecutive proline residues of the MYPPPYY (residues 99–105) sequence of the FG loop of CTLA-4 in complex with tremelimumab adopt an unusual cis-trans-cis conformation and participate in interactions with the antibody28,29,38 (Fig. 7a). The tremelimumab epitope partially occupies the B7-1/2 binding site of CTLA-4, with a larger interface area between CTLA-4 and tremelimumab (buried surface area of 1,802 Å2) than the receptor–ligand interface (1,255 Å2 for CTLA-4/B7-1 and 1,212 Å2 for CTLA-4/B7-2) (Fig. 7b). Therefore, the binding of tremelimumab to CTLA-4 efficiently competes with B7-1/2 binding to CTLA-4, thereby blocking the function of CTLA-4 in cancer.

Discussion

Checkpoint-blocking antibodies have ensured significant advances in cancer therapy and provided a new weapon against cancer. The durability of this immunotherapy largely exceeds that of other forms of anticancer therapy and sometimes enables long-term remission where patients exhibit no clinical signs of cancer for many years. However, monotherapies of these drugs elicit the therapeutic benefit only in a fraction of patients treated. As a critical next step in the progress toward improving clinical responses, combination immunotherapies have been intensively investigated39–41. Combinations of anti-CTLA-4 or anti-PD-1/PD-L1 therapies with other immunotherapies, chemotherapies, targeted therapies, hormonal therapies and radiation therapies are being explored. The combination of anti-CTLA-4 and anti-PD-1 has been the most successful of these combinations tested so far, probably because CTLA-4 and PD-1 modulate the activity of T-cells at different stages of T-cell immunity16,26. Thus, dual blockade of CTLA-4 and PD-1 can be a reasonable and synergistic combination therapy based on their distinct mechanisms of action. In addition, structural studies of therapeutic antibodies to reveal their precise epitopes and therapeutic mechanisms can also facilitate a rational design of combination immunotherapies, as different epitopes and molecular mechanisms of therapeutic antibodies can lead to different therapeutic effects even when therapeutic antibodies target the same molecule42. Pembrolizumab and nivolumab partially share epitopes and three-dimensional (3D) space when binding to PD-1, implying that the mechanisms of antagonism of the two antibodies are highly similar (Supplementary Fig. 4). These results are consistent with earlier clinical data showing that the response rate for both drugs appears similar26, thus, little synergistic therapeutic effect should be expected from a combination of only these two drugs.

CTLA-4 exists as a constitutive homodimer generated by an intermolecular disulphide bond43 (Fig. 8a). Although a single CTLA-4/tremelimumab Fab complex exists in an asymmetric unit, a crystallographic two-fold symmetry generates a homodimeric interface of CTLA-4, which is essentially identical.
to that observed in the structures of free CTLA-4 or CTLA-4 in complex with B7-1, B7-2, and engineered lipocalin 2 (refs 28,29,38,44), implying that tremelimumab binding does not affect the homodimerization of CTLA-4 (Fig. 8b). In the crystal structures of CTLA-4 in complex with B7-1 or B7-2, the crystal packing generates an alternating periodic arrangement in which bivalent CTLA-4 homodimers connect bivalent B7-1/2 homodimers, providing a model describing the assembly of CTLA-4 and B7-1/2 at the interface between a T-cell and cancer cell28,29 (Fig. 8c). In this model, the CTLA-4/B7 complex is expected to span a distance of \( \sim 140 \) Å, which includes the stalk regions connecting these molecules to the opposing cell membranes. This distance is compatible with other ligand–receptor interactions at the central zone of the immunological synapse, suggesting that this oligomeric arrangement of CTLA-4/B7 complex can facilitate the efficient use of low-abundance CTLA-4 at the T-cell surface, thereby promoting the inhibitory signalling of CTLA-4 and decreasing the local concentration of CD28 through simple steric crowding28,29,45. As tremelimumab is an IgG2 antibody, it is capable of binding two CTLA-4 molecules with high avidity through Y-shaped bivalent presentation; the dimension of the CTLA-4/tremelimumab complex is estimated to span 150–190 Å. This distance is incompatible with the oligomeric array of the CTLA-4/B7 complex (Fig. 8d). Therefore, in addition to simple antagonism of the interaction between CTLA-4 and B7-1/2, tremelimumab binding could prevent or disrupt the uniquely organized assembly of the CTLA-4/B7 complex within the immunological synapse.

Despite the clinical success of checkpoint-blocking antibodies, biologics such as therapeutic antibodies have several shortcomings, including the high cost, limited half-life and immunogenicity caused by multiple dosages. As an alternative treatment strategy to overcome the drawbacks of passive antibody-based therapy, several small-molecule immunomodulators targeting the PD-1/PD-L1 axis have been reported46–50. While most of them are based on peptidomimetics, a recent structural study with the first nonpeptidic chemical inhibitor of the PD-1/PD-L1 interaction revealed that this compound binds to PD-L1 and induces dimerization of PD-L1 (ref. 51). Interestingly, the complex structure of CTLA-4/tremelimumab Fab provided insight for the design of a small-molecule modulator that interferes with the CTLA-4/B7 signal pathway. Nine of ten residues in the HCDR3 loop (residues 101–110 of the heavy chain) of tremelimumab are involved in the interaction with CTLA-4 (Fig. 7a). In addition, the binding site of...
the HCDR3 loop overlaps part of the binding site of B7-1/2 on the surface of CTLA-4 (Fig. 9a). Additionally, heavyR101 and heavyY110 are located closely enough to be connected directly by a peptide bond (Fig. 9b). This extensive interaction and the structural features of the HCDR3 loop in the CTLA-4/tremelimumab complex suggest that cyclic peptides based on the HCDR3 sequence may also prevent binding of B7-1/2 to CTLA-4 (Fig. 9c). As the HCDR3 loop conformation in the complex is essentially identical to that of tremelimumab Fab alone, other CDRs and framework regions of tremelimumab likely contribute to the conformation of HCDR3, implying that antibody context is important for the proper display of the tremelimumab HCDR3 (Supplementary Fig. 1c). If this is indeed the case, a cyclic peptide approach would not be so promising because of its intrinsic conformational flexibility. Nonetheless, a cyclic peptide or peptidomimetic approach based on the HCDR3 sequence in combination with the power of phage-display library technology may facilitate the discovery of small-molecule modulators of the CTLA-4/B7 interaction, providing an alternative treatment strategy to overcome the drawbacks of antibody-based drugs.

In summary, the elucidation of the crystal structures of the checkpoint molecules PD-1, PD-L1 and CTLA-4 in complex with the Fab fragments of therapeutic antibodies sheds light on the molecular mechanisms underlying the therapeutic activity of these monoclonal antibodies. In addition, the precise epitopes revealed by the present complex structures should provide useful information for the improvement of current therapeutic antibodies and development of more effective immunotherapeutic combination strategies and small-molecule modulators that can attenuate checkpoint signalling.

**Methods**

**Expression and purification of checkpoint proteins.** Genes encoding the ectodomains of human PD-1 (aa 26–150), human PD-L1 (aa 18–134) and human CTLA-4 (aa 1–126) were subcloned into pET-21a (Novagen), respectively. A Cys to Ser mutation was introduced at position 93 of PD-1 to aid expression and folding. All proteins were expressed in *E. coli* BL21(DE3) as inclusion bodies. The cells were
between a T-cell and cancer cell, imposing an intercellular distance of 
arrangement of bivalent dimers of CTLA-4 and B7-1 at the interface 
cyan) in the crystal. (c) Suggested model for an alternating periodic 
arrangement of bivalent dimers of CTLA-4 and B7-1 at the interface 
between a T-cell and cancer cell, imposing an intercellular distance of 
~140 Å. (d) Suggested model for the bivalent interaction of 
tremelimumab with CTLA-4. Tremelimumab binds two CTLA-4 molecules in 
the vicinity of the two binding site of IgG, spanning 150–190 Å in the 
perpendicular direction from the T-cell membrane.

Figure 8 | Hypothetical model for prevention of an alternating 
arrangement of bivalent dimers of CTLA-4 and B7-1/2 by tremelimumab 
binding. (a) Structure of CTLA-4 dimer (purple) binding to two B7-1 
molecules (green) (PDB code 1i8l). (b) Structure of CTLA-4 dimer (purple) 
binding to two tremelimumab Fab fragments (heavy chain: blue, light chain: 
cyan) in the crystal. (c) Suggested model for an alternating periodic 
arrangement of bivalent dimers of CTLA-4 and B7-1 at the interface 
between a T-cell and cancer cell, imposing an intercellular distance of 
~140 Å. (d) Suggested model for the bivalent interaction of 
tremelimumab with CTLA-4. Tremelimumab binds two CTLA-4 molecules in 
the vicinity of the two binding site of IgG, spanning 150–190 Å in the 
perpendicular direction from the T-cell membrane.

Figure 9 | Suggestion of a cyclic peptide as a modulator of the CTLA-4/B7 
interaction. (a) Surface representations of the CTLA-4 molecule in the 
CTLA-4/B7-1 complex (left, PDB code 1i8l). The B7-1 binding site on the 
surface of CTLA-4 is coloured orange. Surface representations of CTLA-4 in 
the complex structures of CTLA-4/tremelimumab (right). The HCDR3 loop 
binding site on the surface of CTLA-4 is coloured blue. The overlapping 
region between the binding sites of B7-1 and HCDR3 is coloured red on the 
surface of CTLA-4 (centre). (b) Stereoview of the HCDR3 loop in the 
structure of CTLA-4/tremelimumab. The dashed line suggests a peptide 
bond between R101 and Y110 for generating a cyclic peptide. (c) Stereoview of 
a hypothetical model of the interaction between CTLA-4 (electrostatic 
surface representation) and the cyclic peptide based on the HCDR3 
sequence of tremelimumab (yellow stick). The cyclic peptide was generated 
by connecting R101 and Y110 of the HCDR3 sequence. The cyclic peptide 
was then eluted with elution buffer (20 mM Tris, pH 8.0, 200 mM NaCl) by 
sonication overnight. After removing undissolved residue by centrifugation (25,000 g 
for 0.5 h at 4°C), the supernatant containing soluble protein was applied to HisTrap HP column (GE 
Healthcare Life Sciences) and washed with five column volumes of wash buffer 
(8 M urea, 20 mM Tris, pH 8.0, 200 mM NaCl, 50 mM imidazole). The protein was 
then eluted with elution buffer (8 M urea, 20 mM Tris, pH 8.0, 200 mM NaCl, 
400 mM imidazole). The eluted protein was refolded by dialysis three times 
against 20 mM Tris, pH 8.0, 200 mM NaCl and purified further by gel filtration 
chromatography using a HiLoad 16/60 Superdex 200 pg column (GE Healthcare 
Life Sciences). The protein purity was evaluated by reducing and nonreducing SDS–PAGE.

Expression and purification of Fab fragments. The DNA sequences for the Fab 
fragments of pembrolizumab, nivolumab, BMS-936559 and tremelimumab were 
synthesized after codon-optimization for expression in E. coli (Bioneer, Inc). The 
sequences for heavy chain and light chain were cloned into a modified pBAD 
vector, containing the STII signal sequence in each chain for periplasmic secretion and a C-terminal 6His-tag in heavy chain. The plasmid pBAD-Fab was trans- 
formed into E. coli Top10F (Invitrogen). The cells were grown at 37°C in LB 
medium supplemented with 50 μg ml⁻¹ ampicillin until OD₆₀₀ reached 0.6–1.0, and the protein expression was induced with 1 mM IPTG and incubated for 4 h at 37°C. The cells were harvested by centrifugation, 
re-suspended in lysis buffer (20 mM Tris, pH 8.0, 200 mM NaCl) and lysed by 
sonication on ice. Inclusion bodies were recovered by centrifugation (25,000g 
for 0.5 h at 4°C) and solubilized in 8 M urea, 20 mM Tris, pH 8.0, 200 mM NaCl by 
sonication overnight. After removing undissolved residue by centrifugation (25,000g 
for 0.5 h at 4°C), the supernatant containing soluble protein was applied to HisTrap HP column (GE 
Healthcare Life Sciences) and washed with five column volumes of wash buffer 
(8 M urea, 20 mM Tris, pH 8.0, 200 mM NaCl, 50 mM imidazole). The protein was 
then eluted with elution buffer (20 mM Tris, pH 8.0, 200 mM NaCl) and lysed by sonication on ice. After removing cell 
debris by centrifugation (25,000g for 0.5 h at 4°C), the supernatant containing 
soluble protein was applied to HisTrap HP column (GE Healthcare Life Sciences) 
and washed with five column volumes of wash buffer (20 mM Tris, pH 8.0, 
300 mM NaCl, 50 mM imidazole). The protein was then eluted with elution buffer 
(20 mM Tris, pH 8.0, 300 mM NaCl, 400 mM imidazole). The eluted protein was 
concentrated for gel filtration chromatography using a HiLoad 16/60 Superdex 
200 pg column (GE Healthcare Life Sciences). The column had previously been 
equilibrated with gel filtration buffer (20 mM Tris, pH 8.0, 300 mM NaCl). The 
elution profile of the protein showed a single major peak and the protein quality 
was evaluated by reducing and nonreducing SDS–PAGE.
Crystallization and structure determination. Details of the crystallization and structure determination of the nivolumab Fab, tremelimumab Fab, PD-1/pembrolizumab Fab complex, PD-1/nivolumab Fab complex, PD-L1/BMS-936559 Fab complex, CTLA-4/tremelimumab Fab complex are described in Supplementary Methods. Data collection and refinement statistics are summarized in Table 1.

Data availability. The atomic coordinates and structure factors have been deposited in the Protein Data Bank under accession codes 5GGQ, 5GGR, 5GGS, 5GGT, 5GGU and 5GGV. All additional experimental data are available from the corresponding author on request.

Table 1 | Data collection and refinement statistics.

|                          | Nivolumab Fab | Tremelimumab Fab | Pembrolizumab/PD-1 | Nivolumab/PD-1 | BMS-936559/PD-1 | Tremelimumab/CTLA-4 |
|--------------------------|---------------|------------------|--------------------|----------------|-----------------|-------------------|
| Data collection          |               |                  |                    |                |                 |                   |
| X-ray source             | PLS 7A        | PLS 5C           | PLS 7A             | PLS 7A        | PLS 7A          | PLS 5C            |
| Wavelength (Å)           | 1.0000        | 1.0000           | 1.0000             | 1.0000        | 1.0000          | 1.0000            |
| Space group              | P 65 2 2     | C 2 2 2 z       | P 1                | P 2 z         | P 2 z           | P 2 z 2 z         |
| Cell dimensions          | 88.34         | 98.69            | 54.17              | 91.29          | 37.20           | 131.56            |
| a, b, c (Å)              | 215.24        | 184.75           | 104.04             | 134.90         | 164.16          | 118.71            |
| Resolution (Å)           | (1.90)        | (2.30)           | (2.00)             | (3.30)         | (2.80)          | (2.00)            |
| Rmerge (%)               | 8.4 (49.7)    | 10.5 (48.3)      | 5.1 (34.8)         | 11.6 (40.8)    | 9.5 (46.2)      | 7.2 (46.2)        |
| completeness (%)         | 99.8 (99.9)   | 99.8 (98.2)      | 95.2 (95.8)        | 99.0 (98.1)    | 97.3 (97.0)     | 99.3 (98.9)       |
| Redundancy               | 17.2 (10.5)   | 6.1 (3.9)        | 1.9 (1.9)          | 3.4 (3.1)      | 5.9 (5.8)       | 6.6 (6.6)         |
| Refinement               |               |                  |                    |                |                 |                   |
| Resolution (Å)           | 1.90           | 2.30             | 2.00               | 3.30           | 2.80            | 2.00              |
| No. reflections          | 39971          | 42405            | 71844              | 17553          | 14130           | 51708             |
| Rwork/Rfree (%)          | 19.5/22.6     | 18.5/24.3        | 17.9/22.8          | 22.2/26.8      | 21.1/26.9       | 18.1/21.4         |
| No. atoms                |               |                  |                    |                |                 |                   |
| Protein                  | 3187           | 6602             | 8443               | 8256           | 4155            | 4232              |
| Water                    | 311            | 347              | 796                | 0              | 0               | 432               |
| B-factors (Å²)           | 32.8           | 32.1             | 30.7               | 44.5           | 47.8            | 38.7              |
| R.m.s. deviation         | 0.007          | 0.009            | 0.008              | 0.007          | 0.005           | 0.007             |
| Bond lengths (Å)         | 1.152          | 1.222            | 1.165              | 1.286          | 1.058           | 1.081             |
| Bond angles (°)          | 95.91          | 96.82            | 97.33              | 91.85          | 94.76           | 97.46             |
| Ramachandran Favorable   | 3.37           | 3.18             | 2.30               | 7.49           | 4.86            | 2.18              |
| Allowed (%)              | 0.72           | 0.00             | 0.37               | 0.66           | 0.37            | 0.36              |
| Outlier (%)              | 5GGQ           | 5GGU             | 5GGS               | 5GGT           | 5GGV            |                   |

*Values in parentheses are for the outer resolution shell.

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J.Y.L., H.T.L, W.S. and Y.-S.H. designed the experiments. J.Y.L, H.T.L., W.S., J. Chae, J. Choi, S.H.K., H.L., T.W.H., K.Y.P., Y.J.L., S.E.R., J.Y.S. and J.U.L. performed the experiments including expression, purification and crystallization. J.Y.L., H.T.L and W.S. collected the X-ray data, determined and analysed the structures under the guidance of Y.-S.H. Y.-S.H. wrote the paper. All authors discussed the results and commented on the manuscript.

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