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LETTER TO THE EDITOR

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Structural basis for blocking PD-1-mediated immune suppression by therapeutic antibody pembrolizumab

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Dear Editor,

PD-1 is a type I immune inhibitory transmembrane receptor of the CD28 family that modulates the activity of T cells in peripheral tissues [1]. It is expressed in T cells, B cells, monocytes, natural killer cells and many tumor-infiltrating lymphocytes [2]. Binding of PD-1 to its ligands PD-L1 and PD-L2 reduces T-cell activity [3]. Thereby, under normal conditions, the interaction of PD-1 with PD-L1 or PD-L2 prevents excessive lymphocyte activation and maintains immune tolerance to self-antigens by negatively regulating the immune response [3]. However, PD-L1 is often overexpressed in different tumors including lymphoma, melanoma, non-small-cell lung cancer and other types of cancer [2]. As a result, tumor cells attenuate T-cell signaling to evade immune surveillance [4]. Blocking PD-1/PD-L1 interaction has been shown to restore T-cell activation and antitumor response, providing the rationale for therapeutic intervention using PD-1/PD-L1 as target [5]. Currently two monoclonal antibody-based drugs targeting PD-1 are in clinical trials. One is nivolumab or Opdivo from Bristol-Myers Squibb. The other is pembrolizumab or Keytruda, a therapeutic IgG4 antibody developed by Merck.

Crystal structures of mouse PD-1 (mPD-1) in complex with human PD-L1 (hPD-L1), mPD-1 complexed with mouse PD-L2 (mPD-L2) and human PD-1 (hPD-1) in complex with hPD-L1 have revealed the structural basis of PD-1's interaction with its ligands [6-8]. Crystal structure of the full-length pembrolizumab was also reported recently [9]. However, how pembrolizumab specifically recognizes hPD-1 is still unknown. Herein, we report the crystal structure of pembrolizumab Fab (antigen-binding fragment) in complex with hPD-1, revealing the molecular basis for the blockade of hPD-1/hPD-L1 interaction by pembrolizumab.

Crystal structure of the hPD-1/pembrolizumab Fab complex (hPD-1/Fab) was determined at a resolution of 2.9 Å (Supplementary information, Table S1). hPD-1 and pembrolizumab Fab form a 1:1 complex (Figure 1A), consistent with the stoichiometry determined by previous results [10]. hPD-1 is made up of a canonical β-sandwich immunoglobulin variable (IgV) topology with a disulfide bond between Cys^{54} and Cys^{123}. Structural comparison of hPD-1 with apo-hPD-1 (PDB: 3RRQ) and hPD-1 structure extracted from the hPD-1/hPD-L1 complex (PDB: 4ZQK) shows that hPD-1 in the hPD-1/Fab complex resembles the conformation observed in the hPD-1/hPD-L1 complex. The pembrolizumab Fab in the complex exhibits a canonical β-sandwich immunoglobulin fold closely resembling the full-length pembrolizumab antibody (Supplementary information, Figure S1) [9].

The interaction of PD-1 with pembrolizumab Fab buries ~1 774 $Å^2$ surface area, and the hPD-1/Fab interface can be divided into two sub-interfaces. Sub-interface I mainly encompasses the C′D loop of hPD-1 and pembrolizumab Fab's complementary determining regions (CDRs) L1, L3, H2 and four β-strands of framework region (FR), which interact through polar, charged and hydrophobic contacts (Figure 1B). The most notable feature of this sub-interface is that the C′D loop of hPD-1 protrudes into a groove formed by the CDRs and FR of pembrolizumab Fab. Specifically, Asp^{85} of hPD-1 establishes a salt bridge with Arg^{H99} of FR (hereafter residues of the Fab light chain and heavy chain are designated by superscript chain identifiers L and H, respectively). The side chain of Ser^{87} forms hydrogen bond with Arg^{H99} of FR. Interestingly, two arginines Arg^{86} and Arg^{L96} are involved in a T-shaped stacking interaction. The backbone of C′D loop residues Glu^{84}, Ser^{87}, Gln^{88} and Gly^{90} are held in place by hydrogen bonds with side chains of Tyr^{L36}, Tyr^{H35}, Asn^{H59} and Thr^{H58}, respectively. Furthermore, Pro^{89} of hPD-1 inserts into a cavity formed by side chains of Tyr^{H33}, Tyr^{H35}, Asn^{H52} and Asn^{H59} of β-stands 1, 2 and 3, and the main chains of Gly^{H50}, Ile^{H51}, Gly^{H57} and Thr^{H58} of β-stands 1 and 2.

Sub-interface II is dominated by hydrophilic interactions and brings together residues in the C, C′ and F strands of hPD-1 and CDRs L1 and H3 of Fab (Figure 1C). The side chains of Asn^{66} and Lys^{78} of hPD-1 form hydrogen bonds with the backbone groups of Arg^{H102} and Tyr^{H101}, respectively. The side chain of Thr^{76} of hPD-1 is

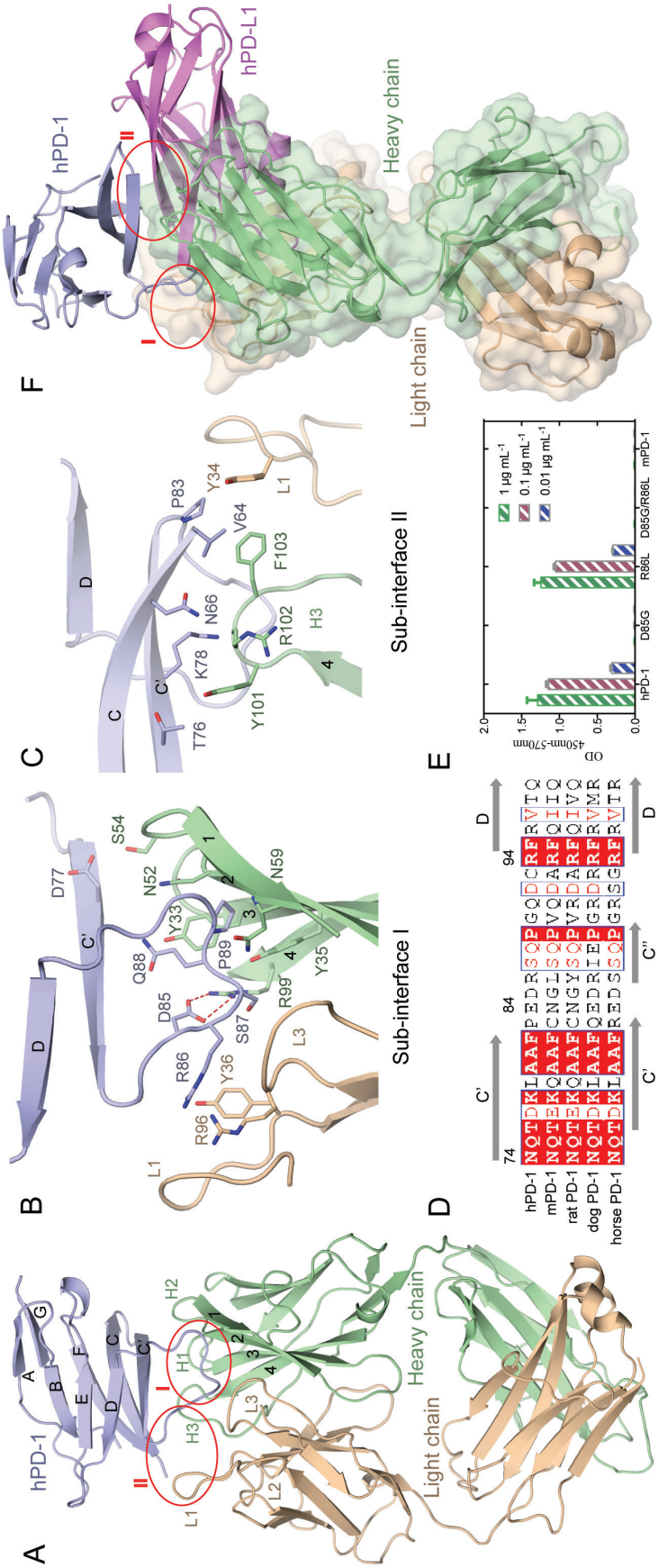

Figure 1 Structural basis for the blockade of hPD-1/hPD-L1 interaction by pembrolizumab. **(A)** Overall structure of the hPD-1/pembrolizumab Fab complex. hPD-1 is shown in light blue, and the light and heavy chains of Fab are in wheat and pale green, respectively. The CDR loops and the β-strands of pembrolizumab that are involved in interactions are labeled. **(B)** View of sub-interface I in hPD-1/pembrolizumab Fab complex. Residues involved in the interaction are shown as sticks and labeled. Hydrogen bonds are shown in dash lines. **(C)** View of sub-interface II in hPD-1/pembrolizumab Fab complex. **(D)** Sequence alignment of the C'D loop in ecto-domains of PD-1. Secondary structural elements of hPD-1 are shown on top of the alignment while those of mPD-1 are shown at the bottom. **(E)** ELISA data showing the binding of pembrolizumab to hPD-1 or hPD-1 mutants, and mPD-1. **(F)** Superposition of the hPD-1/pembrolizumab Fab complex with hPD-1/hPD-L1. hPD-L1 is shown in magenta. For simplicity, only hPD-1 in hPD-1/pembrolizumab Fab is shown in light blue.

hydrogen bonded to the side chain of Tyr^{H101}. In addition, Phe^{H103} of CDR H3 inserts into a hydrophobic pocket formed by Val^{64} and Pro^{83} of hPD-1 and Tyr^{L34} of CDR L1.

The extensive interactions at the hPD-1/Fab interface are consistent with the high binding affinity of pembrolizumab to the hPD-1, with an apparent disassociation constant (K_D) of 27 pM. In the previously published hPD-1 structures (PDB: 3RRQ and 4ZQK), the C′D loop of hPD-1 is disordered and is assumed to be highly flexible [8, 10]. However, the C′D loop of hPD-1 in our hPD-1/Fab complex structure is well ordered, as evidenced by its well-defined electron density, and it contributes to sub-interface I with Fab. Although mPD-1 and hPD-1 share 60% sequence identity and an IgV topology, hPD-1 lacks the additional C″ strand observed in mPD-1 (Figure 1D). Moreover, Asp^{85} and Arg^{86} in hPD-1 are substituted by Gly^{85} and Leu^{86} in mPD-1, respectively. Mutations in hPD-1, D85G and R86L show significant differences in their binding affinities with pembrolizumab. D85G abolishes hPD-1 binding to pembrolizumab as determined by ELISA (Figure 1E). This can be attributed to the disruption of the salt bridge with Arg^{H99} of Fab, which can conceivably impair the PD-1/Fab complex assembly. However, R86L did not affect the binding affinity between hPD-1 and pembrolizumab. These results are consistent with earlier data showing that pembrolizumab displays low binding affinity toward mPD-1 (Patent: WC500190992).

Structural superposition of the hPD-1/pembrolizumab Fab complex and the hPD-1/hPD-L1 complex shows that pembrolizumab Fab and hPD-L1 interact with hPD-1 through overlapping surface regions, suggesting that pembrolizumab and hPD-L1 can exclude each other from binding to hPD-1 (Figure 1F). Although the C′D loop in the sub-interface I contributes predominantly to the binding affinity of permbrolizumab, the C′D loop is disordered in the hPD-1/hPD-L1 complex, suggesting that it is not important for the hPD-1/hPD-L1 interaction. Consistent with this observation, the overlapping regions are mainly located in the sub-interface II, where the antigen-binding site of permbrolizumab Fab largely overlaps with the regions of hPD-L1 that interact with hPD-1.

A second ligand for PD-1 is PD-L2, which shares 34% sequence identity with PD-L1 and exhibits 3-fold higher binding affinity for PD-1 [7]. Given that mPD-L2 and hPD-L2 share a sequence identity of 72%, we modeled the hPD-1/hPD-L2 complex based on the structures of hPD-1/hPD-L1 and mPD-1/mPD-L2. Structural superposition of hPD-1/pembrolizumab Fab complex and modeled hPD-1/hPD-L2 complex suggest that pembrolizumab Fab would also compete with hPD-L2 for binding to hPD-1 (Supplementary information, Figure S2) through overlapping regions similar to those observed between hPD-1/pembrolizumab Fab and hPD-1/hPD-L1. Taken together, these observations suggest a mechanism by which pembrolizumab outcompetes PD-L1 or PD-L2 for binding to hPD-1.

In summary, we have reported the crystal structure of the pembrolizumab Fab in complex with the ectodomain of hPD-1. Pembrolizumab Fab uses its CDRs and FR to interact with the C′D loop of hPD-1, which appears unstructured in previously published reports. The epitope consists of several discontinuous segments of hPD-1, which overlap with the region that interacts with hPD-L1 or hPD-L2, suggesting a mechanism by which pembrolizumab prevents the binding of hPD-L1 or hPD-L2 to hPD-1. These results have implications for the design and improvement of mAb drugs targeting hPD-1.

The atomic coordinates and structure factors for hPD-1/pembrolizumab Fab complex structure have been deposited into Protein Data Bank under the accession code of 5JXE. Additional details of the methods are described in Supplementary information, Data S1.

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(**Supplementary information** is linked to the online version of the paper on the *Cell Research* website.)