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Mutant variants of PD-1 receptor with selective binding to PD-L1 and uses thereof

Abstract

Disclosed are human Programmed Cell Death-1 (PD-1) receptor mutants having selectivity for PD-L1 compared to PD-L2, methods of obtaining the mutants, and uses of the mutants for treatment and imaging.

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Background/Summary

CROSS-REFERENCE TO RELATED APPLICATION (1) This application claims the benefit of U.S. Provisional Patent Application No. 62/736,477, filed on Sep. 26, 2018, the content of which is herein incorporated by reference in its entirety.

BACKGROUND OF THE INVENTION

(1) Throughout this application various publications are referred to in parentheses. Full citations for these references may be found at the end of the specification. The disclosures of these publications are hereby incorporated by reference in their entirety into the subject application to more fully describe the art to which the subject invention pertains.

(2) Lymphocyte activation requires two signals mediated by protein-protein interactions: the T-cell receptor (TCR) interaction with antigenic peptide/MHC complex that provides the specificity of the immune response and an antigen-independent co-modulatory signal that modulates T-cell clonal expansion and acquisition of effector function (1). Engagement of co-stimulatory receptors such as CD28 and ICOS with their cognate B7 family ligands expressed on antigen presenting cells results in T cell activation and proliferation. In contrast, engagement of cytotoxic T lymphocyte associate antigen (CTLA-4), programmed cell death protein (PD-1), and B- and T-lymphocyte attenuator (BTLA) with their corresponding ligands negatively regulate T cell activation (2).

(3) PD-1 is a type I transmembrane protein, composed of an extracellular immunoglobulin variable (IgV) domain, a transmembrane domain, and an intracellular domain with tyrosine-based signaling motifs (3). The PD-1 ligands, PD-L1 (4) and PD-L2 (5), are also type I transmembrane proteins, and possess ectodomains composed of tandem IgV and immunoglobulin constant (IgC) domains, a single pass helical transmembrane region and a cytoplasmic domain (6, 7). T cells, natural killer T cells, B cells, and some myeloid cells express PD-1 receptor (6). PD-L1 is constitutively expressed on mouse immune cells such as T cells, B cells and dendritic cells (DCs) (8). Nonhematopoietic cells can also express PD-1, with expression levels depending on a variety of other stimuli (6). Expression of PD-L2 is limited to DCs, macrophages, and bone marrow-derived mast cells (5).

(4) Engagement of PD-1 with either of its two known ligands, PD-L1 (B7-H1) or PD-L2 (B7-DC) from the B7 family (9), provides inhibitory signals that control autoimmune responses by maintaining immune tolerance to self-antigens (10). PD-1 signaling also makes important contributions to responses against microbial pathogens. Persistent PD-1 expression can cause T cell exhaustion that reduces anti-viral and anti-tumor immune responses, and leads to unfavorable disease progression by inhibiting T-cell proliferation and cytotoxicity, as well as cytokine production (11). Blockade of the PD-1 pathway can reverse T-cell exhaustion and enhance anti-tumor responses (12). Malignant cells utilize a range of mechanisms to evade host immune surveillance, including the overexpression of PD-L1 on their surface, which can result in a highly immune-suppressive milieu in the tumor microenvironment (13, 14). Therapeutic agents, especially monoclonal antibodies (mAbs), have been developed to block the PD-1 signaling pathways (15) and have shown activity against several cancers, with FDA approval for multiple indications (16, 17). Among these, nivolumab and pembrolizumab have shown clinical efficacy for the treatment of metastatic melanoma, non-small cell lung cancer and other malignancies (18). Similarly, re-invigorating T cells through PD-1 blockade can elicit beneficial immune responses in chronic viral infections (19-21). In order to further increase potency, engineered T cells have been combined with other therapeutics targeting the PD-1 signaling pathway (22, 23). However, mAbs have inherent limitations such as antigenicity, poor tissue penetrance due to their large size (˜150 kDa) and detrimental Fc-effector functions that deplete immune cells (24). As a complementary approach to mAbs, PD-1 ectodomains and their engineered variants could be used to interrupt the PD-1 pathway by directly binding to the PD-ligands. Directed evolution via yeast-surface display has been used to engineer a PD-1 variant that specifically antagonizes PD-L1. The resulting PD-1 construct had 10 residues mutated compared to wild type PD-1, resulting in a 15-40 thousand fold increase affinity to PD-L1, while not binding to PD-L2 (25). While possessing remarkable affinity and selectivity, the large number of altered residues could result in undesirable antigenic properties, making variants with the smallest number of mutations desirable. In a recent study, a cross-reactive single mutant (A132L) in PD-1 was reported to enhance binding activity to PD-L1 and PD-L2 by

45- and 30-fold, respectively, compared with wild type PD-1 (26).
(5) Computational protein design methods generate and assess large numbers of sequence variants at predetermined binding surfaces. These approaches dramatically reduce the number of designs for subsequent experimental evaluation (27, 28). Computational design algorithms have been used to design new folds (29), enzymatic functions (30), and novel binding functions (31). Computational approaches for optimizing selectivity often require both positive design considerations to stabilize the desired interactions and negative design considerations to distinguish among a number of sequence and structurally similar competitor molecules (32, 33).
(6) In the context of PD-1 pathway modulation, our goal is to computationally redesign the ligand recognition surface of PD-1 to antagonize the PD-1:PD-L2 interaction, while maintaining or enhancing the affinity of the PD-1:PD-L1 interaction. We utilized our recently developed ProtLID (Protein Ligand Interface Design) method to predict residue-based pharmacophore (rs-pharmacophore) signatures over the binding surface for PD-L1 and PD-L2 (34). We then compared these rs-pharmacophores with the known binding interfaces of PD-1 predicted positions and residue types to alter specificities. In subsequent cell-based assays, we validated a number of these designs. Half of all predicted single mutant PD-1 designs exhibited statistically significantly reduced interaction with PD-L2, nine of which maintained close to wild type interaction with PD-L1, and among these, three designs showed no detectable affinity to PD-L2. These new constructs are both reagents and promising drug leads for modulation of the PD-1 pathway.
(7) The present invention addresses the need for PD-1 receptor mutants having selectivity for PD-LI compared to PD-L2 that can be used for imaging and treatments.
SUMMARY OF THE INVENTION
(8) The present invention provides human Programmed Cell Death-1 (PD-1) receptor mutants comprising mutation N66Q, Y68N, Y68K, Y68R, Y68Q, S73R, Q75N, T76D, T76E, D77E, K78R, G124V, L128V, K131H, I134N or I134F, wherein the mutation is relative to the amino acid sequence set forth in SEQ ID NO:1 or in NCBI Reference Sequence NP 005009.2, and wherein the mutant has selectivity for PD-LI compared to PD-L2. Preferably, the mutant corresponds to a mutated version of at least the first 149 amino acids of SEQ ID NO:1.
(9) Also provided are methods of obtaining the mutants and uses of the mutants for imaging and treating tumors and infections.

Description

BRIEF DESCRIPTION OF THE DRAWINGS
(1) FIG. **1**A-**1**C. Sequence alignments derived from DALI(38) structural superpositions. Pairwise alignment between orthologs of PD1: (A) human and mouse PD-1 (hPD-1 (SEQ ID NO:2) and mPD-1 (SEQ ID NO:3)), and (B) human and mouse PD-L2 (hPD-L2 (SEQ ID NO:4) and mPD-L2 (SEQ ID NO:5)). (C) Alignment between Ig-like variable-type domain of hPD-L1 (SEQ ID NO:6) and hPD-L2 (SEQ ID NO:7). '*' indicates conserved interface residues; '−' refers non-conserved interface residues; '+' indicates a conserved residue that is not part of the interface in mPD-1. All structures and homology models were energetically relaxed in a 25 ns long molecular dynamics simulation prior to superposition. The amino acid numbering refers to the first sequence within each pairwise alignment.
(2) FIG. **2**A-**2**C. Experimental testing of selectivity of PD-1 mutants. (A) Flow cytometric analysis of hPD-1 mutants binding to PD-L1 and PD-L2. A set of 32 human PD-1 mutants were cloned and transiently expressed in suspension HEK 293 cells. Cells were challenged with 0.25 μg recombinant hPD-L1 or hPD-L2 hIgG1 Fc-fusion protein and detected with goat anti-human Alexa 488 secondary antibody. Flow cytometric analysis was used to determine the percent of PD-1 positive cells bound to PD-L1 or PD-L2. The data represents the average of three independent

experiments with error bars showing the standard deviation. (B, C) Titration of hPD-1 mutants shows selective binding to PD-L1. (B) HEK 293 suspension cells transiently expressing WT or mutant PD-1 were challenged with increasing concentrations of hPD-L1 hIgG1 (0.1-200 nM) and FACS analysis was used to determine the percent of PD-1 expressing cells (mCherry) bound to PD-L1 (Alexa 488). For each experiment, the titration data was normalized to wild-type PD-1 binding to the highest concentration of PD-L1 (200 nM). Normalized data was plotted in Graphpad Prism software and EC50s were estimated using the three parameter dose response non-linear regression analysis Y=B.sub.min+(B.sub.max−B.sub.min)/(1+10{circumflex over ( )}((Log EC50-X))). Data shown represents four independent experiments with standard deviation. (B) The same analysis as shown in (B) except titrating hPD-L2 hIgG1 protein. EC50s could not be estimated for Y68R and Y68K (N/A) as no detectable binding was observed within the range of concentrations analyzed.

(3) FIG. **3**A-**3**C. Impact of single point mutations. (A) BeAtMuSiC (1), (B) FOLDX (2), and (C) MutaBind (3) results for assessing the impact of single point mutations. The first column in each pair represents PD-1(PD-1/PD-L1); the second column in each pair represents PD-1(PD-1/PD-L2).

DETAILED DESCRIPTION OF THE INVENTION

(4) The present invention provides a human Programmed Cell Death-1 (PD-1) receptor mutant comprising at least the first 149 consecutive amino acids of SEQ ID NO:1 with mutation N66Q, Y68N, Y68K, Y68R, Y68Q, S73R, Q75N, T76D, T76E, D77E, K78R, G124V, L128V, K131H, I134N or I134F, wherein the mutant has selectivity for PD-LI compared to PD-L2.

(5) The first 149 amino acids of SEQ ID NO:1 covers the soluble first ectodomain of PD1 mutants, i.e. what is shown in the alignment FIG. **1**, the first 149 residiues from the N-terminal or the first IG-V domain (IGV-PD1).

(6) In an embodiment, the mutant comprises or consists of SEQ ID NO:1 with mutation N66Q, Y68N, Y68K, Y68R, Y68Q, S73R, Q75N, T76D, T76E, D77E, K78R, G124V, L128V, K131H, I134N or I134F, wherein the mutant has selectivity for PD-LI compared to PD-L2.

(7) In one embodiment, the mutation is selected from the group consisting of N66Q, Y68N, Y68K, Y68R, Y68Q, S73R, Q75N, T76D, T76E, D77E, K78R, G124V, L128V, K131H, I134N and I134F, wherein the mutant has selectivity for PD-LI compared to PD-L2.

(8) In one embodiment, the mutant is Y68R or Y68K, where the mutant has undetectable PD-L2 binding. In one embodiment, the mutant is Y68N, T76D or T76E, where the mutant has a 4-12 fold selectivity for PD-L1 compared to PD-L2.

(9) In one embodiment, the mutant achieved selectivity by diminishing PD-1 binding to PD-L2.

(10) In a preferred embodiment, the mutation is a single amino acid mutation.

(11) In an embodiment, the un-mutated human full-length PD-1 has the following sequence:

(12) TABLE-US-00001 (SEQ ID NO: 1)

MQIPQAPWPVVWAVLQLGWRPGWFLDSPDRPWNPPTFSPALLVVTEGDN
ATFTCSFSNTSESFVLNWYRMSPSNQTDKLAAFPEDRSQPGQDCRFRVT
QLPNGRDFHMSVVRARRNDSGTYLCGAISLAPKAQIKESLRAELRVTER
RAEVPTAHPSPSPRPAGQFQTLVVGVVGGLLGSLVLLVWVLAVICSRAA
RGTIGARRTGQPLKEDPSAVPVFSVDYGELDFQWREKTPEPPVPCVPEQ
TEYATIVFPSGMGTSSPARRGSADGPRSAQPLRPEDGHCSWPL.

(13) In an embodiment, the mutant is a mutant relative to NCBI Reference Sequence NP_005009.2.

(14) In an embodiment, the mutant comprises the sequence set forth in SEQ ID NO:1 or in NCBI Reference Sequence NP_005009.2, with one of the mutations specified herein. In an embodiment, the mutant consists of the sequence set forth in SEQ ID NO:1 or in NCBI Reference Sequence NP_005009.2, with one of the mutations specified herein.

(15) As described herein, the mutant PD-1 mutant is a not a naturally occurring PD-1 mutant.

(16) The invention further provides a fusion polypeptide comprising any of the PD-1 receptor mutants disclosed herein fused to an immunoglobulin domain polypeptide. Preferably, the mutant is

fused to the immunoglobulin domain polypeptide by a peptide bond between a terminal amino acid of the mutant and a terminal amino acid of the immunoglobulin domain polypeptide. Preferably, the immunoglobulin domain polypeptide comprises an immunoglobulin IgG1 Fc domain. Preferably, the immunoglobulin IgG1 Fc domain is human.

(17) Also provided is a composition comprising any of the PD-1 receptor mutants disclosed herein or any of the fusion polypeptides disclosed herein, and a pharmaceutically acceptable carrier. As used herein, “pharmaceutically acceptable carrier” or “pharmaceutical acceptable excipient” includes any material which, when combined with an active ingredient, allows the ingredient to retain biological activity and is non-reactive with the subject's immune system. Examples include, but are not limited to, any of the standard pharmaceutical carriers such as a phosphate buffered saline solution, water, emulsions such as oil/water emulsion, and various types of wetting agents. Preferred diluents for aerosol or parenteral administration are phosphate buffered saline (PBS) or normal (0.9%) saline. Compositions comprising such carriers are formulated by well known conventional methods (see, for example, Remington's Pharmaceutical Sciences, 18th edition, A. Gennaro, ed., Mack Publishing Co., Easton, PA, 1990; and Remington, The Science and Practice of Pharmacy 20th Ed. Mack Publishing, 2000).

(18) The invention also provides a method of stimulating T cell activation, treating a tumor, or treating an infection in a subject comprising administering to the subject any of the PD-1 receptor mutants disclosed herein or any of the fusion polypeptides disclosed herein, or any of the compositions disclosed herein in an amount effective to stimulate T cell activation, treat a tumor, or treat an infection, respectively, in a subject.

(19) As used herein, to “treat” a tumor means to reduce the size of the tumor or to stabilize the tumor so that it does not increase in size. In an embodiment, the tumor is a tumor of the breast, lung, colon, ovarian, melanoma, bladder, liver, salivary, stomach, gliomas, thyroid, thymus, epithelial, head, or neck. In an embodiment, the tumor is a hematological malignancy. In an embodiment, the tumor is a lymphoma. In an embodiment, the tumor is a myeloma. In an embodiment, the tumor is a multiple myeloma.

(20) As used herein, to “treat” an infection means to reduce a sign or symptom of the infection or to eliminate the infection from the subject. In an embodiment, the infection is a viral Infection. In a further embodiment, the virus is a HIV, HCV, HBV or HTLV. In an embodiment, the infection is a bacterial, fungal, protozoal or parasitic infection. In embodiments, the infection is caused by *Helicobacter pylori*, the fungus *Histoplasma capsulatum*, the parasite *Taenia crassiceps* or *Schistosoma mansoni*, or the protozoa *Leishmania mexicana.*

(21) The invention also provides any of the PD-1 mutants disclosed herein further comprising a radiolabel. Also provided is a method of imaging a PD-L1 positive tumor in a subject comprising administering the radiolabeled PD-1 mutant to the subject, where the mutant binds to the tumor, and imaging the radiolabeled mutant bound to the tumor. Radioisotopes that can be used for radioimmunoimaging include, but are not limited to, 99m-Technetium, 111-Indium, 67-Gallium, 123-Iodine, 124-Iodine, 131-Iodine, 11-Carbon and 18-Fluorine. Imaging modalities that can be used include positron-emission tomography (PET), single-photon emission computed tomography (SPECT), and scintigraphy (gamma scan).

(22) In a preferred embodiment of the methods, the subject is a human.

(23) All combinations of the various elements described herein are within the scope of the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

(24) This invention will be better understood from the Experimental Details, which follow. However, one skilled in the art will readily appreciate that the specific methods and results discussed are merely illustrative of the invention as described more fully in the claims that follow thereafter.

EXPERIMENTAL DETAILS

(25) Introduction

(26) In the context of PD-1 pathway modulation, the present goal was to computationally redesign the ligand recognition surface of PD-1 to antagonize the PD-1:PD-L2 interaction, while maintaining or enhancing the affinity of the PD-1:PD-L1 interaction. We utilized a recently developed ProtLID (Protein Ligand Interface Design) method to predict residue-based pharmacophore (rs-pharmacophore) signatures over the binding surface for PD-L1 and PD-L2 (34). We then compared these rs-pharmacophores with the known binding interfaces of PD-1 predicted positions and residue types to alter specificities. In subsequent cell-based assays, we validated a number of these designs. Half of all predicted single mutant PD-1 designs exhibited statistically significantly reduced interaction with PD-L2, nine of which maintained close to wild type interaction with PD-L1, and among these, three designs showed no detectable affinity to PD-L2. These new constructs are both reagents and promising drug leads for modulation of the PD-1 pathway.

(27) Material and Methods

(28) PD-1, PD-L1, and PD-L2 proteins and their complexes. The crystallographic structures of the human ectodomains of PD-1 and PD-L1 complex (4ZQK) were recently published (35). The hPD-1 and hPD-L2 complex is not available, but the structure of the orthologous mouse mPD-1:mPDL2 (PDB ID: 3BP5) complex has been determined (36). Since hPD-1 and mPD-1 proteins share 64% sequence identity, a computational homology model for the hPD-1:hPD-L2 complex was generated for this study (48-50), using the mPD-1:mPD-L2 complex and hPD1 (5GGS.Y) as the templates.

(29) ProtLID, residue-specific pharmacophore approach for interface design. We recently developed a residue-based pharmacophore (rs-pharmacophore) approach for interface design (ProtLID, Protein Ligand Interface Design) that was used to identify cognate ligand binding partners for given target proteins (34). In this current application, we used ProtLID to generate rs-pharmacophores for the interfaces of ligand proteins (PD-L1 and PD-L2) in order to identify the ideal matching three-dimensional residue pattern signatures (rs-pharmacophore). These rs-pharmacophores were compared with each other and with the wild type receptor interface to identify differences that could be utilized to design ligand-specific single mutant variants of hPD-1. The rs-pharmacophore generation consists the steps indicated below.

(30) Identify interface residues. Interface residues for hPD-L1 (4ZQK.A) and mPD-L2 (3BP5.B) were identified with the CSU program (39): residues interacting between the receptor and ligand were identified if these satisfied CSU classification for legitimate interactions and the nearest atomic distance fell within 4.0 Å (39). Interface residues were required to have at least 1 Å.sup.2 accessible surface area.

(31) Mesh generation over interface residues. A hypothetical mesh was constructed over the interface of the ligands, using a 1 Å distance and probe radius over the solvent accessible region of the interface residues of hPD-L1 and mPD-L2 (51). These mesh points served as starting points for subsequent MD simulations.

(32) Single-residue probe simulation using molecular dynamics. Extensive molecular dynamics (MD) simulations were performed for hPD-L1 and mPD-L2 from each mesh point constructed over their interfaces using AMBER (52) with seven replicas, each with different starting orientations using the 20 amino acid residues as probes. The system was minimized from 5.0 kcal/mol to 0 with 5000 steps using harmonic restraints on heavy atoms and simulated to 30 ps using the Generalized Born implicit solvation model with no periodic boundary condition at 300 K, using Andersen thermal coupling. The system contains either hPD-L1 or mPD-L2, with single-residue probes (uncapped N-terminal N—H and C-terminal C═O). Each probe residue is uniquely defined by one or more side-chain atoms that represent the most characteristic chemical functional group—these were defined as functional atoms (FA). There are 26 FAs to define 18 amino acids (non-specific mainchain interactions were not considered, and consequently neither Gly nor Ala). For example, the amino acid Trp is represented by aromatic ring center (RC_W) and hydrogen-bond donor (NE1_W) (34).

(33) Functional atom preference over the mesh point. The propensity of a FA in the proximity of the mesh point determines residue preferences in various spatial locations. Actual preferences are estimated using the actual to expected (A/E) ratio. The A/E ratio compares the actual FA propensity to the expected FA propensities observed from the similar interaction of all snapshots nearest to the mesh point, and as such implicitly accounts for geometrical artifacts on the molecular surface. In addition, only legitimate molecular interactions were considered such as hydrogen bond acceptor-donor or hydrophobic contacts according to CSU definitions (34).
(34) Match the predicted rs-pharmacophores with the interface residues of PD-1. The calculated rs-pharmacophore for hPD-L1 (4ZQK.A) is then matched with the known residues from the interaction surface of hPD-1 (4ZQK.B) and similarly, the pharmacophores calculated for mPD-L2 (3BP5.B) were matched to mPD-1 (3BP5.A) using CSU (39). When multiple residues are in close proximity, the union of their suggested variants was considered. The rs-pharmacophores generated using hPD-L1 and mPD-L2 (equivalent residues with hPD-L2) were compared with one another and with the observed interface of hPD-1 to select hPD-1 mutants with selectivity for hPD-L1.
(35) Site-directed mutagenesis of human PD-1 variants. The coding sequence for the full-length ectodomain of human PD-1 (Leu 25-Thr 168) was cloned by ligation-independent cloning into a vector that adds the leader sequence from erythropoietin (EPO) and the transmembrane domain from mouse PD-L1 followed by mCherry (hPD-1 Type I mCherry). Site-specific mutagenesis was performed as described previously using high fidelity KOD polymerase (53). After two rounds of primer design, 32 of the 34 predicted mutations were successfully cloned and sequence validated (94% success rate). These mutants were tested for expression by transient transfection of 1 mL suspension HEK 293 cells. Prior to utilization in downstream binding experiments, all of the hPD-1 mutants were shown to express at levels comparable to the parental hPD-1 construct as analyzed by FACS and showed correct membrane localization as observed by fluorescence microscopy.
(36) Analysis of PD-1 variants binding to PD-L1 and PD-L2 by high-throughput flow cytometry. Wild-type and mutant hPD-1 constructs were transiently transfected into 1 mL suspension of HEK 293 cells at a density of 1×10.sup.6 cells/mL in 24-well plates using 0.5 μg plasmid DNA and 2 μg linear PEI. Two days post transfection, cells were counted and diluted to 1×10.sup.6 cells/mL with 1×PBS with 2% BSA. In 96-well V-bottom plates, 100,000 cells were challenged with 0.1 μg of hPD-L1 or hPD-L2 Fc-fusion protein (R&D Systems) for 1 hour at room temperature while shaking in a 96-well plate shaker at 900 rpm. Cells were subsequently pelleted by centrifugation at 500×g and washed with 1×PBS with 2% BSA two times. Goat anti-human Alexa 488 secondary antibody (0.25 μg) was added to the cells and they were incubaed at 4° C. for 45 min. After washing three times, antibody binding was assessed by FACS analysis on a BD Accuri cytometer connected to an Intellicyt Hypercyte auto sampler. Flow data were gated for mCherry positive events (hPD-1 expression) and then sub-gated for hPD-L1/L2 binding (Alexa 488 channel). The experiment was performed in triplicate and the data from each experiment were normalized to wild type PD-1 binding.
(37) For titration experiments, WT and selected PD-1 mutants were transiently transfected as described above. Two days post transfection cells were diluted to 1×10.sup.6 cells/mL with 1×PBS with 2% BSA and 100,000 cells were challenged with increasing concentrations of hPD-L1 hIgG1 or hPD-L2 hIgG1 (R&D Systems) from 0.1-200 nM final concentrations. After binding for 1 hour at room temperature, the cells were washed once with 1×PBS with 2% BSA and goat anti-human Alexa 488 secondary antibody (0.25 μg) was added. After incubation with secondary antibody, the cells were washed twice with 1×PBS with 2% BSA and analyzed by FACS as described above. The percent of PD-1 expressing cells bound to either PD-L1 or PD-L2 was determined and the data normalized to the highest ligand concentration for wild-type PD-1 binding. The EC50s were estimated by plotting the normalized titration data in Graphpad Prism software and fitting the data using the equation for a three-parameter dose response non-linear regression analysis Y=B.sub.min+(B.sub.max−B.sub.min)/(1+10.sup.(LogEC50-X)).

(38) Results
(39) Residue-specific pharmacophore generation. Understanding the intermolecular interactions between PD-1 and its two cognate ligands (PD-L1 and PD-L2) can help selectively block the PD-1 signaling pathway for mechanistic analysis and potentially provide therapeutic leads. We focused on redesigning the human PD-1 (hPD-1) interface to selectively bind to hPD-L1 (positive design), but not to hPD-L2 (negative design), to obtain a selective reagent.
(40) hPD-1 and hPD-L1 contribute 15 and 12 residues, respectively, to the recognition interface (4ZQK) (35) (FIG. **1**, Table 1). As there is no existing structure of the human PD-1:PD-L2 complex, the murine mPD-1:mPD-L2 complex (3BP5) (36) was used as a proxy. Out of the 14 interface residues shared between hPD-1 and mPD-1, 11 residues (N66, S73, Q75, T76, K78, G124, I126, K131, A132, I136, and E136) are identical. In the case of PD-L2, 10 out of 14 interface residues (F21, E28, Q60, S67, I105, W110, D111, Y112, K113, and Y114) are identical between hPD-L2 and mPD-L2 orthologs (FIG. **1**, Table 1). A computational homology model (37) was built for hPD-1:hPD-L2 using hPD1 and mPD-1:mPD-L2 as a template; these orthologs share 64% and 70% overall sequence identity for the entire length of the proteins, respectively. Superposition of the hPD-1 and mPD-1 structures with DALI (38) resulted in C.sub.α-RMSD=1.8 Å over 107 out of 113 residues. Interface residues were identified by the CSU program (39): by this definition, K98 of mPD-1 is not part of the interface of mPD-1:mPD-L2 complex, although K98 of mPD-1 aligns with K131 of hPD-1.
(41) In order to design a PD-L1-specific interface on hPD-1, we used our recently developed computational algorithm, ProtLID, to generate residue-based pharmacophores (rs-pharmacophores) for the hPD-L1 and mPD-L2 interfaces. Rs-pharmacophores are descriptions of idealized complementary interacting surface patches to these ligands, which are obtained through the analysis of single amino acid binding preferences after an extensive molecular dynamics simulation. When the calculated rs-pharmacophore for hPD-L1 is compared with the actual binding residues of hPD-1, 10 out of 15 wild-type residues (67%) were correctly recapitulated (Table 1). Likewise, 11 out of 19 (58%) wild-type binding residues of mPD-1 were recapitulated, of which six (N66, Q75, T76, K78, I126, and E136) were identical to hPD-1 interface residues. Four residues are conserved between the interfaces of hPD-L1 and hPD-L2 (the PD-L2 interface residues were obtained through the comparative model built using the mPD-1:mPD-L2 experimental structure) (Table 1).
(42) Selecting single mutant designs. Differences between the rs-pharmacophores generated for each ligand and the observed interface of wild type hPD-1 suggested residue types and positions to modify for enhanced PD-L1 selectivity (Table 1). One set of design targets consisted of positions where the two ligands (PD-L1 and PD-L2) have different residues interacting with the receptor (PD-1), and there were also differences between the rs-pharmacophores designed for these ligand residues. These differences can be utilized to suggest mutations that selectively prefer only one ligand. For instance, in the case of K133 in hPD-1, the interacting residue in hPD-L1 is Q66, while in both hPD-L2 and mPD-L2 it is S67. The differences between the calculated rs-pharmacophores suggest that hPD-L1 uniquely preferred H and P as interacting partners. After visual inspection of the local structural environment, the K131H variant was selected for testing. Other selections involved cases where both ligands had the same residue type interacting with the receptor, but the calculated rs-pharmacophores, influenced by other residues in the environment, suggested differences in preferences between the two ligands. An example is F19 in hPD-L1 (and the equivalent F21 in mPD-L2), interacting with K78 in hPD-1. The rs-pharmacophore for hPD-L1 had two unique residue preferences, R and T, which are not preferred by PD-L2. After visual inspection, we tested K78R, which in a subsequent cell assay showed selective binding to hPD-L1, relative to hPD-L2 (FIG. **2**.).
(43) Other design elements required more elaboration due to the complex network of interactions between interface residues, which are not readily deconvolved into simple pairwise contacts. These

positions often exhibit “promiscuity”, as they can accommodate a wider range of amino acid substitutions. Once these positions are identified from rs-pharmacophore preferences, they provide a more flexible target environment for exploration. For instance, one of the conserved interface residues is Y123 of hPD-L1, the equivalent of which is Y112 in mPD-L2 (and is also identical in hPD-L2). The calculated rs-pharmacophores for the two ligands suggested similar complementary interacting patches, to accommodate the two “Functional Atoms” of Tyr (aromatic ring center and hydroxyl group). The rs-pharmacophore contains residues that are hydrogen-bond donors or acceptors (DEPNQRHT) (SEQ ID NO:8), hydrophobic (LM), and aromatic (FYW) (Table 1). The wide spectrum of tolerated residues in the rs-pharmacophore is explained by the interacting region on the hPD-1 receptor side, where six residues with diverse properties are found in spatial proximity to Y123 of hPD-L1, including hydrogen-bond acceptors or donors (E136, G124 and T76), hydrophobic (1126, I134), and aromatic (Y68) residues. The corresponding residue in mPD-L2, Y112 interacts with hydrogen-bond acceptors or donors (N35, E103 and T43) and a hydrophobic residue (I101) in the wild type interface of mPD-1. Interestingly, E136 (E103), T76 (T43), and 1134 (I101) are conserved residues between mPD-1 and hPD-1. Once the rs-pharmacophore was rationalized in this local context, a total of 10 mutations were explored in three positions of hPD1. Two of these variants, T76D and T76E, achieved high selectivity for PD-L1 (Table 1).

(44) Another promising location that was revealed by the rs-pharmacophore analysis was position Y68 in hPD-1 of which the equivalent is N35 in mPD-1; however, despite being different residue types, both interact with an Tyr in hPD-L1 (Y123) and mPD-L2, (Y112). Y68 is part of a cluster of interacting residues in hPD-1, whose members include K78, E136, G124, I126, I134, and T76, making the resulting rs-pharmacophores relatively accommodating and suggesting a highly tolerant position ripe for exploration. We explored six mutants for Y68, four of which induced selectivity for PD-L1 (FIG. **2**).

(45) Experimental validation. After excluding all the previously studied mutations (25, 26), we prioritized 34 mutants covering 14 residues in hPD-1 for experimental validation. Site directed mutagenesis was performed, resulting in 32 single point mutations, which were all sequence validated and expressed as GFP fusions presented on the surface of suspension adapted HEK-293 cells. Analysis of the hPD-1 mutants binding to hPD-L1 and hPD-L2 was performed by high-throughput flow cytometry. The percent of hPD-1-expressing cells bound to either hPD-L1 or hPD-L2 was determined and the data normalized to the highest ligand concentration for wild-type hPD-1 binding. Out of the 32 hPD-1 mutants, 16 (N66Q, Y68N, Y68K, Y68R, Y68Q, S73R, Q75N, T76D, T76E, D77E, K78R, G124V, L128V, K131H, I134N, I134F) showed statistically significant ($p<0.05$, two-tailed t-test) increases in selectivity towards PD-L1 (Table 2). Of these, six maintained close to wild type binding interaction to PD-L1. We selected five of these designs for titration experiments, where HEK-293 cells expressing WT or mutant hPD-1 were challenged with hPD-L1 or hPD-L2 expressing cells (FIG. **2**). Two mutants, Y68R and Y68K, showed undetectable PD-L2 binding, while EC50 values for PD-L1 were 1.50 and 4.48 nM, respectively. The other three mutants (Y68N, T76D and T76E) showed a 4-12 fold selectivity for PD-L1. All the successful mutants achieved selectivity by diminishing PD-1 binding to PD-L2, but none of the selectivity was achieved by increasing PD-1 binding to PD-L1 in a statistically significant manner.

(46) Structural insights. We generated comparative protein structure models to gain further insight about mutations that achieved high selectivity between the two ligands. The models of the complexes were subject to 25 ns molecular dynamics simulations using GROMACS (40) to accommodate the rearrangement of local contacts. Interestingly, the Y68 mutant interacts with a highly conserved cluster of residues on hPD-L1 and hPD-L2, which includes D122 (D111 for hPD-L2), Y123 (Y112), K124 (K113). However, the modeling suggests that residue A121 in hPD-L1 (W110 in hPD-L2) is the most relevant for recognition of Y68 of PD-1. When mutated to long, polar side chains (i.e., Y68R and Y68K), a possible steric conflict emerged with W110 of hPD-L2,

despite the otherwise favorable complementary charge interactions. When a shorter side chain is introduced, Y68N, selectivity is still achieved, but to a lesser extent.

(47) When exploring the other most selective site for mutation, hPD-1 T76D or T76E, it appears that a more favorable charged or hydrogen bond interaction is established with R125 and K124 of PD-L1, while the Y114 side chain of hPD-L2 is not suitable to support this mutant.

(48) Correlation with predicted free energy changes. Once experimental data were obtained we also attempted to retrospectively correlate the results with methods that predict the energetic effect of point mutations. We ran three different programs, FoldX (41), Mutabind (42) and BeAtMuSiC (43), all of which returned essentially random predictions (calculated correlations between predicted and measured binding affinity changes are 0.009, 0.017, and 0.032, respectively (FIG. **3**)). These approaches were either unable to distinguish the differential effect of mutations on the two ligands (BeAtMuSiC), or if differences were detected these turned out not to correlate with the observations (FoldX, Mutabind). These results highlight the difficulty of predicting specificity-inducing mutations correctly and highlight the utility of the rs-pharmacophore-based approach described in this work in efficiently capturing these designs.

(49) Discussion

(50) The PD-1 signaling pathway is one of the inhibitory checkpoints that shapes T cell activity for anti-cancer (44) and anti-viral (45) immune responses (15, 44). Highly effective mAbs have been developed to disrupt both sides of the PD-1:PD-L1 interaction for the treatment of cancer (16, 17). As an alternative approach, a re-engineered PD1 with picomolar affinity to hPD-L1 was developed to block the wild type hPD-1:hPD-L1 interaction, with some possible advantages over conventional mAbs (25).

(51) In this study, we utilized the ProtLID computational method (34) to re-engineer the protein binding interface of PD-1 for selective recognition of PD-L1, with the goal of introducing as few mutations as possible. ProtLID reduces the theoretical number of possible mutations to an experimentally manageable set using the concept of pharmacophore elaboration (46). The construction of a high-specificity interface to discriminate among multiple proteins with similar structure from the same superfamily, as in the current work, is highly challenging (47), in this case because PD-1 and its ligands share the same immunoglobulin fold. Interestingly, the most effective mutant designs of PD-1 to induce selectivity involved two residues (Y68 and T76), which interact with a highly conserved cluster of residues in PD-L1 and PD-L2. Retrospective structural analysis can explain the effect of these mutations, but these are hard to predict a priori.

(52) TABLE-US-00002 TABLE 1 human PD-L1/PD1 (4ZQK) mouse PD-L2/PD1 (3BP5) Interface Interface of hPD- Interface Interface of mPD- L1 of hPD1 rs- rs- of mPD1 L2 (4ZQK.A) (4ZQK.B) pharmacophore designs pharmacophore (3BP5.A)* (3BP5.B) PHE19 LYS78 HKNQRTW K78R HKNQW LYS45(78) PHE21 SEQ ID NO: 9 SEQ ID NO: 10 ASP26 GLN75, HKNQRWY Q75N, S73RK HKNQRWY GLN42(75), GLU28 SER73 SEQ ID NO: 11 SEQ ID NO: 12 SER40(73) TYR56 ALA132, HIKLMNPQRVWY I134QEDTNF HNPQRSTWY ALA99(132), GLN60 ILE134 SEQ ID NO: 13 SEQ ID NO: 14 SEQ ID NO: 15 ILE101(134) GLN66 ALA132, HKNPQRTWY K131H KNQRTWY LYS100(133)# SER67 LYS131 SEQ ID NO: 16 SEQ ID NO: 17 ARG113 GLU136 DEPQSY E136QN SEQ ID NO: 18 MET115 ILE126, FIMVWY I126V, DFHIKLMNPQRSTVWY ILE93(126), TRP110 LEU128 SEQ ID NO: 19 L128VMW SEQ ID NO: 20 ALA92(125), ASN33(66), LYS45(78), GLY91(124), MET31(64)# GLY120 GLU84 HPY E84Y ALA121 ASN66, KNQRSTWY N66QE LYS78 SEQ ID NO: 21 ASP122 LYS78, HKNQRTWY Y68KRNQ HKNQRSWY LYS45(78) ASP111 TYR68 SEQ ID NO: 22 SEQ ID NO: 23 SEQ ID NO: 24 TYR123 GLU136, DEFHIKLM.sup.1 I126V, DEFHLMNP.sup.1 GLU103(136), TYR112 GLY124, NPQRTVWY.sup.2 T76DQENSY.sup.1, QRSTVWY.sup.2 ILE101(134), ILE126, .sup.1SEQ ID NO: 25 G124VMY .sup.1SEQ ID NO: 28 THR43(76), ILE134, .sup.2SEQ ID NO: 26 .sup.1SEQ ID NO: 27

.sup.2SEQ ID NO: 29 ASN35(68)# THR76, TYR68 LYS124 ASP77, DENPQSTY D77E DENQST THR43(76) LYS113 THR76 SEQ ID NO: 30 SEQ ID NO: 31 ARG125 GLN75 HNPQSTW HIKLMNPQRSTVWY ASN41(74), TYR114 SEQ ID NO: 32 SEQ ID NO: 33 GLN42(75), GLU103(136), THR43(76)

The first 3 columns list hPD-L1, hPD-1 residues in the interface and the corresponding rs-pharmacophore preferences for PD-L1. The last three columns show, in a similar fashion, the rs-pharmacophores for mPD-L2, and the structurally corresponding interface residues of mPD-1 and mPD-L2. The central column lists single residue mutants of hPD1 that were selected to induce PD-L1 specificity.

(53) TABLE-US-00003 TABLE 2 Two-tailed Mutation AVE-hPD-L1 AVE-hPD-L2 STD-hPD-L1 STD-hPD-L2 t-statistics p-value Y68R 0.876456405 0.033445075 0.008437621 0.010274871 141.781 1.89E−14 Y68K 0.774711472 0.022278096 0.008983203 0.018813393 80.7026 5.54E−10 Y68N 1.060521473 0.108439683 0.099555934 0.09135455 15.7559 2.84E−07 T76D 1.066772515 0.20528398 0.101702917 0.110906348 12.8015 1.40E−06 Y68Q 0.946517574 0.20176295 0.025207873 0.082775811 19.2458 1.12E−05 K78R 0.716545243 0.125063524 0.023788457 0.102307632 12.5917 0.000122932 D77E 0.956794794 1.125776788 0.038664958 0.040052918 −6.78734 0.000140421 T76E 0.9640642 0.641683469 0.041368794 0.079282526 8.06098 0.000190529 K131H 0.994351458 1.579578832 0.092563067 0.155152673 −7.24324 0.000238277 Q75N 0.969896594 1.129497843 0.022302473 0.050712945 −6.44182 0.000937851 N66Q 0.361651172 0.010532588 0.113538667 0.004804627 6.90886 0.00227228 I134N 0.424420142 0.053442429 0.139751747 0.050837972 5.57813 0.00248728 G124V 0.960533228 1.157814602 0.029529666 0.09932396 −4.2572 0.0092079 S73R 0.97075849 1.113224675 0.044996214 0.089809421 −3.17134 0.019782 I134F 0.753900313 1.10854885 0.153483042 0.267588006 −2.57072 0.0400823 L128V 0.881942986 1.027345791 0.024451683 0.112490036 −2.82435 0.0429335 L128W 0.897043527 1.033264785 0.046812027 0.112337649 −2.50286 0.0511656 E136N 0.889324374 0.820234238 0.043052541 0.054451187 2.2256 0.0584235 E136Q 0.861277766 0.967143216 0.074473749 0.079524797 −2.17272 0.0617004 I134Q 0.097854454 0.025154896 0.065523381 0.028632223 2.27339 0.0675694 G124M 0.845121009 0.704707966 0.042961902 0.128279246 2.32087 0.0691911 I126V 0.862978279 0.710723533 0.059453802 0.146729984 2.15044 0.0812971 T76S 0.885344173 0.953666226 0.029152024 0.096908968 −1.50963 0.194933 T76N 0.927660581 0.998189829 0.085646396 0.091255578 −1.26014 0.243271 G124Y 0.650545997 0.557244172 0.128493079 0.186689237 0.920552 0.387512 I134T 0.378917311 0.306791314 0.117036032 0.147679228 0.855899 0.418209 N66E 0.006104358 0.004383021 0.004025551 0.003194503 0.748975 0.476389 T76Y 1.075411178 1.113595086 0.079905355 0.088057786 −0.718051 0.493345 S73K 0.967521585 1.004199987 0.027082062 0.125564458 −0.638492 0.555083 I134D 0.012970022 0.010518663 0.008740301 0.00639797 0.50605 0.627683 T76Q 0.957079559 0.980264494 0.058779294 0.094645654 −0.465324 0.656474 E84Y 0.987208115 0.998721431 0.034309685 0.052567072 −0.410121 0.694178 Control 0.014392837 0.006482458 0.007688471 0.004786447 WT 1 1 0 0

List of tested mutations of PD-1 (first column), five technical replicates of cell-assay-based experiments. The second and third column show average binding, normalized to wild type to PD-L1 and PD-L2. The fourth and fifth columns are the corresponding standard deviations. The sixth and seventh columns are the t-statistics and corresponding two-tailed p-values for differences in PD-1 binding affinity to PD-L1 and PD-L2.

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Claims

1. A human Programmed Cell Death-1 (PD-1) receptor mutant comprising at least the first 149 consecutive amino acids of SEQ ID NO:1 with mutation Y68R or Y68K, wherein the mutant has selectivity for PD-LI compared to PD-L2 and wherein the mutant has undetectable PD-L2 binding.
2. A fusion polypeptide comprising the PD-1 receptor mutant of claim 1 fused to an immunoglobulin domain polypeptide.
3. The fusion polypeptide of claim 2, wherein the mutant is fused to the immunoglobulin domain polypeptide by a peptide bond between a terminal amino acid of the mutant and a terminal amino acid of the immunoglobulin domain polypeptide.
4. The fusion polypeptide of claim 2, wherein the immunoglobulin domain polypeptide comprises an immunoglobulin IgG1 Fc domain.
5. The fusion polypeptide of claim 4, wherein the immunoglobulin IgG1 Fc domain is human.
6. A composition comprising the PD-1 receptor mutant of claim 4, and a pharmaceutically acceptable carrier.
7. The PD-1 receptor mutant of claim 1 further comprising a radiolabel.
8. A method of imaging a PD-L1 positive tumor in a subject comprising administering the

radiolabeled PD-1 receptor mutant of claim 7 to the subject, where the mutant binds to the tumor, and imaging the radiolabeled mutant bound to the tumor.

9. A method of stimulating T cell activation, treating a tumor, or treating an infection in a subject comprising administering to the subject a human Programmed Cell Death-1 (PD-1) receptor mutant comprising at least the first 149 consecutive amino acids of SEQ ID NO: 1 with mutation Y68R or Y68K, wherein the mutant has selectivity for PD-LI compared to PD-L2 and wherein the mutant has undetectable PD-L2 binding, or a fusion polypeptide comprising the PD-1 receptor mutant fused to an immunoglobulin domain polypeptide, or a composition comprising the PD-1 receptor mutant or the fusion polypeptide, in an amount effective to stimulate T cell activation, treat a tumor, or treat an infection, respectively, in a subject.

10. The method of claim 9, wherein the T cell activation comprises cytokine secretion.

11. The method of claim 9, wherein the subject has a tumor.

12. The method of claim 9, wherein the subject has an infection.