PD-1 and PD-1 ligands: from discovery to clinical application

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Abstract

Programmed cell death-1 (PD-1, *Pdcd1*), an immunoreceptor belonging to the CD28/CTLA-4 family negatively regulates antigen receptor signaling by recruiting protein tyrosine phosphatase, SHP-2 upon interacting with either of two ligands, PD-L1 or PD-L2. Because of the wide range of ligand distribution in the body, its biological significance pervades almost every aspect of immune responses including autoimmunity, tumor immunity, infectious immunity, transplantation immunity, allergy and immunological privilege. In this review, we would like to summarize the history of PD-1 research since its discovery and recent findings that suggest promising future for the clinical application of PD-1 agonists and antagonists to various human diseases.

Historical aspects of PD-1, PD-L1 and PD-L2

Discovery of PD-1

In an attempt to identify a gene that induces programmed cell death, Ishida et al. (1) studied two cell lines; LyD9, a hematopoietic progenitor cell line that undergoes programmed cell death upon IL-3 deprivation and 2B4.11, a T cell hybridoma that undergoes programmed cell death by stimulation with phorbol myristate acetate and ionomycin. Because apoptosis of both cells required de novo RNA and protein synthesis, subtractive hybridization was performed to identify the gene that is required for programmed cell death. A cDNA library prepared by subtracting mRNA of resting LyD9 cells from cDNA of dying LyD9 cells was screened by a probe prepared by subtracting mRNA of resting LyD9 cells from cDNA of dying 2B4.11 cells and PD-1 cDNA was identified in 1992 (1). Deduced amino acid sequence predicted that PD-1 is a type I transmembrane protein with a single IgV domain in the extracellular region. However, subsequent experiments did not confirm the direct involvement of PD-1 in programmed cell death of these cell lines (2), and the function of PD-1 remained elusive until spontaneous development of lupus-like autoimmune diseases in PD-1deficient mice ($Pdcd1^{-/-}$ mice) (3, 4).

Augmented immune response and lupus-like syndrome in $Pdcd1^{-/-}$ mice

In 1998, Nishimura *et al.* (3) reported generation of $Pdcd1^{-l-}$ mice, in which exons encoding the transmembrane region

of PD-1 are replaced with neomycin resistance gene. Although previous analyses demonstrated that PD-1 is expressed on activated T and B cells in the periphery and thymocytes undergoing β -selection (2, 5), $Pdcd1^{-/-1}$ mice did not show any drastic immunological phenotypes. Since the genetic heterogeneity of $Pdcd1^{-/-}$ mice with 129SV and C57BL/6 mice-derived chromosomes made it difficult to analyze immunological phenotypes, we backcrossed Pdcd1-/mice on C57BL/6 mice to obtain more stable and reliable results. C57BL/6-*Pdcd1*^{-/-} mice showed mild splenomegaly and B cells from C57BL/6-Pdcd1-/- mice proliferated more extensively than those from wild-type mice upon anti-IgM stimulation. Production of antibodies against T-independent antigen was also enhanced in C57BL/6-Pdcd1^{-/-} mice compared with C57BL/6 wild-type mice (3). Interestingly, about half of the C57BL/6-Pdcd1-/- mice developed lupus-like arthritis and glomerulonephritis in later life (4). These findings suggested that PD-1 negatively regulates the immune responses. However, the precise mechanism for PD-1 regulation was still unknown partly because of the absence of its ligands.

Identification of PD-1 ligands

We generated PD-1–Ig fusion protein that can bind to its ligand. Several expression cloning strategies using PD-1–Ig fusion protein did not allow us to isolate the ligand cDNA. We became aware, through our collaboration on the signal sequence trap screening (6), of the fact that the Biacore

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screening system for the ligand receptor interaction was setup in Genetic Institute. We proposed the collaboration to search the PD-1 ligand by the Biacore assay to Genetic Institute. The group in Genetic Institute tested a new B7 molecule (clone 129) identified by G. J. Freeman in the Harvard University from database in our assay and found clear interaction with PD-1 (7). Engagement of PD-1 with clone 129 inhibited the proliferation and cytokine production of T cells upon stimulation with anti-CD3 antibody and we changed its name from clone 129 to PD-L1 for PD-1 ligand 1 (7, 8). Here it became evident that PD-1 prevents autoimmunity by inhibiting the activation of autoreactive lymphocytes. Our collaboration further identified another ligand, PD-L2 (9). The identification of PD-L1 added PD-1 to the list of CD28 family as its third member (Fig. 1) (10). At the same time, the other groups reported that B7-H1 and B7-DC, which are identical to PD-L1 and PD-L2, respectively, costimulate T cells, the mechanisms of which still remain unknown (11-14).

Structure and expression of PD-1 and its ligands

PD-1

PD-1 is a 50–55-kDa type I transmembrane glycoprotein composed of an IgV-type extracellular domain sharing 21– 33% sequence identity with CTLA-4, CD28 and ICOS. PD-1 lacks the membrane-proximal cysteine residue required for homodimerization of other members of the CD28 family. Actually, structural and biochemical analyses showed that PD-1 is monomeric in solution as well as on cell surface (15). The PD-1 cytoplasmic domain has two tyrosine residues, the membrane-proximal one constitutes an immunoreceptor tyrosine-based inhibitory motif (ITIM) and the other an immunoreceptor tyrosine-based switch motif (ITSM) (16, 17). Because ITIM is widely found in immunoinhibitory receptors including CD72, FcyRIIB and KIR, the membrane-proximal tyrosine residue was supposed to play a central role for the inhibitory function of PD-1. However, in vitro experiments using a murine B cell line expressing various mutants of PD-1 revealed that the tyrosine residue located within ITSM but not ITIM is essential for the inhibitory function of PD-1 (18). Interestingly, the cytoplasmic region of human and murine PD-1 shares only ~60% amino acid identity in contrast to 100% conservation between human and murine CTLA-4, suggesting that PD-1 was less strictly selected in the course of evolution (19). Nonetheless, the amino acid sequence surrounding ITSM was completely conserved between human and mouse, confirming its functional importance (18). Upon antigen stimulation, the tyrosine residue located within ITSM is phosphorylated and recruits the protein tyrosine phosphatase SHP-2, which dephosphorylates downstream effector molecules such as Syk and PI3K in B cells and ZAP70 and CD3ζ in T cells (18, 20, 21). The mechanism of PD-1 signaling was reviewed previously (22).

PD-1 is expressed on double-negative $\alpha\beta$ and $\gamma\delta$ T cells in thymus and induced on peripheral T and B cells upon activation (2, 5). The broader expression of PD-1 contrasts with restricted expression of other CD28 family members to T cells, suggesting that PD-1 regulates a wider spectrum of immune response compared with other CD28 family members. Although most of the expression analyses are directed against PD-1 on cell surface, Raimondi *et al.* (23) reported that PD-1 can also be detected in the cytoplasm of T cells with regulatory function. Currently, it is not clear whether intracellular PD-1 exerts inhibitory function in cytoplasm or activated T cells store PD-1 in the cytoplasm to achieve quick expression of PD-1 upon reactivation. Further analyses may reveal the real role of PD-1 in cytoplasm.



Fig. 1. Summary of CD28 family members and their ligands. Names, expression pattern and function are indicated. Act, expression upon activation; const, constitutive expression.

Zhong et al. proposed an interesting hypothesis as to how PD-1 selectively suppresses aberrant immune responses based on the danger theory (24, 25). They observed that the induction of PD-1 on splenic B cells upon anti-IgM stimulation is suppressed in the presence of so-called danger signals such as LPS and CpG1826. They hypothesized that harmful pathogens accompany with such danger signals and inhibit the induction of PD-1, and thereby anti-infectious immunity escapes from the PD-1-dependent inhibition. In contrast, self-antigens do not couple with danger signals and allow the induction of PD-1, thereby anti-self-responses are suppressed by PD-1. This hypothesis may explain how PD-1 suppresses aberrant immune responses but preserves beneficial immune responses such as anti-bacterial immunity. However, some of the typical danger cytokines like IL-1 α , IL-1 β and tumor necrosis factor α (TNF α) do not suppress the induction of PD-1, while some of the non-danger cytokines like IL-4 suppress the induction of PD-1. Further analyses are needed to test this unique hypothesis.

PD-L1 and PD-L2

PD-L1 and PD-L2 are type I transmembrane glycoproteins composed of IgC- and IgV-type extracellular domains (7, 9, 11, 12). PD-L1 and PD-L2 share \sim 20% amino acid identities with B7.1 and B7.2 that are ligands for CD28 and CTLA-4. PD-L1 and PD-L2 share 40% amino acid identity while human and murine orthologs of PD-L1 or PD-L2 share 70% amino acid identity. The PD-L1 and PD-L2 genes are located in the close proximity on mouse chromosome 19 with only 22-kb interval. This genomic interval is much closer than that of B7-1 and B7-2, which are located on mouse chromosome 16 with 1.8-Mb interval. Both PD-L1 and PD-L2 have short cytoplasmic tails with no known motif for signal transduction, suggesting that these ligands do not transduce any signal upon interaction with PD-1. However, one group reported that cross-linking of PD-L2 induces stimulatory signal in dendritic cells (DCs), resulting in the augmented antigen presentation (26, 27).

A variety of normal tissues express PD-L1 and PD-L2 transcripts, with high levels of expression in placenta, heart, lung and liver, low expression levels in spleen, lymph nodes and thymus and the absence of expression in brain (7, 9, 11, 12). However, the amount of these transcripts does not always correlate with the amount of their proteins (28-30). Especially, the PD-L2 protein is rarely detected in nonlymphoid organs under normal condition. There might be a post-transcriptional regulation as we describe later (31). The expression of PD-L1 in both lymphoid and non-lymphoid tissues suggests that the PD-1-PD-L pathway may modulate immune responses in secondary lymphoid organs as well as in target organs. The expression of PD-L1 and PD-L2 on antigen-presenting cells has been examined most extensively by Yagita and colleagues (29) using murine samples. According to their report, PD-L1 is expressed on resting B cells, T cells, macrophages and DCs. The expression of PD-L1 is further up-regulated on these cells by various stimulation including anti-IgM antibody, LPS and anti-CD40 antibody for B cells, anti-CD3 antibody for T cells, anti-CD40 antibody, LPS, IFNy and granulocyte macrophage colonystimulating factor (GM-CSF) for macrophages and anti-CD40 antibody, IFN γ , IL-4, IL-12 and GM-CSF for DCs. PD-L2 is rarely expressed on resting cells and hardly induced on B cells and T cells. PD-L2 can be induced on macrophages by IL-4 and IFN γ and on DCs by anti-CD40 antibody, GM-CSF, IL-4, IFN γ and IL-12. Loke *et al.* (32) reported that IL-4 induces PD-L2 more strongly than IFN γ , while IFN γ induces PD-L1 more strongly than IL-4 on macrophages, suggesting that T_h1 and T_h2 responses mobilize PD-L1 and PD-L2 differentially. The expression profiles of human PD-L1 and PD-L2 are generally similar to those of mouse (33). PD-L1 and PD-L2 are also expressed on various tumor cell lines and at the site of immune privilege, which we discuss later.

PD-1 deficiency and autoimmunity

Lupus-like phenotypes in C57BL/6-Pdcd1^{-/-} mice

The link between PD-1 deficiency and autoimmunity was initially revealed by the studies on PD-1-deficient mice on the C57BL/6 background, which exhibit hyperactivation of the immune system such as splenomegaly and in vitro augmented proliferation of B cells (4). At 14 months of age, ~50% of C57BL/6-Pdcd1^{-/-} mice suffer from glomerulonephritis, which is similar to human endocapillary proliferative glomerulonephritis. Significant depositions of IgG3 and C3 are detectable in the glomeruli of C57BL/6-Pdcd1^{-/-} mice, however, its auto-antigen is currently unknown. In addition, most of the C57BL/6-Pdcd1^{-/-} mice develop arthritis, which occasionally accompanies an extensive granulomatous inflammation. Unlike most of the lupus-prone mice, common auto-antibodies such as anti-double-strand DNA and rheumatoid factor are hardly detectable in C57BL/6-Pdcd1-/mice. Because the severity of these phenotypes was accelerated by the introduction of lymphoproliferative disorder (Ipr) mutation, PD-1 deficiency and Ipr mutation seem to work synergistically in the development of lupus-like autoimmune diseases (4).

Dilated cardiomyopathy in BALB/c-Pdcd1^{-/-} mice

Dilated cardiomyopathy (DCM) is a myocardial disease characterized by progressive depression of myocardial contractile function and ventricular dilatation. In spite of its rather high frequency (14.0-36.5 in 100 000 people) and high mortality, its pathophysiology is largely unknown and there is no effective therapy except for the heart transplantation. When Pdcd1^{-/-} mice were backcrossed on BALB/c background, BALB/c-Pdcd1-/- mice started to die from 5 weeks and ~50% of BALB/c-Pdcd1^{-/-} mice died by 5 months of severe congestive heart failure. From echocardiographic examinations, the movement of cardiac walls is decreased with enlarged left ventricular spaces both in diastolic and systolic phases, resulting in the reduction of ejection fraction to 14.9% on average (34). Overall, the cardiac function of BALB/c-Pdcd1^{-/-} mice is impaired in a manner similar to human DCM. The dilated heart showed inflammation to various degrees with prominent deposition of immune complex on the surface of cardiomyocytes. In addition, all the sera from DCM-Pdcd1-/- mice contain hightiter auto-antibodies against cardiac troponin I (cTnI).

Injection of mAbs against cTnI has been shown to enlarge end-systolic and end-diastolic volume of left ventricle 3-fold and 1.3-fold, respectively, and slow the velocity of pressure development, which reflects the contractile function, to \sim 60% in 12 weeks. Although these changes are rather mild compared with the phenotypes of BALB/c-Pdcd1-/- mice, some of the anti-cTnl antibody-injected mice develop more severe disease in 40 weeks. The ejection fraction of such mice is <15%. Because there is almost no infiltration of lymphocytes in hearts of anti-cTnI antibody-injected mice, autoantibodies against cTnI are considered to be responsible for the DCM in Pdcd1-/- mice (35, 36). Recently, Goser et al. (37) reported that immunization with cTnI but not with cTnT induced severe myocarditis in BALB/c and A/J mice, confirming the pathogenic role of autoimmune response against cTnl.

cTnl is a member of the troponin complex of heart, which regulates the contraction of cardiac muscle. Mutations in the cTnl gene have been found in familial DCM and hypertrophic cardiomyopathy (38, 39). So, the subcellular localization of cTnl is believed to be restricted to cytoplasm. However, anti-cTnl antibody is depositing on the surface of cardiomyocytes in $Pdcd1^{-l-}$ mice and three different mAbs against cTnI have been confirmed to stain the surface of cardiomyocytes in normal heart by immunoelectronmicroscopic analysis, indicating that cTnl is also expressed on the surface as well as in the sarcomere of cardiomvocvtes. Moreover, addition of anti-cTnl antibodies augments voltage-dependent Ca²⁺ current of cardiomyocytes as much as 1.4-fold in 5 min in vitro, suggesting that cTnI on the surface of cardiomyocytes is involved in an unidentified function to regulate the magnitude of Ca2+ current. Taken together, it is likely that anti-cTnl antibodies in Pdcd1-/- mice induce DCM by chronic enhancement of Ca²⁺ current of cardiomyocvtes (35).

In accordance with these findings, Ig-adsorption therapy has been reported to have beneficial effects on hemodynamic parameters of human DCM patients in short-term (3 months) and long-term (1 year) follow-up studies in Germany as well as in Japan (40–43). It is tempting to speculate that the improvement of cardiac function by immunoadsorption therapy is mediated by the removal of the anti-cTnI autoantibodies. Further analyses are needed to determine the real targets of the pathogenic auto-antibodies in human DCM and establish the immunoadsorption of antigen-specific auto-antibodies, which may not require Ig substitution and is safer in many respects.

Acute type I diabetes in NOD-Pdcd1^{-/-} mice

Non-obese diabetic (NOD) mouse is a useful animal model of type I diabetes with many common features with human type I diabetes (44, 45). The incidence of type I diabetes of NOD mice is affected by various environmental and genetic factors. Overall, 40–80% of the female NOD mice and 10–40% of the male NOD mice develop diabetes by 30 weeks. Introduction of PD-1 deficiency dramatically accelerated the onset and incidence of diabetes and all the female and male NOD-*Pdcd1^{-/-}* mice developed diabetes by 10 weeks with more severe insulitis (46). Interestingly, PD-L1 but not PD-L2

is highly expressed on β cells in pancreatic islet with insulitis. In PD-1-sufficient NOD mice, infiltrating lymphocytes form a cluster surrounding islets and rarely invade the islets as though there is a barrier between the islets and lymphocytes, with strong PD-L1 expression on the β cells exactly adjacent to lymphocytes, suggesting that PD-L1 on β cells may serve as a barrier to suppress the effector function of diabetogenic T cells. In NOD-Pdcd1^{-/-} mice, this barrier is lost and lymphocytes invade deeply inside islets despite augmented PD-L1 expression on β cells. As a result, NOD-Pdcd1^{-/-} mice develop type I diabetes more rapidly than the original NOD mice with massive destruction of the islets. In addition to β cells, PD-L1 is also expressed on DCs in the islets of NOD-Pdcd1^{-/-} mice. Because T cells are more strongly activated in the islets than in draining lymph nodes, PD-1-PD-L1 interaction seems to inhibit the in situ priming/ activation of T cells as well. In agreement with these observations, antibody blockade of PD-1-PD-L pathway in prediabetic NOD mice induces type I diabetes within 10 days (47). Later, Keir et al. (48) clearly demonstrated that PD-L1 on β cells but not on lymphoid cells is critical for delaying diabetes and maintaining peripheral tolerance using NOD- $Pdcd1lg1^{-/-}$ mice which also develop acute diabetes like NOD-*Pdcd1*^{-/-} mice.

Bilateral hydronephrosis in BALB/c-Fcgr2b^{-/-}Pdcd1^{-/-} mice

Although it is now clear that PD-1 inhibits adverse immune responses to prevent autoimmunity, a major question remains; how many inhibitory receptors exist in the immune system and how do they cooperate? To answer this question, the possible cooperation between PD-1 and FcyRIIB (low-affinity type IIb Fc receptor for IgG, Fcgr2b) in the regulation of autoimmunity has been analyzed (49). FcyRIIB is an inhibitory Fc receptor and Fcgr2b^{-/-} mice develop systemic lupus erythematosus (SLE)-like syndrome spontaneously on a C57BL/6 but not on a BALB/c background (50-52). When BALB/c-Pdcd1^{-/-} mice are crossed with BALB/c-Fcgr2b^{-/-} mice, approximately one-third of the BALB/c-Fcgr2b-/-Pdcd1^{-/-} mice develop autoimmune hydronephrosis with concomitant production of anti-urothelial auto-antibodies. In addition, ~15% of the BALB/c-Fcgr2b-1-Pdcd1-1- mice produce anti-nuclear auto-antibodies. In contrast, the frequency of the autoimmune cardiomyopathy and the production of anti-parietal cell auto-antibody, which are observed in BALB/c-Pdcd1^{-/-} mice, are not affected by the additional FcyRIIB deficiency (49). Therefore, PD-1 seems to regulate autoimmune responses synergistically with or independently of FcyRIIB depending on the antigens involved.

Association of PD-1 dysregulation with human autoimmune diseases

Single-nucleotide polymorphisms of PD-1 gene and autoimmune diseases

Based on these animal experiments, single-nucleotide polymorphisms (SNPs) on human PD-1 gene were vigorously searched and analyzed for the linkage with various autoimmune diseases (Table 1). To date, >30 SNPs have been identified in human PD-1 gene (53, 60, 61, 63). Prokunina

et al. reported that the allele A of a SNP named PD1.3 (PD1.3A) in intron 4 is associated with the development of SLE in Europeans (relative risk = 2.6) and Mexicans (relative risk = 3.5) but not African-American. To date, the PD1.3A allele has been reported to link with the development of lupus nephritis, type I diabetes and progressive multiple sclerosis (61, 63). The PD1.3 locates on the binding site for the runtrelated transcription factor 1 (RUNX1) and PD1.3A interferes the binding of RUNX1 resulting in the impaired induction of PD-1. RUNX1 seems to regulate the transcription of other genes as well, which are involved in the autoimmune diseases. Helms et al. reported that a SNP on putative RUNX1binding site, which locates between SLC9A3R1 and NAT9 is associated with the development of psoriasis (45, 67). Tokuhiro et al. (68) reported that a SNP on the RUNX1-binding site of SLC22A4, which encodes an organic cation transporter is associated with the development of rheumatoid arthritis (RA). They further identified that a SNP in the intron 6 of RUNX1 gene itself is associated with the development of RA. However, opposite results have also been reported in Spanish populations that the PD1.3A allele is rather less frequent among SLE patients in Spain with statistical significance (54).

Later, a SNP in the exon 5 of PD-1 gene (PD-1.5T) has also been reported to associate with the development of RA

but not SLE among Chinese people in Taiwan (55). Recently, a Korean group reported that another SNP in exon 5 (PD1.9T) associates with ankylosing spondylitis (64). More recently, a group in Taiwan reported the association of a SNP in PD-L2 gene with SLE (58). These findings suggest that the effect of SNPs in PD-1 gene may vary depending on the genetic background, just like the effect of PD-1 deficiency in different strains of mice. Therefore, it is essential to analyze various immunoregulatory SNPs in combination to fully understand the genetic pathology of autoimmune diseases. However, because disease promoting SNPs are generally less frequent and the number of possible combination is enormous, multivariable studies of SNPs are still rather impractical.

Expression of PD-1 in human autoimmune diseases

Augmented PD-1 expression has been found on synovial fluid T cells in RA and salivary T cells in Sjogren's syndrome, suggesting that PD-1 may actually exert its regulatory function in target organs (Table 2) (77–79). Recently, Wan *et al.* (80) found a soluble PD-1 which is produced by the alternative splicing of exon 3 encoding the transmembrane region in sera as well as synovial fluid of patients with RA. Interestingly, the concentration of soluble PD-1 in the sera but not

Disease	Gene	Population	Association	Reference
SLE	PD-1	Nordic, Mexican	Yes	(53)
	PD-1	Spanish	Yes (opposite)	(54)
	PD-1	Taiwan	No	(55)
	PD-1	Taiwan	Yes	(56)
	PD-L1, PD-L2	Swedish, Mexican, Argentina	No	(57)
	PD-L2	Taiwan	Yes	(58)
Rheumatoid arthritis	PD-1	Taiwan	Yes	(55)
	PD-1	Swedish	Yes	(59)
	PD-1	Hong Kong	Yes	(60)
Type I diabetes	PD-1	Danish	Yes	(61)
51	PD-1	Japanese	Yes	(62)
Multiple sclerosis	PD-1	German	Yes	(63)
Ankylosing spondylitis	PD-1	Korean	Yes	(64)
Myocardial infarction	PD-1	Swedish	Yes	(65)
Allergy	PD-1	Australian, British	Yes	(66)

Table 2. Aberrant PD-1 and PD-	1 expression in human	infectious and	autoimmune diseas	ses
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Protein	Population	Reference
PD-1	CD8 T cell	(69–71)
PD-L1	Monocyte, B cells	(72)
PD-1	CD8 T cells	(73, 74)
PD-L1	Monocytes	(75)
PD-L1	Gastric epithelium	(76)
		(- <i>i</i>
PD-1. PD-L1	T cells and salivary epithelium	(77, 78)
PD-1	Synovial CD4 T cells	(79)
Soluble PD-1	Synovial and peripheral T cells	(80)
PD-1	Lymphocytes	(81)
PD-L1, PD-L2	DC, KC, sinusoidal endothelium	
	Protein PD-1 PD-L1 PD-L1 PD-L1 PD-L1 PD-L1 PD-1 PD-1 Soluble PD-1 PD-1 PD-1 PD-1 PD-1 PD-1 PD-1 PD-L1, PD-L2	ProteinPopulationPD-1CD8 T cellPD-L1Monocyte, B cellsPD-1CD8 T cellsPD-1CD8 T cellsPD-L1MonocytesPD-L1Gastric epitheliumPD-1, PD-L1T cells and salivary epitheliumPD-1, PD-L1Synovial CD4 T cellsSoluble PD-1Synovial and peripheral T cellsPD-1, PD-L2DC, KC, sinusoidal endothelium

HCV, hepatitis C virus; *H. pylori, Helicobacter pylori*; SS, Sjogren's syndrome; AIH, autoimmune hepatitis; PBC, primary biliary cirrhosis and KC, Kupffer cell.

synovial fluid positively correlated with the amount of rheumatoid factor in the sera and TNF α in the synovial fluid. Because soluble PD-1 can functionally block the regulatory effect of membrane-bound PD-1 on T cell activation, it is possible that soluble PD-1 attenuates the PD-1 pathway and worsens the disease.

Therapy of autoimmune diseases by the manipulation of PD-1 signal

Based on these observations, a trial to cure autoimmune diseases by PD-L1-expressing DCs was carried out. Hirata et al. elaborated a method to generate DC from embryonic stem (ES) cells, because genetic modification is more easily performed on ES cells than freshly isolated cells that are the most popular source of DCs (82, 83). First, ES cells were transfected with expression vectors for PD-L1 and human invariant chain with peptide epitope of myelin oligodendrocyte glycoprotein (MOG) in the class II-associated invariant chain peptide region to allow the presentation of MOG peptide in the context of MHC class II. Double-transfectant ES cells were allowed to differentiate into DCs by co-culturing with OP9 feeder cells in the presence of GM-CSF (ES-DC-PD-L1/ MOG). Intra-peritoneal injection of ES-DC-PD-L1/MOG but not control DC (ES-DC/MOG) before or after the immunization with MOG peptide drastically reduced the inflammation of spinal cord as well as the severity of clinical experimental encephalomyelitis (EAE). T cells from ES-DC-PD-L1/MOG pre-treated mice were anergic and unresponsive to ex vivo re-stimulation with MOG peptide but not with irrelevant antigen. Because the transfer of these anergic T cells into naive mice did not prevent the subsequent induction of EAE, it is postulated that the therapeutic effect of PD-L1-expressing DCs is likely due to the induction of anergy on antigenspecific T cells rather than the induction of T cells with regulatory function.

More recently, Ding et al. (84) tried to cure lupus-like syndrome in BXSB mice by delivering PD-1 signal using recombinant adenovirus expressing full-length mouse PD-L1 gene (Ad.PD-L1). Intravenous injection of Ad.PD-L1 partially prevented the development of nephritis as evidenced by the lower frequency of proteinuria, reduced amount of serum anti-dsDNA IgG and better renal pathology. They also observed a synergistic effect of Ad.PD-L1 and anti-ICOS-Lblocking antibody on the suppression of lupus-like syndrome in BXSB mice. Because they detected strong PD-L1 expression on renal proximal tubular epithelial cells after Ad.PD-L1 injection, they attributed the protective effect of Ad.PD-L1 to the suppression of autoreactive T cells at the target organ. Further analyses may enable us to establish a new therapeutic modality for autoimmune diseases by manipulating the inhibitory function of PD-1.

PD-1 plays critical roles in the immunological tolerance

Impaired tolerance induction in Pdcd1^{-/-} mice

The spontaneous development of autoimmune diseases by $Pdcd1^{-l-}$ mice implies that PD-1 is involved in the establishment and/or maintenance of immunological self-tolerance. Analyses on transgenic mice of 2C-TCR, which recognize

H-2L^d-bearing cells revealed that PD-1 deficiency abrogates peripheral but not central tolerance of T cells. In the autoreactive genetic background (H-2^{b/d}), 2C⁺ autoreactive T cells are negatively selected in the thymus with a few escaped cells migrating to the periphery. The number of mature 2C⁺ T cells in the thymus is unchanged by the PD-1 deficiency, indicating normal central tolerance in the thymus. In PD-1-sufficient mice, autoreactive 2C⁺ T cells in the periphery express PD-1 on their surface and show naive phenotype. However, in 2C-Pdcd1^{-/-}H-2^{b/d} mice, 2C⁺ T cells are activated and the mice die of a graft-versus-host-like disease ~10 weeks of age (4). PD-1 is thus postulated to function mainly in peripheral tolerance rather than in central tolerance.

Probst et al. further examined the role of PD-1 in the induction of peripheral tolerance using dendritic cell-specific inducible expression of T cell epitopes by recombination (DI-ETER) mice, in which two dominant T cell epitopes of lymphocytic choriomeningitis virus (LCMV), GP(33-41) and NP(396-404), can be inducibly presented on CD11c⁺ cells by tamoxifen treatment. In this system, induced presentation of these antigens on resting DCs prior to LCMV infection efficiently induces tolerance of antigen-specific CD8 T cells in a manner independent from regulatory T cells. In mixed bone marrow chimeras of RAG-deficient DIETER, B6-thy-1.1 and B6-thy-1.2- $Pdcd1^{-/-}$ mice, tamoxifen pre-treatment prior to LCMV infection efficiently induces tolerance on thy-1.1⁺ PD-1-sufficient CD8 T cells but not on thy-1.2⁺ PD-1-deficient T cells. Because the CD8 T cell response against another immunodominant epitope of LCMV, NP(276-286), is unchanged between PD-1-sufficient and PD-1-deficient cells by tamoxifen pre-treatment, the loss of tolerance is not due to a non-specific activation of PD-1-deficient T cells. Because the injection of CTLA-4-blocking antibody further increases the number of antigen-specific PD-1-deficient CD8 T cells, CTLA-4 and PD-1 seem to play non-redundant roles in the induction of CD8 T cell tolerance by resting DCs (85.86).

The role of PD-1 in the regulation of T cell tolerance is also analyzed in vitro. In an attempt to find a molecule that requlates the T cell stimulatory function of DC, Selenko-Gebauer et al. (87) generated mAbs against DC. One of the antibodies, DF272 enhanced the T cell stimulatory function of DC by blocking PD-L1. Moreover, DF272 could reactivate T cells that had been anergized by IL-10-treated DC, suggesting that PD-1 plays a critical role not only in the induction but also in the maintenance of T cell anergy. Recently, Nurieva et al. (88) analyzed the role of positive and negative costimulatory molecules in the induction of T cell tolerance and found that antigen stimulation in the absence of CD28 and ICOS signals induces tolerance in naive T cells. However, the lack of positive co-stimulation was not enough for the induction of tolerance but negative co-stimulation was required because blocking antibodies against PD-1, B7-H3 or B7-H4 reverted the tolerance induction. T cells stimulated in the absence of CD28 and ICOS signals expressed reduced amount of GATA-3, T-bet and Eomes whereas they expressed high amount of Grail, Itch and Cbl-b, E3 ubiquitin ligases that have been shown to play essential roles in T cell tolerance (89). PD-1 blockade suppresses the expression of Grail, Itch and CbI-b and promotes the expression of GATA-3, T-bet and Eomes, resulting in the augmented proliferation and secretion of effector cytokines (88). These results suggest that PD-1 actively induces T cell tolerance by inducing Grail, Itch and CbI-b in the absence of strong positive co-stimulation. Further analyses on the role of PD-1 in the immunological tolerance may reveal more precise mechanisms of autoimmune diseases in $Pdcd1^{-/-}$ mice.

PD-1-PD-L system in feto-maternal tolerance

PD-Ls are also expressed in placenta, which led us to speculate that feto-maternal tolerance is accomplished by PD-1-PD-L-dependent inhibition of the maternal immune system. Guleira et al. (90) provided concrete data that support this idea using a murine abortion model. PD-Ls are strongly induced in placenta under conditions of abortion-prone allogeneic mating (B6 \times CBA) but not syngeneic mating (CBA \times CBA). Treatment with PD-L1-blocking antibody augmented the abortion rate in the abortion-prone allogeneic (18% and 86% in the absence and the presence of anti-PD-L1 Ab, respectively) but not syngeneic mating. Because the augmentation of abortion was coupled with the increase of T cell infiltration in placenta, it is likely that PD-L1-expressing decidual cells inhibit virtually all T cells with PD-1 expression that have entered the placenta in a promiscuous manner to make the placenta neglected by immune surveillance (91). Further analyses may reveal the role of PD-1-PD-L pathway in other sites of immune privilege such as the eye, testis and brain.

PD-1–PD-L pathway in transplantation immunity

Critical role of PD-1 in the transplantation immunity is also reported by several groups. Hancock *et al.* (92) reported that the survival of cardiac allograft can be prolonged by potentiating PD-1-dependent inhibition by a chimeric molecule of PD-L1 and Fc portion of Ig. They also demonstrated that the permanent survival of islet allograft induced by CCR2 blockade in conjunction with rapamycin is dependent on PD-1–PD-L1 pathway (92). Recently, the same group unraveled a beautiful job-sharing between PD-1 and B and T lym-

phocyte attenuator (BTLA), a new family member of CD28 family with inhibitory function (Fig. 1), using cardiac transplantation models (93, 94). Partially MHC-mismatched cardiac allografts (B6.C-H2^{bm12} to C57BL/6-H2^b) show strong induction of BTLA but not PD-1 mRNA and survive >100 days. In accordance with BTLA induction, blocking of BTLA but not PD-1 prompts the rapid rejection of allografts. In contrast, fully MHC-mismatched cardiac allografts (BALB/c-H2^d to C57BL/6-H2^b) are rejected within 10 days despite the induction of both BTLA and PD-1. Blocking of PD-1 accelerates rejection, whereas blocking of BTLA unexpectedly prolongs allograft survival by enhancing PD-1 expression on CD4 and CD8 T cells. When T cells are stimulated with allogeneic DCs in vitro, BTLA is primarily induced at low stimulator to responder (S:R = 1:40) ratio. However, as S:R ratio increases, PD-1 is strongly up-regulated and most of the T cells express PD-1 but not BTLA at high S:R ratio (S:R = 1:5). Therefore, it is likely that PD-1 suppresses stronger and more chronic immune responses than BTLA.

Tumor cells escape the immune response by expressing PD-1 ligands

As mentioned above, the expression of PD-L1 and PD-L2 is also found on various tumor cells. Iwai *et al.* and Hirano *et al.* have shown using PD-L1-over-expressing P815 mastocytoma cell line that PD-L1 on tumor cells suppresses the cytolytic activity of CD8 T cells (95, 96). To date, using various systems, it has been shown that tumor eradication can be accelerated by PD-1–PD-L blockade. The different systems used include antibody blockade of PD-1 and PD-L1, DNA vaccination of the extracellular region of PD-1, $Pdcd1^{-/-}$ mice, tumor-specific T cell clones, TCR transgenic mice and human autologous T cells (97–100). PD-1 blockade has also been shown to suppress tumor metastasis using melanoma and colon cancer cell lines (101).

Recently, a strong correlation between PD-L expression on tumor cells and negative prognosis has been demonstrated for human cancer patients (Table 3). Thompson *et al.* (105) have analyzed the expression of PD-L1 on clinical specimens of renal cell carcinoma and found that patients with

Table 3.	Expression	of PD-L1	and PD-L2 in	human cancer	tissues
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Organ	PD-L1 or PD-L2	Prognostic value	Method	Reference
Lung, ovary, skin, colon	PD-L1	ND	IHC(f)	(102)
Glioma	PD-L1	ND	IHC(f)	(103)
Bladder, breast, colon etc. ^a	PD-L1	ND	IHC(f)	(33)
SCCHN	PD-L1	ND	IHC(f)	(98)
Lung	PD-L1	No	IHC(f)	(104)
Renal cell	PD-L1	Yes	IHC(f, p)	(105, 106)
Esophagus	PD-L1, PD-L2	Yes (L1 and L2)	RT-PCR	(107)
Stomach	PD-L1	Yes	IHC(p)	(108)
Breast	PD-L1	ND	IHC(f)	(109)
Oral squamous cell	PD-L1	ND	IHC(f)	(110)
Urothelial cell	PD-L1	Yes	IHC(f)	(111)
Ovary	PD-L1, PD-L2	Yes (L1), no (L2)	IHC(p)	(112)
Pancreas	PD-L1, PD-L2	Yes (L1), no (L2)	IHC(f)	(113)
Renal cell	PD-1	Yes	IHC(f)	(114)

ND, not determined; IHC(f), immunohistochemistry of frozen specimens; SCCHN, squamous cell carcinoma of the head and neck; IHC(p), immunohistochemistry of paraffin-embedded specimen and RT, reverse transcription. ^aThymoma, lymphoma, cervix, endometrium, gallbladder, larynx, liver, lung, ovary, salivary, stomach and thyroid.

high tumor and/or lymphocyte PD-L1 levels are 4.5 times more likely to die from their cancer than patients exhibiting low levels of PD-L1 expression. Hamanishi *et al.* (112) have analyzed the expression of PD-L1 and PD-L2 in human ovarian cancer using paraffin-embedded specimen and found that PD-L1-positive patients have a significantly poorer prognosis than negative patients. Moreover, patients with tumors positive for both PD-L1 and PD-L2 show dramatically lower survival rate than patients with tumors negative for both of these ligands (46 versus 83% for 5-year survival). Interestingly, PD-L1 expression is negatively correlated with the number of intra-epithelial CD8 tumor infiltrating T cells, suggesting that PD-L1 on tumor cells suppresses the invasion of tumor-specific T cells *in situ*.

More recently, Parsa et al. (31) reported that the expression of PD-L1 protein but not its transcript is augmented by the loss of phosphatase and tensin homolog (PTEN), a tumor suppressor gene, using glioma cell lines as well as primary glioblastoma samples. They further demonstrated that the post-transcriptional augmentation of PD-L1 expression is due to the facilitated recruitment of the PD-L1 transcript to the polysome by the S6 kinase 1 activated by PI3K-AktmTOR pathway, in which PTEN plays a regulatory role. Actually, glioma cell lines that do not express PTEN were resistant to lytic activity of tumor-specific T cells and the addition of blocking antibodies against PD-1 partially restored the lytic activity. Therefore, in addition to other well-documented mechanisms, PTEN loss may confer tumor aggressiveness by augmenting the expression level of PD-L1 resulting in the tumor immune evasion (115).

Therefore, it is likely that PD-1–PD-L-dependent immunoinhibition is hijacked by tumor cells to evade the host immune system both in mouse and human. In this regard, the finding that sporadic immunogenic tumors in SV40 T antigen transgenic mice induced anergy of tumor-specific T cells indicates the importance of anergy induction for immune evasion by tumor cells (116). It is tempting to speculate that these immunogenic tumors may induce anergy of tumorspecific T cells by expressing PD-Ls on their surface. PD-1– PD-L blockade may thus revert the immunocompromised status of tumor-bearing hosts and activate the host immune system to eradicate tumors.

Immune anergy against viral infection is induced by PD-1 expression

The first evidence for the PD-1-dependent suppression of anti-viral immunity was made by Iwai *et al.* (117), in which $Pdcd1^{-/-}$ mice were challenged with lacZ-expressing adenovirus as a model virus. At day 7 after intravenous injection of lacZ-expressing adenovirus, most of the hepatocytes are infected with lacZ-expressing adenovirus in wild-type mice, whereas lacZ-positive cells are only sparsely observed in $Pdcd1^{-/-}$ mice. Because massive T cell infiltration is observed in the liver of $Pdcd1^{-/-}$ mice, adenovirus is most likely eliminated by T cells. By day 30 after infection, no lacZ-positive cells are observed in the liver of $Pdcd1^{-/-}$ mice and hepatocytes are fully regenerated. The source of PD-L1 in this system is most likely liver non-parenchymal cells (liver sinusoidal endothelial cells and Kupffer cells) as these cells express PD-L1 constitutively. It is likely that lacZexpressing adenovirus preferentially colonizes the liver of wild-type mice, where the immune response is partially suppressed by PD-L1-expressing cells.

In addition to colonizing PD-L1-abundant organs like liver, viruses seem to induce PD-L1 on DCs to paralyze host immune system. Human rhinovirus (HRV) infection is one of the most frequent causes of the common cold. In HRV infection, pathogen-specific immune responses appear to be hindered or dysregulated in the respiratory tract, which makes patients susceptible to secondary infection by bacteria leading to bronchitis and pneumonia. Kirchberger et al. (118) attribute this hindered immune response to the functional change of DCs, which is dependent on PD-1-PD-L1 and sialoadhesin. They have co-cultured T cells with allogeneic DCs pre-treated with or without HRV and monitored the proliferative response of T cells. HRV-treated DCs fail to induce T cell proliferation but instead induce promiscuous hypoproliferative state, which is not reversed by addition of exogenous IL-2. HRV treatment does not alter the expression level of co-stimulatory molecules such as CD40, CD58, B7.1 and B7.2. However, a dramatic increase in PD-L1 and sialoadhesin on DCs is induced by HRV treatment, and the addition of blocking antibodies against PD-L1 and sialoadhesin completely restores the stimulatory function of HRV-treated DCs. Therefore, PD-1-PD-L1-dependent inhibition seems to play a central role in the HRV-induced T cell anergy.

Recently, PD-1 has been shown to be expressed on virusspecific CD8 T cells that are exhausted by continuous encounter with viral antigen in chronic viral infection. Ahmed and colleagues dissect the function of PD-1 in acute and chronic viral infection using two different strains of LCMV; the Armstrong strain that is cleared within a week and clone 13 strain that establishes a persistent infection (119, 120). In the chronic phase of LCMV-clone 13 infection, a substantial population of LCMV-specific CD8 T cells are functionally impaired or exhausted. PD-1 is highly expressed on these exhausted LCMV-specific CD8 T cells and PD-1 blockade during the chronic phase of infection efficiently reanimated exhausted CD8 T cells and promoted clearance of the persisting viruses. In contrast, in acute LCMV-Armstrong infection, PD-1 expression is transiently induced and declines guickly to the basal level. The viruses are cleared in a week and blocking the PD-1-PD-L1 interaction did not affect the number of LCMV-specific CD8 T cells.

Subsequently, three groups simultaneously reported that PD-1 is highly expressed on HIV-specific CD8 T cells in HIV-infected individuals (Table 2) (69–71). Day *et al.* analyzed the expression of PD-1 on HIV-specific CD8 T cells in clade-C-infected people who were naive to anti-HIV treatments in South Africa. They found that PD-1 is significantly up-regulated on HIV-specific CD8 T cells and that the expression level is correlated with predictors of diseases progression, higher viral load and the reduced CD4 T cell count. In accordance with the finding by Barber *et al.* in mouse model, PD-1 expressing CD8 T cells are functionally exhausted and PD-1 blockade partially restored its function. Interestingly, CD4 T cells from same donors also expressed higher amount of PD-1 and its expression level showed correlation with predictors of disease progression. Although all three



Fig. 2. Clinical application of PD-1 agonists and antagonists. PD-1 agonists that suppress adverse immune responses may be useful in treating autoimmune diseases, allergy and transplant rejection. PD-1 antagonists that augment immune response may be useful in cancer and infectious diseases.

papers report that PD-1 blockade can augment the proliferation and cytokine production of CD8 T cells upon stimulation with HIV-peptide *in vitro*, there are several discrepancies. Day *et al.* and Trautmann *et al.* observed an inverse correlation between PD-1 expression and T cell function, suggesting that PD-1-expressing cells are functionally exhausted, whereas Petrovas *et al.* found no functional difference between PD-1 high and low CD8 T cells. Further analyses are needed to understand the real contribution of PD-1-dependent immune suppression in HIV infection. In addition to HIV infection, Urbani *et al.* and Radziewicz *et al.* reported that PD-1-expressing exhausted CD8 T cells are also found in hepatitis C virus-infected patients (73, 74). These findings gave a bright prospect for the future application of PD-1 blocker for chronic infectious diseases.

Although clinical trials on the blocking antibody against CTLA-4 and CTLA-4-Ig fusion protein (abatacept) have proved their efficacy in the treatment of cancer and RA, respectively (121–124), manipulations of co-stimulatory signals can result in tragic consequences (125). Actually, PD-L1 knockout mice succumb to LCMV-clone 13 infection with immunopathologic damage in a week (120). This is in apparent contradiction to the beneficial effect of blocking the PD-1-PD-L1 interaction during the chronic phase of LCMV-clone 13 infection. Although $Pdcd1^{-/-}$ mice also develop severe hepatitis at the initial phase of adenovirus infection, Pdcd1-/mice recover guickly and their hepatocytes fully regenerate within 30 days (117). That opposite long-term effects observed in PD-L1 and PD-1 knockout mice are probably due to a difference in the capacity for regeneration of the affected organs and the replication rate of the viruses in the two models

There may be a limited period of time during which the host immune system can eliminate the virus without damaging host tissue. When the host immune system cannot eliminate viruses within that period of time, PD-1 expression is increased to suppress an excessive immune response that would lead to tissue destruction. The recent report by Isogawa *et al.* (126) indicating that the anti-viral response oscillates in response to hepatitis B virus (HBV) infection

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may also support this scenario. HBV-specific CD8 T cells from an immune donor rapidly migrate into the liver, the site of HBV replication, and secrete IFN_γ which leads to the suppression of HBV replication within 24 h. However, following this initial phase, CD8 T cells lose the ability to produce IFN_γ and instead begin to produce granzyme B, which leads to the acquisition of cytolytic activity against infected hepatocytes. Because PD-1 expression is observed at the same time as the emergence of granzyme B-positive T cells and persists after the disappearance of viral gene expression at day 7, PD-1 is thought to regulate the oscillatory response of HBV-specific CD8 T cells. Further analyses may reveal the involvement of PD-1 in cytokine production, cytolytic activity and lifespan of anti-viral T cells in acute and chronic viral infections.

Concluding remarks

Since the discovery of PD-1 in 1992, the biological function of PD-1 remained mystery for many years. Generation of Pdcd1-/- mice and the discovery of its ligands turned around the situation and the function of PD-1 was unveiled thick and fast in these 5 years. Consequently, it became clear that PD-1 plays critical roles in the regulation of autoimmunity, tumor immunity, infectious immunity, transplantation immunity, allergy and immune privilege. The development of autoimmune diseases by *Pdcd1^{-/-}* mice especially enchanted clinicians and promoted clinical research as well. Currently, many groups are trying to generate not only PD-1 antagonists for the treatment of cancer and infectious diseases but also PD-1 agonists for the treatment of autoimmune diseases, allergy and transplant rejection (Fig. 2). Among these, humanized antibody against human PD-1 was approved by Food and Drug Administration of the United States as an investigational new drug in August 1, 2006. Clinical trials will test its clinical efficacy on cancer and infectious diseases.

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Abbreviations

B and T lymphocyte attenuator
cardiac troponin I
dendritic cell
dilated cardiomyopathy
dendritic cell-specific inducible expression of T cell epitopes by recombination
experimental encephalomyelitis
embryonic stem
granulocyte macrophage colony-stimulating factor
hepatitis B virus
human rhinovirus

ITIM	immunoreceptor tyrosine-based inhibitory motif
ITSM	immunoreceptor tyrosine-based switch motif
LCMV	lymphocytic choriomeningitis virus
lpr	lymphoproliferative disorder
MOG	myelin oligodendrocyte glycoprotein
NOD	non-obese diabetic
PD-1	programmed cell death-1
PTEN	phosphatase and tensin homolog
RA	rheumatoid arthritis
RUNX1	runt-related transcription factor 1
SLE	systemic lupus erythematosus
SNP	single-nucleotide polymorphism
TNFα	tumor necrosis factor a

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