Establishment of NOD-Pdcd1<sup>−/−</sup> mice as an efficient animal model of type I diabetes

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Mice deficient in programmed cell death 1 (PD-1, Pdcd1), an immunoinhibitory receptor belonging to the CD28/cytotoxic T lymphocyte-associated antigen-4 family, spontaneously develop lupus-like autoimmune disease and autoimmune dilated cardiomyopathy on C57BL/6 and BALB/c backgrounds, respectively. However, how PD-1 deficiency induces different forms of autoimmune diseases on these two strains was unknown. Here, we report that PD-1 deficiency specifically accelerates autoimmune predisposition of the background strain, leading to the induction of different forms of autoimmune diseases depending on the genetic background of the strain. Using NOD-Pdcd1<sup>−/−</sup> mice as an efficient animal model of type I diabetes, we screened diabetes-susceptible loci by genetic linkage analysis. The diabetic incidence of NOD-Pdcd1<sup>−/−</sup> mice was controlled by five genetic loci, including three known recessive loci [Idd (insulin-dependent diabetes) 1, Idd17, and Idd20] and two previously unidentified dominant loci [Iddp (Idd under PD-1 deficiency) 1 and Iddp2].

autoimmunity | coreceptor | Idd locus | Th1 | linkage analysis

Although many diabetes-susceptible loci have been identified by using NOD mice, the identification of their responsible genes and/or the analyses of the immunological function of each locus have not been carried out smoothly. The difficulty is likely due in part to the late onset and the low penetrance of type I diabetes in NOD mice (40–70% and 20–40% at 30 weeks of age for females and males, respectively) and also to the involvement of many genes. Therefore, the establishment of a better animal model of type I diabetes is required for efficient and refined genetic analyses of type I diabetes. In addition, the low penetrance of the disease in the NOD mouse made the linkage analyses possible only with BC1 (backcross 1) progenies by backcrossing F1 mice on NOD mice, by which dominant loci could not be analyzed (7).

Programmed cell death 1 (PD-1, Pdcd1), an immunoreceptor belonging to the CD28/cytotoxic T lymphocyte-associated antigen-4 family, provides negative costimulation by recruiting a protein tyrosine phosphatase, SHP-2 (src homology 2 domain-containing tyrosine phosphatase 2), upon interaction with its ligands, PD-L1 and PD-L2 (8–10). Negative costimulation is required to suppress inappropriate immune responses such as autoimmune and sustained inflammation. PD-1 knockout mice (Pdcd1<sup>−/−</sup> mice) develop various autoimmune diseases depending on the genetic background. C57BL/6-Pdcd1<sup>−/−</sup> mice develop lupus-like glomerulonephritis and arthritis (11); BALB/c-Pdcd1<sup>−/−</sup> mice produce anti-cardiac troponin I autoantibodies, which are responsible for dilated cardiomyopathy (12, 13). The development of different kinds of autoimmune disease on different genetic backgrounds by PD-1 deficiency lead us to assume that PD-1 deficiency may exaggerate the genetic predisposition of autoimmune diseases such as diabetes in NOD mice.

Here, we report that NOD-Pdcd1<sup>−/−</sup> mice developed type I diabetes by 11 weeks with complete penetrance. Genetic linkage analyses on BC1 backcross progenies of NOD-Pdcd1<sup>−/−</sup> mice revealed that only 3 loci showed significant association with the diabetic incidence among the 28 Idd loci described previously using original NOD mice. Using NOD-Pdcd1<sup>−/−</sup> mice, we found that 20% of F2 intercross progenies developed diabetes by 15 weeks of age, allowing us to identify two previously unrecognized dominant susceptible loci, Iddp (Idd under PD-1 deficiency) 1 and Iddp2.

Materials and Methods

Animals. NOD/ShiJic mice were purchased from Japan Clea (Hamamatsu, Japan). NOD-Pdcd1<sup>−/−</sup> mice were generated by backcrossing C57BL/6-Pdcd1<sup>−/−</sup> mice (11) on NOD WT mice five to eight times. All experiments except for the diabetes incidence experiment (Fig. 1 a and b) were done with eighth-generation mice. F2 progenies were generated by crossing (NOD-Pdcd1<sup>−/−</sup> × C57BL/6-Pdcd1<sup>−/−</sup>)F1 mice with F1 mice. BC1 progenies were generated by crossing (NOD-Pdcd1<sup>−/−</sup> × C57BL/6-Pdcd1<sup>−/−</sup>)F1 mice with NOD-Pdcd1<sup>−/−</sup> mice. Pdcd1<sup>−/−</sup> mice were chosen for subsequent analyses. All animals were maintained under specific pathogen-free conditions at the Institute of Laboratory Animals at the Graduate School of Medicine, Kyoto University, and all mouse protocols were approved by the Institute of Laboratory Animals.

Histological Analysis. Insulitis was scored according to the published criteria (14). Briefly, grade 1 represents periinsulitis with 20% of the area of each islet, grade 2 represents moderate insulitis with 40–70% of the area of each islet, and grade 3 represents severe insulitis with mononuclear cell infiltration in >50% of the area of each islet. Sialoadenitis was scored accord-

Abbreviations: PD-1, programmed cell death 1; NOD, nonobese diabetic; Idd, insulin-dependent diabetes; Iddp, Idd under PD-1 deficiency; BC1, backcross 1; Iod, logarithm of odds; Th, T helper; DC, dendritic cell.

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and NOD-Pdcd1-deficient female mice were backcrossed C57BL/6-Pdcd1−/− mice on NOD mice for more than five generations and were monitored for the incidence of diabetes. As shown in Fig. 1a, some of the female NOD-Pdcd1−/− mice became diabetic from 5 weeks of age, and the penetration of the disease reached 100% by 10 weeks. The diabetic phenotypes of the female PD-1-deficient NOD-Lpr mice were milder, starting from 13 weeks of age and reaching 60% penetration by 24 weeks of age. By contrast, female NOD-WT littermates started to become diabetic from 17 weeks of age, and only 30% of them developed diabetes by 24 weeks of age. The acceleration of NOD diabetes by PD-1 deficiency is more evident in male animals (Fig. 1b). None of male NOD mice developed diabetes by 24 weeks of age, whereas male NOD-Pdcd1−/− mice began to develop diabetes from 5 weeks of age, and all of them became sick by 11 weeks. Again, the PD-1 heterozygotes showed milder but significant acceleration of diabetic incidence. Male NOD-Pdcd1−/− mice started to develop diabetes from 5 weeks of age, and all of them became sick by 11 weeks. Again, the PD-1 heterozygotes showed milder but significant acceleration of diabetic incidence. Male NOD-Pdcd1−/− mice started to
become diabetic from 13 weeks of age, and 40% of them developed diabetes by 24 weeks of age. Thus, the onset and frequency of type 1 diabetes in NOD mice were highly accelerated by the disruption of the PD-1 gene.

We next examined insulitis in NOD-Pdcd1−/− mice. Mild periinsulitis was detectable from 2 weeks of age, and destructive insulitis was already found in 21% of the islets by 4 weeks of age in female NOD-Pdcd1−/− mice (Fig. 1c and data not shown). At 6 weeks, 82% of the islets were affected with severe insulitis (grades 2 and 3) in female NOD-Pdcd1−/− mice, whereas <1% of the islets were affected with severe insulitis in female NOD WT mice (Fig. 1c and e). Mean insulitis scores of NOD-Pdcd1−/−, NOD-Pdcd1+/−, and NOD WT mice at 6 weeks were 0.4, 0.7, and 2.5, respectively (Fig. 1d). The results indicate that more severe insulitis, leading to earlier destruction of beta cells, is responsible for the early onset of diabetes in NOD-Pdcd1−/− and NOD-Pdcd1+/− mice.

NOD mice also develop sialoadenitis, similar to the human Sjögren’s syndrome (19). As shown in Fig. 1e, sialoadenitis was also accelerated by PD-1 deficiency. The acceleration was more evident in parotid glands than in submandibular glands (mean score 5.2 in female NOD-Pdcd1−/− mice vs. 2.4 in female NOD WT mice, P = 0.05).

Increased Invasion of CD8+ T Cells in NOD-Pdcd1−/− Mice. In addition to the increase in the number of lymphocytes in islets of NOD-Pdcd1−/− mice (Fig. 1c and d), CD4+ and CD8+ T cells seemed to be more invasive; they were always found deeper inside islets in NOD-Pdcd1−/− mice than in NOD WT mice at the same degrees of inflammation stage (Fig. 2a) and higher frequency of type I diabetes in NOD mice were highly accelerated by the disruption of the PD-1 gene. The acceleration was more evident in parotid glands than in submandibular glands (mean score 5.2 in female NOD-Pdcd1−/− mice vs. 2.4 in female NOD WT mice, P = 0.05).

It was reported that PD-L1, but not PD-L2, was up-regulated on beta cells (Left) and glucagon-producing alpha cells (Right) in prediabetic NOD WT mice. Red signal represents IgM+ B cells (Left) and CD11c+ DCs (Right). Green signal represents PD-L1+ cells. (d) PD-L1 is expressed on beta cells (Left) and glucagon-producing alpha cells (Right) in prediabetic NOD WT mice. Red signal represents insulin+ beta cells (Left) and glucagon+ alpha cells (Right). Green signal represents PD-L1+ cells. (d and e) CD8/CD4 ratio of islet infiltrates. (d) Representative FACS profiles of islet infiltrates are shown for prediabetic NOD WT and NOD-Pdcd1−/− mice. (e) Mean CD8/CD4 ratios are shown. Error bars represent standard error.

It was reported that PD-L1, but not PD-L2, was up-regulated on islets upon insulitis in NOD WT mice by immunohistochemistry (21, 22). Because the cell types that express PD-L1 were not determined in the previous reports, we performed immunohistofluorescence using monoclonal antibodies against PD-L1 and PD-L2. PD-L1 was expressed strongly on beta cells as well as CD11c+ dendritic cells (DCs) and moderately on alpha cells, whereas T cells and B cells did not express PD-L1 (Fig. 2a–c).

Interestingly, the expression of PD-L1 on beta cells was stronger at the boundary with T cells than inside of islets in NOD WT mice, suggesting that beta cells may suppress activities of PD-1-expressing T cells at the boundary to prevent the invasion of T cells into islets (Fig. 2a). Actually, the expression of PD-1 was detected on a small fraction of infiltrating CD4+ and CD8+ T cells of NOD WT mice and up-regulated by in vivo blockade of PD-L1 using anti-PD-L1 antibody, suggesting that the expression of PD-1 is not stable in the presence of a continuous interaction with its ligand (data not shown). The expression of PD-L1 was detected more highly and diffusely on islets in NOD-Pdcd1−/− mice than in NOD WT mice (Fig. 1b). As shown in Fig. 1c, sialoadenitis was also accelerated by PD-1 deficiency.
mice, which is probably due to more severe insulitis in NOD-Pdcd1KO mice. Similar to NOD WT mice, PD-L1 was expressed on beta cells, CD11c+ DCs, and alpha cells but not on T cells and B cells in NOD-Pdcd1KO mice (data not shown). The expression of PD-L2 was not detected in islets of NOD-Pdcd1KO mice, consistent with the reports on NOD WT mice (refs. 21 and 22 and data not shown).

Islet T Cell Infiltrates Are Strongly Polarized Toward T Helper (Th) 1. Accumulating evidence indicates that Th1 cytokines exacerbate diabetes, whereas Th2 cytokines protect the development of diabetes in NOD mice (23). Thus, we analyzed the Th1/Th2 balance of islet infiltrates in NOD-Pdcd1KO mice. Infiltrating T cells from disease stage-matched prediabetic pancreata of NOD WT (12–15 weeks) and NOD-Pdcd1KO (5–7 weeks) mice were isolated and stimulated in vitro for 5 h with phorbol 12-myristate 13-acetate/ionomycin to enhance the cytokine production. The numbers of recovered cells were not different between prediabetic NOD-Pdcd1KO and NOD WT mice in accordance with the matched stages of the insulitis in these mice. As shown in Fig. 3 a and b, a far larger fraction of T cells from NOD-Pdcd1KO islets produced IFN-γ than those from NOD WT islets. The percentage of IL-4-producing cells was not changed in NOD-Pdcd1KO islets, compared with NOD WT islets. These results indicate that Th1 response is enhanced in the NOD pancreas in the absence of PD-1. Because the increase of IFN-γ-producing cells was not observed in spleen and pancreatic lymph nodes (Fig. 3c), the Th1 polarization seems to occur specifically on diabetogenic T cells in islets in NOD-Pdcd1KO mice, which should have been suppressed by the interaction of PD-1 on diabetogenic T cells with PD-L1 on beta cells and/or tissue-resident antigen-presenting cells in NOD WT mice (Fig. 2 a–c).

Only 3 of 28 Idd Loci Showed Association with Diabetic Incidence by Linkage Analyses Using NOD-Pdcd1KO Mice. Because NOD-Pdcd1KO mice have better features for the type I diabetes model than NOD mice (quick onset and complete penetration), we made intercrosses of NOD-Pdcd1KO mice and C57BL/6-Pdcd1KO mice and performed linkage analysis. We made 201 BC1 progenies by backcrossing (NOD-Pdcd1KO × C57BL/6-Pdcd1KO)F1 mice on NOD-Pdcd1KO mice and monitored for the incidence of diabetes for 24 weeks. Seventy-four microsatellite markers were used for the first screening (mean interval = 18.5 cm). As shown in Fig. 4 a–d, ~40% of BC1 progenies developed diabetes by 15 weeks of age. Genetic linkage analyses on BC1 progenies revealed that development of type I diabetes was strongly associated with the D17mit239 and D3mit244 polymorphic markers and weakly associated with the D6mit263 polymorphic marker, which reside in close proximity with Idd11, Idd17, and Idd20, respectively (Fig. 4 b–d). By contrast, all of the other known Idd loci showed no association with the development of type I diabetes in NOD-Pdcd1KO mice.

Identification of Previously Unrecognized Dominant Loci by Genome-Wide Screening for Dominant Diabetes-Susceptible Loci. Because 2% intercross progenies of NOD WT mice with either of the strains rarely develop diabetes, dominant diabetes-susceptible loci have not been analyzed throughout the genome (7). Because of the high diabetic incidence in NOD-Pdcd1KO mice, dominant diabetes-susceptible genes were screened by F2 crosses. As shown in Fig. 5 a, ~20% of the (NOD-Pdcd1KO × C57BL/6-Pdcd1KO) F2 progenies developed diabetes by 15 weeks of age. Genetic linkage analyses for dominant loci on 184 F2 progenies using MAPMANAGER QTX. Arrows indicate the positions of microsatellite markers used for genotyping. The lod score is given on the y axis. Dashed lines indicate the suggestive lod score of 1.9 and the significant lod score of 3.3 (31).

![Fig. 3.](image)

![Fig. 4.](image)

**Fig. 3.** Th1 response is augmented in the islets of NOD-Pdcd1KO mice. (a and b) Islet-infiltrating T cells were collected and examined for the production of IFN-γ and IL-4. (a) Representative FACS profiles of NOD WT (Left) and NOD-Pdcd1KO (Right) mice are shown. (b) Mean percentage of IFN-γ-producing cells (Left) and IL-4-producing cells (Right) are shown. White bars represent NOD WT mice and black bars represent NOD-Pdcd1KO mice. Error bars represent standard error. (c) Production of IFN-γ (Left) and IL-4 (Right) by splenocytes (spl) and pancreatic lymph node cells (pLN) was examined as above. Error bars represent standard error.

**Fig. 4.** Genetic association of Idd1, Idd17, and Idd20 with diabetic incidence of NOD-Pdcd1KO mice. (a) Diabetic incidence of BC1 backcross progenies (NOD-Pdcd1KO × C57BL/6-Pdcd1KO)F1 × NOD-Pdcd1KO. Filled circles, open circles, and triangles represent cumulative diabetic incidence of NOD-Pdcd1KO and C57BL/6-Pdcd1KO mice and their BC1 progenies, respectively. (b–d) Logarithm of odds (lod) score plots for individual chromosomes containing the putative locus controlling diabetic incidence in BC1 progenies. The length of each chromosome is adjusted to the same size, although the centimorgan length of each chromosome varies. Genetic maps were constructed by using the program qtx. Arrows indicate the positions of microsatellite markers used for genotyping. The lod score is given on the y axis. Dashed lines indicate the suggestive lod score of 1.9 and the significant lod score of 3.3 (31).
are destined to be partially immunodeficient, because their islet antigen. The islet antigen-specific TCR transgenic mice and the reduced titer of serum autoantibodies against GAD65, activity of regulatory T cells. However, these mice seem to have the acceleration of diabetes to the impaired production and regulation did not show any positive association in the current linkage analyses on PD-1-deficient mice. However, Idd loci that showed positive association in the current study may be involved in determination of organ specificity toward beta cells in pancreatic islets.

Strong acceleration of diabetes by PD-1 deficiency enabled us to identify previously unrecognized diabetes-susceptible loci (designated Iddp1 and Iddp2), which functioned in a dominant fashion. According to the criteria of statistical significance proposed by Lander and Kruglyak (31), dominant loci analyzed with F2 progenies should have lod scores of $>2.0$ for suggestive linkage and $3.4$ for significant linkage (31). Although Iddp1 showed only suggestive linkage in the current analysis on 184 F2 progenies, lack of diabetic incidence among F2 progenies homozgyous for C57BL/6 at Iddp1 (1 of 44) implies the high necessity of this locus for diabetic incidence. Because the diabetic incidences of F2 progenies heterozygous or homozygous for NOD at Iddp1 (29.4% and 21.8%, respectively) are only slightly higher than those of total F2 progenies (20.7%), the sufficiency of this locus for diabetic incidence seems to be not so high, which may be a reason for the low lod value for this locus. Hence, Iddp1 seems to have a unique property with its essential but not sufficient role in the diabetic incidence of NOD-Pdcd1$^{-/-}$ mice. The neighboring region of Iddp1 contains several genes involved in immune responses, such as caspase 6, small inducible cytokine subfamily E member 1 (casy1), NF-$\kappa$B1, p50, complement component factor I (efi), B cell leukemia/lymphoma 10 (Bcl10), and prostaglandin F receptor. This region has also been reported to have a linkage with susceptibility to experimental allergic encephalomyelitis (eae10) and resistance to systemic lupus erythematosus (Sles3) using SJL/J and NZW mice, respectively (32, 33).

Iddp2 showed a significant association with the diabetic incidence of NOD-Pdcd1$^{-/-}$ mice. F2 progenies homozygous for C57BL/6 at Iddp2 were protected from diabetes completely (0 of 53), which assures the high necessity of this locus for diabetic incidence. Compared with Iddp1, Iddp2 showed the dosage effect on the diabetic incidence (0, 20.1, and 45.5% for B6/B6, B6/NOD, and NOD/NOD, respectively), suggesting moderate sufficiency of Iddp2 on the diabetic incidence of NOD-Pdcd1$^{-/-}$ mice. The comparable region of Iddp2 on the human chromosome locates on the short arm of chromosome 19, which contains a type I diabetes-susceptible locus found in sibling pair families from the United Kingdom (19p13) (34). Although the linkage in the human study is not so strong (maximum lod score = 1.7), there might be a responsible gene common in human and mouse. The candidate genes for the Iddp2 locus include IL-27, vavl, complement component 3, SH3 domain GRB2-like 1 (sh3g1l), regulatory factor times 2 (rft2), TNF ligand superfamily members 7, 9, and 14 (CD70, 4-1BB ligand, and LIGHT, respectively), and thyroid hormone receptor interactor 10 (trip10). Although database searches revealed the existence of some missense mutations and deletions in some of the listed genes of NOD mice, currently it is not clear which of the candidate genes are responsible for these loci. Generation of congenic mice for these regions may allow us to identify responsible gene(s) in these loci. These congenic mice may also give us an answer to whether these loci are involved in the diabetic incidence of original NOD mice or just modify the autoimmune response in the absence of PD-1.

Iddp1 and Iddp2 appear to be essential for the development of diabetes.

**Discussion**

Here, we report that NOD-Pdcd1$^{-/-}$ mice develop type I diabetes by 11 weeks of age, with 100% penetration and marked Th1 polarization of islet infiltrates. Augmentation of Th1, but not Th2, response in NOD-Pdcd1$^{-/-}$ mice is consistent with the observation on PD-L1-deficient (Pdcd1lg1$^{-/-}$) mice. Latchman et al. (24) reported that DCs from Pdcd1lg1$^{-/-}$ mice induced the augmented production of IFN-$\gamma$ but not IL-4 in an allogenic mixed lymphocyte reaction compared with DCs from WT mice (24). Several other genetic manipulations on NOD mice have been shown to boost occurrence of diabetes to nearly 100%, including B7.1/B7.2 double knockout and islet antigen-specific T cell receptor (TCR) transgenesis (25–27). In the case of B7.1/B7.2 double-knockout mice, Salomon et al. (26) attributed the acceleration of diabetes to the impaired production and activity of regulatory T cells. However, these mice seem to have a wide range of defects in the immune system, as evidenced by the reduced production of both Th1 and Th2 cytokines by T cells and the reduced titer of serum autoantibodies against GAD65, an islet antigen. The islet antigen-specific TCR transgenic mice are destined to be partially immunodeficient, because their CD8$^+$ T cells have a single specificity. Compared with these mice, the immune system of NOD-Pdcd1$^{-/-}$ mice are not deviated as we have discussed above; therefore, NOD-Pdcd1$^{-/-}$ mice may serve as a useful animal model to analyze the cellular and genetic pathology of type I diabetes.

The requirement of Idd1, Idd17, and Idd20 loci for the diabetic incidence of NOD-Pdcd1$^{-/-}$ mice suggests that diabetic incidence in NOD WT and NOD-Pdcd1$^{-/-}$ mice is regulated by similar polymorphic genes, at least in part. However, the other Idd loci did not show association with diabetic phenotypes in NOD-Pdcd1$^{-/-}$ mice. Many of the Idd loci are supposed to regulate any of the following immune responses: differentiation and expansion of the autoreactive lymphocytes, migration of these autoreactive cells into the pancreas, in situ activation of these cells, and destruction of beta cells. Previously, we have reported that PD-1 deficiency augments general immune responses by facilitating the beta selection of T cells in thymus (28), augmenting the activation and proliferation of T and B cells (11, 20) and enhancing the cytotoxic activity of CD8$^+$ T cells (29, 30). PD-1 deficiency may thus overcome the absence of some of the Idd loci, which contribute to the diabetic incidence by enhancing general immune responses. If this is the case, it is reasonable that some of the Idd loci associated with the general immune regulation did not show any positive association in the current linkage analyses on PD-1-deficient mice.
Serreze et al. (35) analyzed the (NOD \times NOR)F2 progenies and found Idd13, which functioned in a dominant fashion. However, only 11.6% of the whole mouse chromosome could have been analyzed in their study, because the NOR genome shares \approx88.4% identity with the NOD genome. McAleer et al. (36) analyzed the (NOD \times NON.H2\textsuperscript{b})F2 progenies and found Idd14 and Idd15, which also functioned in a dominant fashion. Because NON mice have been segregated from the same colony as NOD mice as a nonidiabetic line, their linkage analysis also could not have covered the whole mouse genome. According to their reports, regions around Iddp1 and Iddp2 are derived from NOD mice in NOR mice, and the region around Idd2p is derived from NOD mice in NON.H2\textsuperscript{b} mice. Therefore, they could not have identified Iddp1 and Iddp2 as dominant loci in their analyses. In the current study, Idd13, Idd14, and Idd15 did not show positive association. It is probable that the strong enhancement of immune response by PD-1 deficiency overcomes the absence of these Idd loci, as we have discussed for recessive loci. The usage of different strains for control may also explain the negative association of Idd14 and Idd15.

Ancsari et al. (21) reported that the injection of antibodies against PD-1 or PD-L1, but not PD-L2, into NOD mice accelerated the insulitis and subsequent development of diabetes, suggesting the negative regulatory role of PD-1/PD-L1 in the diabetic incidence of the NOD mouse. Consistent with this report, current findings clearly demonstrated that the PD-1 deficiency accelerates insulitis and subsequent diabetes in the NOD mouse. However, Subudhi et al. (37) found costimulatory function of PD-L1 in the autoimmune response against islet antigens by generating PD-L1 transgenic mice under the rat insulin promoter, which spontaneously developed insulitis and subsequently diabetes. One possible explanation for this discrepancy may be a dominant-negative effect of transgenic PD-L1, which may bind but not transduce signal for some unknown reason.

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