Blockade of the Programmed Death-1 (PD1) Pathway Undermines Potent Genetic Protection from Type 1 Diabetes

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Abstract

Aims/Hypothesis: Inhibition of PD1-PDL1 signaling in NOD mice accelerates onset of type 1 diabetes implicating this pathway in suppressing the emergence of pancreatic beta cell reactive T-cells. However, the molecular mechanism by which PD1 signaling protects from type 1 diabetes is not clear. We hypothesized that differential susceptibility of Idd mouse strains to type 1 diabetes when challenged with anti PDL1 will identify genomic loci that collaborate with PD1 signaling in suppressing type 1 diabetes.

Methods: Anti PDL1 was administered to NOD and various Idd mouse strains at 10 weeks of age and onset of disease was monitored by measuring blood glucose levels. Additionally, histological evaluation of the pancreas was performed to determine degree of insulitis. Statistical analysis of the data was performed using Log-Rank and Student’s t-test.

Results: Blockade of PDL1 rapidly precipitated type 1 diabetes in nearly all NOD Idd congenic strains tested, despite the fact that all are moderately (Idd5, Idd3 and Idd10/18) or highly (Idd3/10/18 and Idd9) protected from spontaneous type 1 diabetes by virtue of their protective Idd genes. Only the Idd3/5 strain, which is nearly 100% protected from spontaneous disease, remained normoglycemic following PDL1 blockade.

Conclusions: These results indicate that multiple Idd loci collaborate with PD1 signaling. Anti PDL1 treatment undermines a large portion of the genetic protection mediated by Idd genes in the NOD model of type 1 diabetes. Basal insulitis correlated with higher susceptibility to type 1 diabetes. These findings have important implications since the PD1 pathway is a target for immunotherapy.

Introduction

Type 1 diabetes is a multi-factorial autoimmune disease resulting from the destruction of pancreatic beta cells by autoreactive T cells. Both environmental factors and variations in multiple genetic loci have been implicated in the etiology of type 1 diabetes. The NOD mouse recapitulates many features of human type 1 diabetes and is used extensively as an experimental model.

Programmed death-1 (PD1) and its ligand PDL1 have been shown to play an important role in regulating T cell activation and peripheral tolerance. The PD1- PDL1 pathway is being explored for developing therapies against recurrent solid tumors and infectious diseases (such as HIV), since blocking the pathway results in an increased immune response against tumors and infections [1–3].

We and others have shown that PD1-PDL1 interaction is critical for the regulation of CD4 and CD8 autoreactive T cells involved in the development of type 1 diabetes [4,5]. Further, while PD1 deficiency resulted in lupus-like symptoms in C57BL6 or BALB/c mice, it led to accelerated onset and frequency of type 1 diabetes in NOD mice [6].

In the NOD mouse model, blockade of PD1-PDL1 pathway results in accelerated onset of autoimmune diabetes, raising concern that immunotherapy by such blockade could increase...
susceptibility to autoimmune diseases, particularly in individuals harboring susceptibility alleles. To date, numerous MHC-linked and non-MHC-linked genes and genetic regions influencing the susceptibility to autoimmune diseases have been identified in humans, rats, and mice. In insulin dependent type 1 diabetes, many genes implicated in the control of glycemia have also been described in the NOD Idd congenic mouse strains. Congenic NOD strains have genetic loci from diabetes resistant parental strains inserted (introgressed) into their genome (reviewed in [7]).

In recent years, NOD H2-Ag7 and H2-Emull MHC class II genes have been unequivocally identified as susceptibility genes within Idd2 [8]. Additionally, accumulated data support the existence of particular susceptibility genes within other Idd regions.

Idd3 is the most well studied Idd region [9-11]. Protective alleles in Idd3 reduce type 1 diabetes frequency and B2 and B21 are the prime candidate genes. The protective effects of Idd3 are evident in multiple cell types including antigen-presenting cells, effector T cells and regulatory (FoxP3+) T cells which are critical for maintaining immune cell homeostasis [12,13].

The prime candidate gene for Idd10 is Cd104 whose expression on regulatory T cells and dendritic cells is affected in NOD/B6 polymorphisms [14]. Vav3, which encodes a guanine nucleotide exchange factor important for signaling in immune cells, is the only complete gene present in the 604 kb Idd18.1 region on chromosome 4. Gene expression evidence indicates that alteration of Vav3 expression is an etiological factor in the development of autoimmune beta-cell destruction in NOD mice, making it the most likely candidate [15]. The Idd5 region is composed of at least 5 sub-regions. Idd5 contributes to islet-specific CD8 T cell tolerance and to loss of CD4 T-cell tolerance between both lymphocytic and non-lymphocytic compartments [9,16,17]. Candidate genes for Idd5 sub-regions include Cldn4 for Idd5.1 [18], Skil1a1 for Idd5.2 [19] and Acadl for Idd5.3 [20]. The Idd9 region on chromosome 4 is composed of at least three separate intervals, Idd9.1, Idd9.2, and Idd9.3 and numerous candidate genes are present. Fine mapping of type 1 diabetes regions Idd9.1 and Idd9.2 revealed further genetic complexity [21]. The Idd9.1 sub-region has been shown to influence regulatory T cells and iNKT cells [22,23]. Idd9.2 and Idd9.3 have been linked to limit the expansion of islet specific autoreactive CD8 T cells [24]. The Idd9.3 candidate gene encodes 4-1bb, which is important for CD4 and CD8 T cell activation [25]. The Idd9 locus has also been previously described to play a role in homing of islet-specific T cells [26]. Overall, Idd9 mice display profound resistance to diabetes even though nearly all develop insulin.

In this study, we made use of four loci on Chromosome 3, four on Chromosome 1, and three on Chromosome 4 to determine which Idd regions conferring resistance to type 1 diabetes remain so in the presence of anti PDL1 negative co-stimulatory blockade. We show that blockade of the PD1-PDL1 interaction results in accelerated onset of type 1 diabetes in all the NOD Idd strains except NOD Idd3/3. Additionally, basal insulin levels correlated with higher susceptibility to type 1 diabetes induction by anti PDL1 treatment.

Methods

Mice

Female NOD mice were obtained from Taconic (Germantown, NY, USA). NOD congenics were obtained through the Taconic Emerging Models program; NOD.B10-Idd9.1/9.2/9.3 (line 905) [27], NOD.B10-Idd9.1 (line 1565) [22], NOD.B10-Idd9.2 (line 1566) [22], NOD.B10-Idd9.3 (line 1106) [22], NOD.B6-Idd10/18 (line 7754) [14,15,27-30], NOD.B10-Idd5.1 Idd5.2 Idd5.3 (line 1094) [31], NOD.B10-Idd5.1 (line 2193) [31], NOD.B10-Idd5.2 (line 6146) [31], NOD.B10-Idd5.3 (line 6360) [32], NOD.B6-Idd3/ Idd10/18 (line 1530) [15,27], NOD.B6-Idd3 (line 1098) [12,27], NOD.B6-Idd3 B10-Idd5 (line 6109) [27], NOD.B10-Idd5.2 Idd5.3 (line 1395) [32] and NOD.B10-Idd5.2 Idd5.3 Idd3 (lines 7380) and 9245, data combined in this study [33]. The NOD congenic strains will be referred to by their Idd numbers without adding NOD before the designated Idd region. When referring to congenic mice containing two or more Idd loci, the loci will be separated by slashes. For example, Idd3.1 Idd9.2 Idd9.3 (line 905) mice will be referred to as Idd9.1/9.2/9.3 for simplicity.

Antibodies and Treatment Protocol

Anti mouse PDL1 mAb (MIH6, rat IgG2a) was generated as previously described, [35] and was manufactured by BioXCell (West Lebanon, NH, USA). Rat IgG (Sigma-Aldrich, St.Louis, MO, USA) served as a control. Anti PDL1 was injected in PBS i.p.; 500 μg on day 0, followed by 250 μg on days 2, 4, 6, 8, and 10 unless indicated otherwise. Mice were 10 weeks of age at the start of treatment.

Monitoring for Diabetes

The onset of type 1 diabetes was defined as a random blood glucose reading of 250 mg/dl or greater for three consecutive days. Blood glucose levels were measured daily for the first two weeks followed by 2–3 times per week by One Touch Ultra meter and One Touch Ultra test strips (LifeScan, Milpitas, CA, USA).

Histology

Pancreases were fixed in 10% neutral buffered formalin in PBS for 16 h and transferred to 70% ethanol before being embedded in paraffin. Tissue sections were stained with H&E (Dana Farber Cancer Institute’s Research Pathology Core, Boston, MA, USA) and insulin was stained using a primary antibody for insulin and an anti-rabbit secondary antibody (Cedarlane, Burlington, NC, USA) and was developed with 0.05% 3,3’ diaminobenzidine tetrahydrochloride (DAB) and 0.001% hydrogen peroxide. Sections were then counterstained with hematoxylin (Dana Farber Cancer Institute’s Research Pathology Core, Boston, MA, USA) and coverslipped in Permount. For the analysis of MHC-II expression, sections were stained using a primary antibody for MHC-II (Dako, Santa Barbara, CA) and an anti-rabbit secondary antibody (Cedarlane, Burlington, NC, USA) and was developed with DAB. Sections were then counterstained with hematoxylin (Dana Farber Cancer Institute’s Research Pathology Core, Boston, MA, USA) and coverslipped in Permount.

Adoptive transfer of BDC2.5 TCR-transgenic cells

Anti-CD25 mAb (clone 7D4, ATCC, Manassas, VA, USA) and rabbit complement (Cedarlane, Burlington, NC, USA) were incubated with splenocytes of BDC2.5 TCR Tg mice at 37°C for 45 min to remove CD25+ cells (technique described in [36]). Remaining cells were labeled with 7.5 μM CFSE (Invitrogen, Carlsbad, CA, USA) and was manufactured by BioXCell (West Lebanon, NH, USA). Cells were injected i.v. into the tail veins of pre-diabetic 8–10 week old female NOD and age matched Idd3/10/18 mice. Mice received 500 μg of either anti PDL1 mAb or rat IgG one day before transfer (day 0), and 250 μg...
on days 2 and 4. Pancreatic LN and spleens were harvested on day 6 and cells were stained for CD4, Vβ4 (KT4, BD Biosciences, USA), labeled with CFSE and analyzed by flow cytometry.

Adoptive transfer of NY8.3 TCR transgenic cells

Splenocytes from NY8.3-NOD TCR Tg mice were used for adoptive transfer studies. Splenocytes were enriched for CD8+ T cells using the CD8+ T cell untouched isolation kit II (Miltenyi, Auburn, CA, USA). One million CD8+ T cells were injected iv. into the tail veins of pre-diabetic 8–10 week old female NOD and age matched Idd3/10/18 mice. The recipients received 500 µg of either anti PDL1 mAb or IgG Ab one day before transfer (day 0), and 250 µg on days 2 and 4. The pancreatic lymph node and spleen were harvested on day 6 and the cells were acquired by flow cytometry for CFSE labeling.

RNA extraction and Real time PCR of pancreas tissue

Pancreas tissue from Idd9 mice was stored in RNAlater solution (Ambion, Austin, TX, USA) and total RNA was extracted using the RNeasy Mini kit (Qiagen, Gaithersburg, MD, USA). RNA was redissolved in RNase-free water and the yield quantified by spectrophotometry. Equal amounts of RNA were used for quantitative real time PCR. First strand cDNA synthesis was performed using Superscript III (Invitrogen, Grand Island, NY, USA). All reactions were run in triplicates in an ABI Prism 7300 (Applied Biosystems, Foster City, CA, USA) and normalized to GAPDH. For a list of primers used, see Electronic Supplemental Material.

Statistical Analysis

Kaplan-Meier survival analysis was performed to compare the frequency of diabetes in sub-congenic strains using the Log-Rank test. Differences in insulitis between congeneric strains were analyzed by unpaired two-tailed Student’s t test. A p value of < 0.05 was considered significant.

Results

Diabetes-resistant NOD Idd strains develop diabetes upon anti PDL1 treatment

In order to determine if blocking the PD1-PDL1 pathway would induce autoimmune diabetes in mice genetically protected from developing the disease, strains of mice protected from type 1 diabetes because they carry protective genes derived from B6 and B10 mice, were treated with anti PDL1 mAb. We tested the following 14 NOD congeneric strains to examine the genetic protection due to a range of genes and gene combinations which can possibly contribute to resist the precipitation of type 1 diabetes following PDL1 blockade: Idd3, Idd10/18, Idd3/10/18, Idd5 (which includes the subcongenic regions of Idd5.1, Idd5.2, and Idd5.3), Idd5.1, Idd5.2, Idd5.3, Idd5.2/5.3, Idd9, Idd9.1, Idd9.2 and Idd9.3. The incidence of diabetes for females from these 14 strains at 28 to 30 weeks of age are 5% (Idd3/10/18, Idd5, Idd3/Idd5.2/Idd5.3 and Idd9), 15–40% (Idd3, Idd9.1 and Idd5), and 45–65% (Idd5.1, Idd5.2, Idd5.3, Idd5.2/Idd5.3, Idd9.1, Idd9.2, Idd9.3, and Idd10/18). Throughout the time period of defining the Idd regions using congeneric strains that are resistant to type 1 diabetes (1990 to 2010) the NOD female diabetes incidence has ranged from 70–90% at 28 to 30 weeks of age.

Idd5

Untreated Idd5 mice have a 40% cumulative incidence of diabetes at 28 to 30 weeks of age [37]. With anti PDL1 treatment, 10-week old Idd5 mice started to develop the disease by day 10, and after 30 days, 62.5% had developed type 1 diabetes (Figure 1a, Table 1, 2). The sub-congenic strains Idd5.1, Idd5.2, Idd5.2/5.3 and Idd5.3 showed a higher onset of diabetes than day 7. The Idd5.3 strain had the highest cumulative incidence of type 1 diabetes following anti PDL1 treatment, with 90% of the mice developing diabetes by day 30 (P = 0.0140 Idd5 vs Idd5.3), followed by Idd5.2 with 80% cumulative incidence (P = 0.0194 Idd5 vs Idd5.2). Idd5.1 developed diabetes with a 66% cumulative incidence. The combination of two sub-congenic strains in Idd5.2/5.3 developed type 1 diabetes with a cumulative incidence of 65% (Figure 1a, Table 1, 2). Of the control NOD mice treated with anti PDL1 93% developed type 1 diabetes by day 21. None of the control NOD mice developed type 1 diabetes during the course of the experiment (Figure 1a, Table 1, 2).

Idd9, Idd9.1, Idd9.2, Idd9.3

Idd9 mice receiving anti PDL1 treatment developed type 1 diabetes with a cumulative incidence of 46% between days 6 and 16. The sub-congenic strain Idd9.2 showed a reduced cumulative incidence of diabetes at 56% (between days 4–16), whereas the Idd9.1 and Idd9.3 strains had a much higher cumulative incidence with 95% and 90% respectively (onset from day 3 to day 22), which is quite similar to 93% type 1 diabetes in anti PDL1 treated NOD mice (between days 4–12) (Figure 1b, Table 1, 2). As the Idd9.2 strain had the lowest cumulative incidence among Idd9 subcongeneric strains, we deduced that this sub-congenic strain must be associated with the protective allele in the Idd9 congenic interval.

Idd3, Idd10/18 and Idd3/10/18

Twenty percent of Idd3 mice spontaneously develop type 1 diabetes within 7–8 months [37]. Upon anti PDL1 administration, 50% of the mice developed type 1 diabetes between days 6 and 16 (Figure 1c). Idd10/18 mice have a 50% occurrence of spontaneous diabetes, and with anti PDL1 treatment 94% of mice developed the disease between days 4 to 27. The Idd3/10/18 strain develops diabetes with 31% incidence upon anti PDL1 treatment (days 4 to 28), which is ~6-fold greater than the spontaneous incidence at the age of 7–8 months (Figure 1c, Table 1, 2).

Idd3/Idd5 and Idd3/5.2/5.3

The Idd3/5 strain has protective alleles at both Idd3 and Idd5 and only 1% of mice develop spontaneous diabetes by 7–8 months of age [38]. Anti PDL1 treatment did not induce diabetes in Idd3/5 mice as 100% of them stayed non-diabetic over the course of 30 days PDL1 blockade (Figure 1d). The Idd3/5.2/5.3 (without protective alleles at the Idd5.1 sub-region) strain that is also almost completely protected from spontaneous diabetes shows susceptibility to treatment with anti PDL1, and 15% of the mice developed diabetes by day 30 (Figure 1d, Table 1, 2).

Insulitis in anti PDL1 treated congenic strains

One of the hallmarks of developing type 1 diabetes is the presence of infiltrating lymphocytes in the pancreas. Idd congenic strains have made it possible to identify checkpoints of disease progression. Ninety percent of Idd9 mice show evidence of islet insulitis, but only 3% develop diabetes spontaneously [21,37]. Analysis of insulitis scores of the Idd9 strains revealed that NOD congenics that were partially (Idd 9.2, Idd 3/10/18) or fully (Idd 9.3/
5) protected from anti PDL1 accelerated diabetes had lower basal insulitis scores (Idd 9.2 (0.18±0.11), Idd3/10/18 (0.24±0.059) and Idd3/5 (0.0±0.0) compared to the almost unprotected Idd9.1 (1.2±0.3), Idd9.3 (0.82±0.14) and the NOD (1.23±0.24) mice. These results were statistically significant Idd9.2 vs Idd9.1, Idd9.3, NOD p value 0.0016, 0.0031 and 0.0007, respectively; Idd3/10/18 vs Idd9.1, Idd9.3, NOD p value <0.0001, 0.0002 and <0.0001, respectively; Idd3/5 vs Idd9.1, Idd9.3, NOD p value 0.0047, 0.0014 and 0.0018 respectively).

Clearly, there seems to be a direct link between basal insulitis levels and the incidence of anti PDL1 induced accelerated diabetes. Interestingly, Idd9.2 mice that turned diabetic showed similarly high insulitis scores (3.31±0.25) upon anti PDL1 treatment as treated NOD mice (3.25±0.119), while the Idd9.2 mice which stayed non-diabetic had almost no pancreatic infiltrates and low insulitis scores (0.38±0.17) (Figure 2).

**Cytokine and chemokine profile in Idd9 subcongenic mice**

The cumulative incidence of diabetes following anti PDL1 treatment for Idd9.2 was different from that of Idd9.1 and Idd9.3 mice. Nearly 100% of Idd9.1 and Idd9.3 mice while only 56% of Idd9.2 mice developed type 1 diabetes following anti PDL1 treatment. Basal insulitis was also lower in Idd9.2 versus Idd9.1 and Idd9.3 mice. Therefore we sought to determine if any cytokines or chemokines were differentially expressed in these three sub-congenic lines. Real time PCR of pancreas tissue of anti PDL1 treated mice showed that diabetic Idd9.2 mice had lower expression of IFN-γ, TNF-α, CCR2, RANTES (CCL5) and MIP-1α (CCL3) as compared to diabetic Idd9.1 and Idd9.3 mice (Figure 3a-f). MIP-1α up-regulation has been associated with progression to type 1 diabetes [39]. Idd9.2 mice also had lower cytokine and chemokine levels than the Idd9.1 and Idd9.3 substrains. These studies show that a low level of insulitis as observed in Idd9.2 correlates with lower levels of cytokines even
when diabetes develops in some of these mice following PDL1 blockade. It remains to be seen if the quality of insulitis in Idd9 and Idd9.2 mice is different from that present in the Idd9.1 and Idd9.3 substrains.

We performed an adoptive transfer of transgenic T cells and analyzed their proliferation rates to identify differences between the congenic strains undergoing anti PDL1 blockade. Adoptive transfer of CFSE-labeled BDC2.5 Tg CD4^+ T cells and NY8.3 CD8^+ T cells in pancreatic LN of Idd3/10/18 mice following anti PDL1 treatment. NY8.3 CD8^+ Tg T cells divided more frequently as portrayed by an increase in the number of CFSE-diluted CD8^+ T cells in Idd3/10/18 mice that received anti PDL1 antibody as compared to mice that received control IgG. These data show that both auto-reactive CD4 as well as CD8 T cells expand in Idd3/10/18 mice following anti PDL1 treatment, similar to that of untreated and anti PDL1 treated NOD mice.

### Discussion

More than 38 Idd regions from resistant strains that confer protection in the NOD model, have been described to date [7,40].

<table>
<thead>
<tr>
<th>Comparison of Idd Strains</th>
<th>p-value</th>
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<tbody>
<tr>
<td>Idd5 vs. Idd5.1</td>
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<td>Idd5 vs. Idd5.2</td>
<td>p = 0.0194</td>
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<tr>
<td>Idd5 vs. Idd5.3</td>
<td>p = 0.0140</td>
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<tr>
<td>Idd5.1 vs. Idd5.2/5.3</td>
<td>p = 0.0039</td>
</tr>
<tr>
<td>Idd9 vs. Idd9.1</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>Idd9 vs. Idd9.2</td>
<td>p = 0.0209</td>
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<tr>
<td>Idd9 vs. Idd9.3</td>
<td>p &lt; 0.001</td>
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<tr>
<td>Idd9.2 vs. Idd9.1</td>
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<tr>
<td>Idd9.2 vs. Idd9.3</td>
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</tr>
<tr>
<td>Idd3 vs. Idd10/18</td>
<td>p = 0.0103</td>
</tr>
<tr>
<td>Idd3/10/18 vs.</td>
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<td>Idd10/18</td>
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<tr>
<td>Idd3 vs. Idd3/10/18</td>
<td>p = 0.2531</td>
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<tr>
<td>Idd3/5 vs Idd3/5.2/5.3</td>
<td>p = 0.2298</td>
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### Table 2. Spontaneous incidence of type 1 diabetes at 7 months of age compared to anti PDL1 treatment of 10 week old mice.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Line</th>
<th>Cumulative Incidence of spontaneous type 1 diabetes</th>
<th>Cumulative Incidence of type 1 diabetes with aPDL1 treatment starting at 10 weeks</th>
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</thead>
<tbody>
<tr>
<td>NOD.B10-Idd5.1</td>
<td>1094</td>
<td>40%</td>
<td>62.5%</td>
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<td>Idd5.2 Idd5.3</td>
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<td></td>
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<tr>
<td>NOD.B10-Idd5.1</td>
<td>2193</td>
<td>62%</td>
<td>66%</td>
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<td>NOD.B10-Idd5.2</td>
<td>6146</td>
<td>38%</td>
<td>80%</td>
</tr>
<tr>
<td>NOD.B10-Idd5.3</td>
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<td>69%</td>
<td>90%</td>
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<tr>
<td>NOD.B10-Idd5.2</td>
<td>1595</td>
<td>25%</td>
<td>65%</td>
</tr>
<tr>
<td>Idd5.1</td>
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<td></td>
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<tr>
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<td>35%</td>
<td>95%</td>
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<td>1566</td>
<td>55%</td>
<td>56%</td>
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<td>90%</td>
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<td>7754</td>
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<td>94%</td>
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<td>31%</td>
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<td>15%</td>
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<tr>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NOD</td>
<td>70–90%</td>
<td>93%</td>
<td></td>
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</table>

Table 1. Statistical Significance Figure 1.

Table 2. Spontaneous incidence of type 1 diabetes at 7 months of age compared to anti PDL1 treatment of 10 week old mice.

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doi:10.1371/journal.pone.0089561.t002
Results are expressed as Mean ± SD. P-values are expressed as * (P < 0.05), ** (P < 0.01), *** (P < 0.0001). # In case of the Idd3/10/18 mice, treatment with anti PDL1 resulted in 94% of diabetes incidence.

In the current study we examined the effects of 9 Idd regions, alone and in combination, on accelerated type 1 diabetes following anti PDL1 treatment. These congenic and subcongenic mice have multiple protective alleles that mediate varying degrees of resistance to type 1 diabetes. We chose the NOD Idd congenics (Idd3/5, Idd9, Idd10/10/18 and Idd3/5.2/5.3) that are almost completely protected from spontaneous diabetes occurrence due to allelic interactions between the candidate genes present in the loci and subloci. We also looked at mouse strains that are variations of the above-mentioned strains, containing the individual locus or a combination of loci, where the disease progression is either moderate (Idd3, Idd9.1, Idd5) or relatively high but always lower than that of the NOD parental strain (Idd5.1, Idd5.2, Idd5.3, Idd9.1, Idd9.2, Idd9.3, Idd10/10/18). Frequency of diabetes in these mice strains range from <5% to 65%.

PDL1 blockade is known to accelerate diabetes precipitation in NOD mice. Our aim was to determine if the interaction between the protective Idd loci in the different NOD congenics affects diabetes induction by PDL1 blockade. All except one among the NOD congenic strains tested here, Idd3/5, developed accelerated type 1 diabetes following anti PDL1 treatment. Our data show that PDL1 blockade is not enough to induce accelerated diabetes in the NOD Idd3/5 congenic mouse strain that contains alleles for Il2, Cita4, Slek11a1 and Acdl. The interaction between these alleles is able to protect the mice from diabetes induction by PDL1 blockade. We do not observe this in the case of any other congenic strains. This is probably because Cita4 and Il2both modulate the survival and function of Treg cell population and the blockade of PDL1-PDL1 pathway is not enough to limit the ability of these Tregs, and tolerance is maintained in the Idd3/5 congenic mice. It is also worth mentioning that PDL1 and Cita4mediated tolerance induction functions through two distinct pathways. Allelic interaction of Cita4 with the other candidate genes in the Idd3/5 strain is able to overcome the effect of anti PDL1 treatment and maintain tolerance in these congenic mice. Slek11a1 plays an important part in antigen presenting function of DCs and may play a role in inducing tolerance to self antigens. Acdl is proposed to have a significant role in T cell function and survival by altering fatty acid metabolism. We therefore suggest that the combination of these four candidate genes and their interaction renders the Idd3/5 congenics resistant to diabetes induction by PDL1 blockade. The Idd3/5 mice have also been shown to be resistant to other experimental autoimmunity [41].

The Idd10/18 strain has an insulin rate of 78%, and ~50% rate of spontaneous diabetes development [37], in contrast to the Idd3/5 strain which shows greater protection, with 19% of mice developing insulitis and 7% developing type 1 diabetes. The Idd10/18 strain demonstrates a median protection against anti PDL1 accelerated diabetes development with a 31% cumulative incidence as opposed to a cumulative incidence of 94% in Idd10/10/18, and 0% in the Idd3/10/18 strain following treatment. The Idd3/10/18 strain is almost completely protected from diabetes, similar to the Idd3/5 strain. However, the effect of PDL1 blockade results in significantly different outcomes in respect to diabetes induction. These data also imply that in the absence of negative costimulation by the PDL1-PDL1 pathway, CTLA4 possibly maintains the self tolerance with the help of IL-2 (one of the candidate genes in Idd3) in case of the Idd3/5 strain. And IL-2 alone is not sufficient to prevent anti PDL1 mediated accelerated diabetes in case of the Idd10/10/18 strain. Similarly in Idd3/5.2 and Idd3/5.3 strains where the congenic Idd5.1 locus containing the CTLA4 gene is absent, IL-2 alone cannot prevent diabetes induced by PDL1 blockade.

The Idd9 strain including sub-congenic 9.1, 9.2 and 9.3 shows high levels of pancreatic infiltrates (90%), but does not develop diabetes at a high rate (3%) [21]. Nonetheless, this strain develops insulin autoantibodies [27]. The Idd9.1 region was identified to control type 1 diabetes development through TNF-α [42]. Idd9.2 and Idd9.3 regions were found to be responsible for preventing the expansion of islet specific CD8+ T cells, providing an explanation for the dichotomy of high insulitis incidence and a low rate of actual diabetes development in the Idd9 strain [24].

Remarkably, a profound increase of diabetes incidence (3% to 50%) was observed after PDL1 blockade in Idd9 congenic mice. We studied the effect of anti PDL1 on Idd9 subcongenics (Idd9.1, Idd9.2 and Idd9.3). Interestingly, almost 100% of Idd9.1 and Idd9.3 mice developed accelerated diabetes following anti PDL1 treatment, in contrast to the Idd9.2 strain, which was partially protected (56% became diabetic). Findings in the Idd9 strain suggest that in mice that already have infiltrating lymphocytes in target organs at 10 weeks of age, like Idd9.1 and Idd9.3 strains, diabetes development is exacerbated following anti PDL1 treatment. Idd9.1 and Idd9.3 strains which develop accelerated diabetes, also show slightly higher scores of insulitis. Idd9.2 mice had significantly less pancreatic infiltrates and only 56% developed diabetes after anti PDL1 treatment.

The Idd9 mice strain similar to Idd3/5 and Idd3/10/18, is also resistant to spontaneous diabetes occurrence. However, the level of insulitis is much higher in the Idd9 strain in comparison to that of Idd3/5 and Idd3/10/18. The difference in the pathogenicity of the
Idd9.2

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FoxP3 expression between Idd9 vs. Idd9.1 on day 0 and 250 of pancreatic infiltrates in the anti PDL1 induced diabetes. We also observe a very low frequency and Idd9.1 (approximately 50%) in the by the fact that the strains of frequency of autoreactive CD8* T cells in comparison to the NOD mice [43].

Further, CD4* Type II NKT cells were shown as regulators of diabetes and it was shown that these cells were sufficient in down-regulating diabetes, promoting activity of CD4* BDC2.5 tg T cells in vivo. Interestingly, blockade of ICOS and PDL1 was found to negate the regulatory effect of the CD4* Type II NKT cells in the pancreatic lymph node leading to a sudden development of diabetes [44].

We used the Idd3/10/18 strain to further dissect anti PDL1 mediated diabetes. BDC2.5 Tg CD4* T cells were transferred into NOD and Idd3/10/18 mice treated with anti PDL1. Similar rates of T cell proliferation were observed in pancreatic LNs of both strains. Corresponding to CD4* TCR Tg T cells tested above, CD8* 8.3 TCR Tg* T cells divided more frequently, as seen by an increase in the number of CFSE-diluted CD8* T cells in Idd3/10/18 mice (and NOD mice) that received anti PDL1 antibody as compared to mice that received control IgG. These data are similar to our findings in regular NOD mice [4] and suggest that

disease in the Idd9 strain is attributed to the Th2 type cellular response induced by the TNFR superfamily gene cluster present in the Idd9 locus. The combination of three subloci Idd9.1, Idd9.2 and Idd 9.3 renders this strain resistant to the disease even though individually the candidate genes are susceptible. PDL1 blockade negates this interaction and in the absence of any other functional negative costimulatory pathway, disease progression is accelerated and pathogenicity is altered. Among the three subloci of Idd9, Idd9.2 is considered the most potent region in providing protection against the disease by restraining autoreactive CD8* T cells. Blockade of PDL1 is known to cause CD8* T cell exhaustion. This explains the similarity in the anti PDL1 induced diabetes incidence (approximately 50%) in the Idd9 and Idd9.2 strains; whereas the Idd9.1 and Idd9.3 strains are completely susceptible (90-95%) to anti PDL1 induced diabetes. We also observe a very low frequency of pancreatic infiltrates in the Idd9.2 strain. This can be explained by the fact that the strains of Idd9 and its sub regions have a low frequency of autoreactive CD8* T cells in comparison to the NOD mice. Further, the genes in the Idd9 sub regions prevent a massive expansion of these autoreactive CD8* T cells during disease onset and progression [25]. However, extensive insulitis was observed in the group of Idd9.2 mice that become diabetic after PDL1 blockade suggesting that in some of the mice the low frequency of autoreactive cells can expand when this regulatory pathway is inhibited. The decreased cytokine and chemokine production in Idd9 and Idd9.2 mice may also be related to the low frequency of autoreactive T cells [25] that affects the quality of the infiltrating cells following PDL1 blockade as compared to mice not having protective alleles at Idd9.2.

Our study using treatment with anti PDL1 mAb indicates that sufficient numbers of effector cells are present in these congenic strains to mediate type 1 diabetes. The rapid onset of diabetes in some of the Idd congenic strains is probably due to auto-aggressive memory/effector T cells that are suddenly set free when PDL1 is blocked, as has been shown in a study in NOD mice [43].

Figure 3. Quantitative PCR detection of cytokine, chemokine and transcription factor levels in pancreas tissue after anti PDL1 treatment in Idd9(n = 3) and subcongenics Idd9.1(n = 3), Idd9.2 (n = 5), Idd9.3 (n = 5). Mice (10 weeks old) were treated with 500 µg anti PDL1 on day 0 and 250 µg anti PDL1 on days 2, 4, 6, 8 and 10 by i.p. injection. Pancreas tissue was harvested on day 30 or when mice had turned diabetic. Horizontal lines show median value. P-values are expressed as * (P < 0.05), ** (P < 0.01), *** (P < 0.001) in figure. a): IFN-γ: Idd9.1 vs. Idd9.2 p = 0.0039; Idd9.1 vs. Idd9.3 p = 0.063. b): CCR2: Idd9 vs. Idd9.1 p = 0.0003; Idd9.1 vs. Idd9.2 p < 0.0001; Idd9.2 vs. Idd9.3 p = 0.0431; Idd9 vs. Idd9.3 p = 0.0183. c): RANTES: Idd9 vs. Idd9.1 p = 0.022; Idd9.1 vs. Idd9.2 p = 0.0016; Idd9.2 vs. Idd9.3 p = 0.0284; Idd9 vs. Idd9.3 p = 0.0362. d): No significant differences in Foxp3 expression between Idd9 and subcongenics. e): MIP-1α: Idd9 vs. Idd9.1 p = 0.0257; Idd9.2 vs. Idd9.1 p = 0.0017. f): TNF-α: Idd9 vs. Idd9.1 p = 0.019; Idd9.2 vs. Idd9.1 p = 0.0004. doi:10.1371/journal.pone.0089561.g003

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the lower susceptibility of Idd3/10/18 mice to develop type 1 diabetes following PDL1 blockade is probably not dependent on expansion of CD4 and CD8 T cells, rather that anti PDL1 treatment likely affects behavior of these cells which contributes to lowering susceptibility for developing disease. The role of PDL1 expression in the pancreas and its effect on resistance in this strain remains to be further investigated.

Correlation between the level of basal insulitis and the development of anti PDL1 induced diabetes also proved to be true in the case of Idd3/5 mice to which anti PDL1 was administered. These mice stayed diabetes free. The Idd3/5 strain exhibits profound resistance, has the lowest spontaneous diabetes incidence, and also shows the lowest levels of insulitis among all Idd congenics.

Prominent genes associated with Idd3/5 congenic strain are Il2 and Il21 from Idd3, and Cita4 from Idd5 regions [37]. The role of IL-2 in diabetes has been previously demonstrated. NOD mice express less IL-2 than diabetes resistant mouse strains [45], and low dose IL-2 administered at the onset of type 1 diabetes can reverse established disease in NOD mice [46]. This mechanism has been attributed to an increase in regulatory T cell numbers in the pancreas, and to increased expression of FoxP3, CD25, CTLA-4, ICOS and GITR [46]. Lower levels of IL-2 were found to have an impact on antigen presenting cells like DCs, since low IL-2 levels correlated with higher numbers of DCs and increased T cell stimulation and activation. The cellular mechanism of protection from T1D in Idd3/5 congenic mice strain is already defined by Hamilton-Williams et al. [9].

Further analysis of the Idd3/5 region showed that removal of protective alleles at a subcongenic region from the Idd3/5 region as in the Idd3/5.2/5.3 strain results in a 15% incidence of disease upon anti PDL1 treatment in contrast to no incidence of disease in the Idd3/5 group, which supports the role of Cita4 at the Idd3.1 locus in preventing diabetes in a concerted interplay with Idd3. Although Idd3/5.2/5.3 mice do not develop diabetes, an increase of insulitis in Idd3/5.2/5.3 mice as compared to Idd3/5 has been reported [33]. These observations support the hypothesis that the ability of PDL1 to accelerate diabetes relies on some minimal amount of effector cell accumulation that is normally manifested as at least a mild insulitis.

**Conclusion**

Taken together, our data show that PDL1 blockade destroys the genetic protection mediated by different protective alleles. We show a link between occurrence of insulitis and disease susceptibility through a break of tolerance induced by anti PDL1. We suggest that the presence of a functional CTLA4 allele is probably
responsible to prevent disease susceptibility induced by anti PDL1.

 Increased understanding of the mechanisms of gene-gene interaction, and discovering additional traits that play a role in type 1 diabetes will help identify novel treatments of this disease. The PD1-PDL1 pathway is currently studied for developing therapy for cancer and infectious diseases including HIV, since blockade of this pathway results in increased immune responses against tumor cells [1,47,48] and infectious agents [3,49,50]. However, we show that blockade of this pathway interrupts critical tolerance mechanisms that operate to prevent autoimmune diabetes. Acceleration of diabetes following PD1-PDL1 pathway blockade to treat disease underscores the need for caution before proceeding to a widespread use of this form of treatment, especially when used in combination with antiCTLA-4 (Ipilimumab) that is currently approved for use in melanoma. A combined blockade of CTLA4 and PD1-PDL1 will in all probability shift the balance from an effective immune response towards autoimmunity. It is important to note that our group had earlier shown that type 1 diabetes resistant NOR mice, which are congenic for the MHC locus to the NOD mice, did not develop diabetes following anti PDL1 treatment [51]. The fact that these congenic mice were protected against type 1 diabetes post-anti PDL1 treatment suggests that PDL1 blockade may still prove suitable in human patients without HLA alleles associated with autoimmune disease such as type 1 diabetes.

 Future research should focus on strategies to exploit enhanced immune responses by blocking the PD-PDL1 pathway and at the same time prevent the development of autoimmune disease as a consequence.

Author Contributions
Conceived and designed the experiments: IG. Performed the experiments: NMK AJK BZ LA. Analyzed the data: NMK ST AJK BZ. Wrote the paper: NMK ST. Reviewed and Edited the manuscript: MHS DLG LSW IG DR AR. Guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis: IG.

Figure 5. Proliferation of adoptively transferred BDC2.5 Tg CD4 T and NY8.3 Tg CD8 T cells in pancreatic LN and spleen of anti PDL1 treated NOD and Idd3/10/18 mice. a) Collective data from 3 out of 8 experiments of the percentage of CFSE-dividing cells (gated on CD4+ V[b]4+) are shown. Horizontal lines express mean value. For CD4+ T cells pLN, NOD, control vs. treated p = 0.0276; pLN, Idd3/10/18, control vs. treated p = 0.0002. b) A representative experiment from 3 performed is shown. For CD8 T cells pLN, Idd3/10/18 control vs. treated p = 0.0321, spleen Idd3/10/18 control vs. treated p = 0.0185.

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