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Anti-CD3 treatment cures PDL1 –/–.NOD mice of diabetes but precipitates fatal myocarditis

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Abstract

Anti-CD3 mAb is an effective therapy that can reverse diabetes in NOD mice and has therapeutic potential in patients with type 1 diabetes (T1D). We administered anti-CD3 to PDL1 –/–.NOD mice in order to determine whether this treatment would reverse the development of diabetes in these mice. Mice injected with anti-CD3 neonatally were protected from T1D. However, all of these anti-CD3 treated PDL1 –/–.NOD mice developed a wasting disease between 12–20 weeks of age with sudden deterioration and weight loss, leading to death within 3–5 days of development of illness. Histology revealed severe inflammation in the heart and skeletal muscles. These results suggest that deficiency of PDL1 in NOD background has the potential to lead to immune-mediated tissue damage in organs other than the pancreas, but this cannot be appreciated in PDL1 –/–.NOD mice as the mice develop T1D at an early age and die from diabetes prior to manifesting other autoimmune diseases.

INTRODUCTION

Several tolerance mechanisms prevent inappropriate activation of self-reactive T cells, and autoimmunity [1]. The balance between positive and negative costimulatory signals delivered to T cells after antigenic challenge determines the fate of T cells and the outcome of an immune response, resulting in T cell activation or tolerance [2; 3]. The inhibitory costimulatory molecule programmed death-1 (PD-1) and its ligands, PDL1 and PDL2, have been shown to play an important role in regulating T cell activation and peripheral tolerance [4; 5; 6; 7]. PDL1 is expressed on resting T cells, B cells, dendritic cells (DCs), and macrophages and is further up regulated on these cells upon their activation [2; 3]. PDL1 is also expressed on parenchymal cells, including vascular endothelial cells and pancreatic islet cells [8; 9; 10; 11; 12].

Preclinical, as well as clinical studies, have shown that a monoclonal antibody against CD3 could reverse hyperglycemia at presentation and can protect against recurrent disease [13; 14; 15]. The mechanism of action of the anti-CD3 monoclonal antibody may involve direct

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effects on pathogenic T cells, the induction of populations of regulatory cells, or effects on other immune cell types [15; 16; 17; 18]. For example, in an established disease model of relapsing-remitting EAE, anti-CD3 antibody induced tolerance was suggested to be linked with the ability of phagocytes to induce apoptosis mediated by Tgf-beta [16]. It has also been shown by You et al., that adaptive Tgf-b dependent Tregs could be induced from peripheral CD4⁺CD25⁻ T lymphocytes by anti-CD3 immunotherapy, resulting in the restoration of self-tolerance [17].

PD-1 deficiency leads to features of lupus including the development of anti-DNA antibodies in C57BL/6 mice [19; 20]. In the NOD mouse model we have previously shown that PD-1 interaction with PDL1 is critically important in the regulation of autoimmune diabetes [12]. Consistent with these observations are recent reports by our group and others demonstrating that PD-1 or PDL1 deficiency accelerates the onset and frequency of type 1 diabetes in NOD mice, with strong Th1 polarization of islet infiltrating cells [21; 22]. PDL1 deficient mice develop accelerated diabetes between 4–6 weeks of age. To determine whether anti-CD3 could prevent or delay diabetes in PDL1^{-/-}.NOD mice, neonatal PDL1^{-/-}.NOD mice and their littermate controls were injected with anti-CD3 at day 10 and 12 days of age and followed for incidence of diabetes. PDL1^{-/-}.NOD mice were protected from autoimmune diabetes. However, they developed a wasting disease between 12–20 weeks of age which was characterized by myocarditis with massive infiltration of T cells, B cells and macrophages. Neither NOD mice heterozygous for PDL1 deficiency nor WT littermate controls manifested this disease. PDL1^{-/-} mice on B6 background given anti-CD3 neonatally did not develop myocarditis or any other immunopathology. Our data suggest that PDL1^{-/-} mice on NOD background, if protected from diabetes, have the potential to develop immunopathology in a different organ.

MATERIALS AND METHODS

Mice

PDL1 deficient mice on NOD background (PDL1^{-/-}.NOD) get accelerated diabetes as early as 4 weeks of age [22]. They were hence maintained in our colony by mating PDL1 heterozygous females with PDL1 heterozygous males.

Antibodies and treatment protocol

Neonatal PDL1 deficient mice and littermate controls were injected with 250 ug each of anti-CD3 mAb (clone 2C11) i.p. on day 10 and 12 of age. The injections were given in a volume of 50ul with a 30-gauge syringe. Hamster IgG was used as isotype control for each experiment. Non-mitogenic antibody anti-CD3 F(ab')₂ was obtained from Bioexpress. It was injected to neonatal PDL1 deficient and littermate controls at 50ug/injection for 5 consecutive days. An isotype F(ab')₂ was used as control.

Monitoring of mice

Mice were monitored daily for glucose measurement and for general health. The mice were measured for glucose by urine analysis till they were 4 weeks of age. They were thereafter monitored for glucose in the blood.

Histology

Upon development of weight loss and deterioration of mouse condition, mice were sacrificed and various organs were fixed in 10% phosphate-buffered formalin, dehydrated, and embedded in paraffin for histopathological analysis. Five-micron paraffin tissue sections were stained with haematoxylin and eosin for microscopic analysis. All slides were examined by at least one pathologist in a blinded fashion. Immunohistochemistry was

performed using antibodies to detect various infiltrating cells. Frozen sections were fixed in cold acetone, washed in phosphate buffered saline (PBS) and quenched in 0.3% hydrogen peroxide in PBS. The sections were then washed in PBS, blocked with normal serum and then incubated with primary antibody for T cells, B cells, macrophages and neutrophils (anti-CD3, B220, Mac-2 and Gr-1 respectively) overnight at 4°C. After washing with PBS, slides were incubated with biotinylated antibody for 40 min at room temperature. Slides were incubated with avidin-conjugated peroxidase (Vectastain ABC vector Elite kit) and developed with DAB (3,3'-diaminobenzidine). The tissue sections were then lightly counterstained with hematoxylin and positive staining was indicated by a red-brown coloration as described before [23]. Isotype-matched antibodies (Rat IgG2a or Rat IgG2b) were used as negative control.

Flow cytometric Analysis

Heart and spleen were removed from PDL1^{-/-}.NOD mice and littermate controls when the mice developed weight loss and deteriorating condition. Spleens were processed to prepare single cell suspensions. Hearts were washed briefly in cold PBS and diced before digestion in media containing collagenase IV (Roche) and DNase I (Sigma) for 1 hour. After preparing the cells in a single cell suspension, the leukocytes were separated using a Percoll equilibrium density gradient with a higher density of 1.095 and a lower density of 1.030. The cells were then washed twice and counted. To study the phenotype of splenocytes and cells infiltrating the heart, cells were stained with anti-CD4-percp, anti-CD8-APC and anti-F4/80-PE (for staining macrophages) mAb. All Abs and reagents for flow cytometric analysis were purchased from BD Pharmingen or ebiosciences. Cells were analyzed on a FACSCalibur™ (Becton Dickinson, San Jose, CA) using CELLQuest software™ (Becton Dickinson, San Jose, CA). Depletion of various leukocytes following anti-CD3 was determined at 1 week, 2 week, 8 week, 12 week and 16 week post anti-CD3 mAb treatment was determined by Flow cytometric analysis on blood.

Luminex cytokine assay—For cytokine analysis, blood was collected from mice one day after the last injection of anti-CD3 or anti-CD3 F(ab')₂. Serum was separated and the multibead array Luminex (Linco Research Inc., St. Charles, MO, USA) was used to quantify the cytokines, according to the manufacturer's instruction. A Luminex 100 IS instrument (Biosource, Nivelles, Belgium) with the Star Station acquisition program (v2 Applied Cytometry Systems, Sheffield, UK) was used to process the data. All samples were run in duplicate wells.

RESULTS AND DISCUSSION

Anti-CD3 administration to neonatal PDL1^{-/-}.NOD mice protects them from development of diabetes

Preclinical studies have shown that systemic treatment with anti-CD3 mAb can prevent diabetes [13; 14; 24]. Harold et al., have shown that treatment with the anti-CD3 monoclonal antibody results in improved C-peptide responses and clinical parameters in type 1 diabetes for at least 2 years in the absence of continued immunosuppressive medications [15]. Long term follow up showed that anti-CD3 preserves insulin production for up to 5 years [15].

Oral administration of anti-CD3 mAb can suppress STZ-induced diabetes and experimental autoimmune encephalitis [25]. The mechanisms by which anti-CD3 can afford protection from these autoimmune diseases are of great interest. It has been proposed to involve direct effects on pathogenic T cells and the induction of populations of regulatory cells [15]. In view of the important role of PDL1 in controlling tolerance and autoimmunity, we

investigated whether anti-CD3 could prevent the development of diabetes or ameliorate diabetes in these PDL1^{-/-}.NOD mice.

Neonatal PDL1^{-/-}.NOD mice were given anti-CD3 (250 µg each, i.p.) on days 10 and 12 of age and followed for onset of diabetes. All PDL1^{-/-}.NOD mice (12/12) treated with anti-CD3 were protected from diabetes until 24 weeks of age, whereas all untreated PDL1^{-/-}.NOD mice developed diabetes between 4 to 8 weeks of age (Figure 1). These findings indicate that administration of anti-CD3 to neonatal PDL1^{-/-}.NOD mice results in prevention of diabetes in these mice. There was between 75–100 fold reduction in number of CD3, CD4 and CD8 T cells following anti-CD3 treatment. There was interestingly a compensatory increase in F4/80+ macrophages (up to 300 fold) following this decrease in T cells (Figure 2).

Anti-CD3 treated PDL1^{-/-}.NOD mice develop myocarditis

Although PDL1^{-/-}.NOD mice injected with anti-CD3 were protected from diabetes, they developed a wasting disease which resulted in a loss of >30% of their body weight with sudden deterioration and death over a 3 to 5 day period. Most mice developed this wasting disease between 12–20 weeks of age, but one mouse (1/12) developed this wasting disease at 30 weeks of age. Histological and immunohistologic analyses revealed that these anti-CD3 treated PDL1^{-/-}.NOD mice developed severe myocarditis associated with massive infiltration of T cells, B cells and macrophages in the heart (Figure 3 a,b,c). Flow cytometric analyses of infiltrating cells revealed increased percentages of CD3+ T cells in the hearts of these sick mice as compared to anti-CD3 treated wild type (WT) littermate control NOD mice, which showed no evidence of cardiac pathology (Figure 3e). However, F4/80+ (macrophages) (16.4% in KO versus 19.9% in WT, ns) and CD19+ (B cells) (11.24% in KO versus 13.79% in WT, ns) cells were similar in hearts of anti-CD3 treated PDL1^{-/-}.NOD as compared to WT littermate controls; this difference in flow cytometry versus histology data for macrophages and B cells could be related to the timing of response. The major infiltrate comprised of T cells which could be appreciated both with histological as well as flow cytometry findings. The skeletal muscles also had inflammatory infiltrates, however consisting mainly of B cells (Figure 3d). Even though there was a preferential infiltration of B cells into skeletal muscle there was no evidence of antibody-mediated pathology in the muscle as investigated by immunostaining for neutrophils with Gr-1.

In addition, inflammation in stomach and salivary gland was also observed in some (3/12) of these mice. The numbers of T cells and macrophages were similar in number in the spleens of anti-CD3 treated WT and PDL1^{-/-}.NOD mice (data not shown).

Analysis of PBMCs showed that PDL1^{-/-}.NOD mice with wasting disease exhibited mild leukocytosis and monocytosis in their blood samples, as compared to WT controls.

Administration of anti-CD3 to PDL1^{-/-} mice on B6 background does not lead to precipitation of myocarditis or any other inflammatory disease

In order to determine if this was a general effect of anti-CD3 treatment in mice with PDL1 deficiency, we gave anti-CD3 to PDL1^{-/-} mice on B6 background at days 10 and 12 of age, analogous to our studies in PDL1^{-/-}.NOD mice. Anti-CD3 treated C57BL/6 PDL1^{-/-} mice stayed healthy and did not develop any signs of wasting disease when followed for a 30-week period. Histopathology analysis revealed no evidence of inflammatory infiltrates in the heart, skeletal muscle or any other organs (data not shown).

Cytokine analysis in the serum of anti-CD3 treated mice

It has been shown that mitogenic anti-CD3 treatment can cause a cytokine storm in the host. We confirmed these findings in our model. Anti-CD3 treated PDL1^{-/-}.NOD mice had high levels of IFN- γ , IL-6, IL-17 and IL-10 (Figure 3f).

Treatment with non-mitogenic anti-CD3 F(ab')₂

Because of this systemic effect of a cytokine storm following mitogenic antibody treatment, recent studies have made use of the non-mitogenic form of anti-CD3, the F(ab')₂ fragment, for studies in type 1 diabetes. We injected neonatal PDL1^{-/-}.NOD mice with anti-CD3 F(ab')₂ (50 μ g/injection) for 5 consecutive days and followed the mice for diabetes and evidence of myocarditis. Interestingly, this treatment did not prevent precipitation of diabetes in the PDL1^{-/-}.NOD mice but it did protect the heterozygotes (PDL1^{+/-}.NOD; hets) and wildtypes (PDL1^{+/+}.NOD; WT) to some extent (Figure 4). In order to determine if these mice developed myocarditis, tissues were analyzed by histological methods. Our data show that none of the PDL1^{-/-}.NOD mice that developed diabetes developed myocarditis or infiltrates in any other organ (data not shown). This is in contrast to our data with mitogenic anti-CD3; treatment with which resulted in protection of all the PDL1^{-/-}.NOD mice against type 1 diabetes but resulted in development of myocarditis. There is also no cytokine storm upon injection of non-mitogenic anti-CD3 F(ab')₂ systemically as shown by luminex analysis (Figure 3f).

In the present study, we investigated whether anti-CD3 administration, the only therapeutic option known to have effect in preclinical and clinical setting, could prevent diabetes in PDL1^{-/-}.NOD mice. Whereas PDL1^{-/-}.NOD mice develop diabetes beginning at 4 to 6 weeks of age, none of the anti-CD3 treated PDL1^{-/-}.NOD mice developed diabetes. However, these anti-CD3 treated PDL1^{-/-}.NOD mice developed fatal myocarditis. These findings demonstrate that when diabetes is prevented in PDL1^{-/-}.NOD mice, these mice can develop other inflammatory/autoimmune diseases. The development of another inflammatory/autoimmune disease in the anti-CD3 treated PDL1^{-/-}.NOD mice may be related to dysregulation of tolerance mechanisms in NOD mice lacking PDL1. The genetic background of the mouse, in this case NOD, is important to manifest such disease, since PDL1^{-/-} mice on autoimmune resistant B6 background did not exhibit any inflammation following anti-CD3 administration. In addition to autoimmune diabetes, the NOD strain is genetically prone to developing other autoimmune diseases targeting other organs such as the salivary glands and thyroid. It has been shown by Bluestone's group that NOD mice deficient for the costimulatory molecule B7.2 were protected from autoimmune diabetes but spontaneously developed an autoimmune peripheral neuropathy beginning at 20–25 wk of age [26]. However in another study, treatment of patients with new onset Type 1 diabetes with a single course of anti-CD3 mAb resulted in generation of C-peptide despite various adverse reactions but myocarditis as an adverse reaction was not reported. It is also not known if these subjects who received anti-CD3 and were protected from diabetes for 5 years had any deficiency in PDL1 or any other costimulatory molecule.

Our findings suggest that subjects who are given mitogenic anti-CD3 for amelioration of type 1 diabetes should be followed closely for signs of inflammation or autoimmune disease.

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Research Highlights

- We administered anti-CD3 to PDL1^{-/-}.NOD mice in order to determine whether anti-CD3 mAb, an effective therapy that can reverse diabetes in NOD mice, would reverse the development of diabetes in these mice. All mice injected with anti-CD3 neonatally were protected from T1D.
- Interestingly, all of these anti-CD3 treated PDL1^{-/-}.NOD mice developed a wasting disease between 12–20 weeks of age with sudden deterioration and weight loss, leading to death within 3–5 days of development of illness. Histology revealed severe inflammation in the heart and skeletal muscles.
- These results suggest that deficiency of PDL1 in NOD background has the potential to lead to immune-mediated tissue damage in organs other than the pancreas, but this cannot be appreciated in PDL1^{-/-}.NOD mice as the mice develop T1D at an early age and die from diabetes prior to manifesting other autoimmune diseases.
- Our findings suggest that subjects who are given mitogenic anti-CD3 for amelioration of type 1 diabetes should be followed closely for signs of inflammation or autoimmune disease.

Figure 1.

a) Anti-CD3 treatment protects PDL1^{-/-}.NOD neonatal mice from diabetes. PDL1^{-/-} mice at 10–12 days of age were injected with anti-CD3 and followed for blood glucose levels once a week. A group of PDL1^{-/-} mice were given Hamster IgG as control and these mice developed accelerated diabetes in a manner similar to that reported earlier. b) The same group of anti-CD3 treated PDL1^{-/-} mice protected from mice develop myocarditis later in life. PDL1^{-/-} mice on B6 background did not develop myocarditis.

Figure 2.

Anti-CD3 treatment results in depletion of CD3, CD4 and CD8 T cells. In order to confirm that this treatment with anti-CD3 resulted in long term depletion of T cells, blood from the treated mice was tested till 16 week by staining for CD3, CD4, CD8 and F4/80 population by Flow cytometry. Depletion data from 1 and 2 week post treatment is shown. Depletion was maintained up to 16 weeks.

Figure 3e

Figure 3f

Figure 3.

Anti-CD3 treated PDL1^{-/-}.NOD mice are protected from diabetes but develop myocarditis.

a) Representative H&E staining showing infiltration of immune cells in the heart. Infiltrate in the heart mainly comprises of (b) T cells and (c) macrophages. (d) Representative H&E staining showing that skeletal muscles are mainly infiltrated with B cells. (e) Flow cytometry data showing an increase in pan-leukocytes (CD45) and T (CD3) cells in infiltrates in the heart of PDL1^{-/-}.NOD mice. (f) Systemic increase in cytokines as measured by Luminex in anti-CD3 treated PDL1^{-/-}.NOD is shown here. Significantly increased IFN-g (p=0.036), IL-6 (p=0.001), IL-17 (p=0.001) and IL-10 (p=0.0445) in PDL1^{-/-}.NOD mice treated with mitogenic anti-CD3 as compared to anti-CD3 F(ab')₂ or isotype controls.

Figure 4.

Anti-CD3 F(ab')₂ treatment results in delay in precipitation of diabetes in PDL1 het (PDL1^{+/-}.NOD) and WT (PDL1^{+/+}.NOD) but not PDL1^{-/-}.NOD mice. PDL1^{-/-}.NOD mice and littermate controls at 10–12 days of age were injected with anti-CD3 F(ab')₂ for 5 consecutive days (50ug/injection) and followed for blood glucose levels once a week. A group of mice were given Isotype F(ab')₂ IgG as control and monitored for development of diabetes. There was a delay in precipitation of diabetes in mice injected with anti-CD3 F(ab')₂ in PDL1 het and PDL1 WT group but not in PDL1^{-/-}.NOD mice.