The Effect of Regulatory T Cell Depletion on the Spectrum of Organ-Specific Autoimmune Diseases in Non-Obese Diabetic Mice at Different Ages

Running title: Effector and regulatory T cells in NOD mice

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Abstract

The non-obese diabetic (NOD) mouse spontaneously develops several autoimmune diseases, including type 1 diabetes and to a lesser extent thyroiditis and sialitis. Imbalance between effector T cells (Teffs) and regulatory T cells (Tregs) has recently been proposed as a mechanism for the disease pathogenesis in NOD mice, but previous studies have shown the various outcomes by different timing and methods of Treg-depletion. This study was therefore designed to compare the consequences of Treg-depletion by the same method (anti-CD25 antibody) on the spectrum of organ-specific autoimmune diseases in NOD mice of different ages. Treg-depletion by anti-CD25 antibody at 10 days of age accelerated development of all three diseases we examined (insulitis/diabetes, thyroiditis and sialitis); Treg-depletion at 4 weeks of age accelerated only diabetes but not thyroiditis or sialitis; and Treg-depletion at 12 weeks of age hastened only development of thyroiditis and exhibited little influence on diabetes or sialitis. Increased levels of insulin autoantibodies (IAA) were however observed in mice depleted of Tregs at 10 days of age, not in those at 4 weeks. Thus, the consequences of Treg-depletion on the spectrum of organ-specific autoimmune diseases depend on the timing of anti-CD25 antibody injection in NOD mice. Aging gradually tips balance between Teffs and Tregs toward Teff-dominance for diabetes, but this balance for thyroiditis and sialitis likely alters more intricately. Our data also suggest that the levels of IAA are not necessarily correlated with diabetes development.

Key words

type 1 diabetes, thyroiditis, sialitis, regulatory T cells, NOD mice

Author Disclosure Statement

The authors have nothing to disclose.

Introduction

The non-obese diabetic (NOD) mouse is characterized by spontaneous development of several autoimmune diseases, including type 1 diabetes and to a lesser extent thyroiditis and sialitis. Autoimmune destruction of insulin-producing β cells in the pancreatic islets of Langerhans has long been thought to be $CD4^+$ T-cell mediated [1, 2]. $CD4^+$ T cells can be largely divided into two categories; effector (Teffs) and regulatory (Tregs) T cells, which positively and negatively, respectively, regulate immune responses. Naturally occurring $CD4^+CD25^+FoxP3^+$ T cells (Tregs) have recently emerged as a predominant Treg population mediating peripheral self-tolerance [3]. This type of Tregs represents 5-10 % of thymic or peripheral $CD4^+$ T cells in mice and man. The fork head-family transcription factor FoxP3 is essential for generation and maintenance of Tregs [4]. Tregs were originally reported to be generated in the thymus and then migrate to the periphery, but the recent studies show that $CD4^+CD25^-$ T cells can be converted to $CD4^+CD25^+$ Tregs *in vivo* [5-8] or *in vitro* [9, 10].

Imbalance between Tregs and Teffs has recently been proposed as one of the mechanisms for the onset of type 1 diabetes in NOD mice [11]. However, there exists controversial data regarding numerical and functional alterations of Teffs and Tregs in these mice. First, although NOD mice were originally reported to be deficient in Tregs [12], other reports showed comparable Treg numbers in the periphery between NOD and non-autoimmune mouse strains [11, 13, 14]. Second, age-dependent waning of suppressor function of Tregs in the pancreatic lymph nodes and spleen was also proposed to be a mechanism for diabetes development [15, 16], but more recent studies demonstrated normal suppressor function of Tregs throughout life [14]. Furthermore, another mechanism, that is, progressively enhanced pathogenecity of Teffs [11] or Teff-resistance to Treg-suppression was also proposed [16, 17].

Experimental depletion of Tregs can be achieved by multiple means; for example, genetic disruption of Foxp3, IL-2 or CD28-B7 pathway [18-20] and anti-CD25 [21] or anti-IL-2

antibodies [22], all of which exacerbated autoimmunity. However, the spectrum of the organs affected appears to be different with these different methods even in the same mouse strain. For example, in NOD mice, FoxP3-deficeint scurfy mice developed inflammation in numerous organs including lung, liver, skin *etc*, but not exocrine or endocrine organs at 2 weeks of age, and die within 3 weeks [23]. On the other hand, depletion of Tregs by antibody neutralization of IL-2 starting at 10 days of age led to acute development of diabetes in parallel with the inflammation of the other organs such as nervous system (inducing clinically evident ataxia and paralysis), and endocrine (thyroid) and exocrine (lachrymal and salivary) glands [22]. However, Treg-depletion by anti-CD25 antibody at 4 weeks of age induces only acceleration of diabetes, but clinical neuropathy was not observed [24]. Thus, sites of inflammation induced by Treg-depletion in NOD mice only partially overlap in these reports. These differences may be attributed to distinct methods (genetic disruption of FoxP3 or antibodies to IL-2 or CD25) or ages (congenital, 10 days or 4 weeks) of Treg-depletion. Therefore, this study was designed to compare the consequences of Treg-depletion by the same method (anti-CD25 antibody) on the spectrum of organ-specific autoimmune diseases in NOD mice at different ages.

Materials and methods

Mice

Male and female NOD mice, 3–4 weeks of age, were purchased from Clea Japan (Tokyo, Japan). All mice were kept under specific pathogen-free conditions at the Laboratory Animal Center for Biomedical Research of Nagasaki University, and were housed in an air conditioned room with a 12-hour light-darkness cycle. Animal care and all experimental procedures were performed in accordance with the Guideline for Animal Experimentation of Nagasaki University with approval of the Institutional Animal Care and Use Committee.

Monitoring blood glucose levels

The blood glucose levels were monitored once a week with a Glutest-Ace meter (Sanwa Kagaku, Nagoya, Japan) starting at 8 weeks of age. Mice with blood glucose levels above 250 mg/dl for 2 consecutive measurements were considered diabetic.

Purification and injection of anti-CD25 antibody (PC61)

Anti-CD25 monoclonal antibody was purified from ascites of mice intraperitoneally (ip) injected with hybridoma PC61 using a HiTrapTM protein G HP column (Amersham, Piscataway, NJ) as previously described [24]. Anti-CD25 antibody was ip injected into mice at 10 days (250 μg/mouse), 4 weeks and 12 weeks (500 μg/mouse) of ages.

Flow cytometry

Single cell suspensions of SPCs were prepared from spleens of NOD mice. Red cells were lysed in the ammonium chloride buffer. The cells were resuspended in PBS and stained with PE or FITC-conjugated anti-CD4 (GK1.5), anti-CD25 (7D4) and/or anti-FoxP3 (FJK-16s; Foxp3 staining kit) (all from e-Bioscience, San Diego, CA), and analyzed on a FACSCanto II flow cytometry using FACS Diva software (BD Biosciences, San Diego, CA). Note that the binding site of 7D4 on CD25 is different from that of PC61.

Measurement of insulin autoantibody (IAA)

Mice were bled at 4, 8, 10 and 12 weeks of ages and sera were stored at -20 C until use. The levels of IAA in sera were determined by using a 96-well filtration plate micro IAA assay [25]. The index over 0.01 was considered as positive.

Histology

For histological examinations, mice were sacrificed when a half or more of mice developed

diabetes in each group. Thus, the time intervals between anti-CD25 antibody injection and euthanization were 9.4 ± 1.1 (mean ± S.D.), 7.9 ± 0.4 and 22.2 ± 1.8 weeks in mice treated at 10 days, 4 weeks and 12 weeks of ages, respectively.

Pancreata, thyroid tissues and salivary glands in these mice were histologically analyzed by fixing in 10% formalin and staining with hematoxylin and eosin (H & E). For each organ, infiltration was scored according to the following criteria in non-continuous three sections. [Insulitis] grade 0, no lymphocyte infiltration; grade 1, islets with lymphocyte infiltration in less than 25% of their area; grade 2, 25–50% of the islet area infiltrated; grade 3, 50–75% of the islet area infiltrated; grade 4, more than 75% infiltrated or small retracted islets [24, 25].
[Thyroiditis] grade 0, no lymphocyte infiltration; grade 1, less than 10 % lymphocytic infiltration; grade 2, 10 to 30 % lymphocytic infiltration; grade 3, 30 to 50 % lymphocytic infiltration; grade 4, greater than 50 % lymphocytic infiltration [26].
[Sialitis] one infiltrate is defined as 50 or more lymphocytes in the cluster. The score for each mouse is the total infiltrates present in 30 mm^2 of the histological sections.

Immunohistochemistry was also performed in pancreata to examine immune cells infiltrated into the islets. Pancreata were embedded in Tissue-Tek OCT compound (Sakura Finetechnical, Tokyo, Japan) and stained with anti-CD4 (SC-13573, Santa Cruz Biotechnologies, Santa Cruz, CA), anti-CD8 (CBL1318, Chemicon International, Temecula, CA), and anti-CD19 (SM018P, Acris Antibodies GmbH, Herford, Germany) antibodies and Vectastatin Vector Kit (PK-4000, Vector laboratory, Burlingame, CA). For each staining, positively stained cells were scored in more than five islets.

Statistical analysis

Group differences were performed with the Student‘s *t* test or chi square test and differences between Kaplan–Meier survival curves were estimated by the log rank test. *P* values less than 0.05 were considered statistically significant.

Results

$CD4^+CD25^+$ Tregs/$CD4^+$ T cells in NOD mice after administration of anti-CD25 antibody

To clarify the consequences of Treg-depletion using the same standard method on development of various autoimmune diseases in NOD mice of different ages, anti-CD25 antibody (PC61), a widely used means to deplete Tregs, was employed. To determine the efficacy of anti-CD25 antibody, we ip administered anti-CD25 antibody to 10 day-, 4 week- and 12 week-old NOD mice and determined the percentages of $CD4^+CD25^+$ Tregs/$CD4^+$ T cells in the splenocytes. Representative flow cytometric data in the PBS-treated (control) and anti-CD25 antibody-treated mice are shown in Fig. 1A. The percentages of $CD4^+CD25^+$ Tregs/$CD4^+$ T cells was substantially declined at least for the first 3 weeks, and then gradually returned to the normal levels in 8 weeks in all 3 groups (Fig. 1B). Those of $CD4^+CD25^-$ Teffs/$CD4^+$ T cells were however unchanged during this experimental course (data not shown).

In addition, these data also clearly shows that the percentages of $CD4^+CD25^+$ Tregs/$CD4^+$ T cells were not altered from 10 days to 15 weeks of ages in the control NOD mice, indicating no age-dependent decline of the frequency of Tregs in NOD mice as previously reported in some [11, 13, 14], but not all [12], articles.

Diabetes onset and IAA appearance after administration of anti-CD25 antibody

NOD mice generally develop diabetes after 12 weeks of ages with the incidence being higher in female [27]. In the mice treated with anti-CD25 antibody at 10 days of age (the d10-depleted mice) and 4 weeks of age (the wk4-depleted mice), the diabetes onset were significantly accelerated in both sexes (Fig. 2A & B). For example, the incidences of diabetes were 80 to 90 % in the d10-depleted mice and 60 to 80 % in the wk4-depleted mice, as compared to 0 to 10 % in the control mice at 16 weeks of age. However, no significant acceleration of diabetes development was observed in the wk12-depleted mice (Fig. 2C).

Turing to the IAA, in the control NOD mice, the IAA index became positive at 8 weeks of

age and then gradually increased in females, and was at borderline levels at 8 to 12 weeks of age in males (Fig. 3). Treg-depletion at 10 days of age accelerated the appearance of the IAA, and enhanced the tiers and/or the positivity of the IAA in both sexes as compared to the control mice (Fig. 3A & B), clearly demonstrating that Treg-depletion exacerbated both diabetes and the IAA titers in the d10-depleted mice. Therefore, earlier appearance and higher titers/positivity of the IAA were also expected in the wk4-depleted mice, because diabetes onset was accelerated in these mice as shown in Fig. 2. However, contrary to our expectation, no significant difference was observed in the IAA index between the wk4-depleted and the control mice except at 10 weeks of age (Fig. 3C & D).

Lymphocyte infiltration to pancreata, thyroid glands and salivary glands after administration of anti-CD25 antibody

Consistent with the aforementioned data on diabetes (Fig. 2), the severity of insulitis was significantly higher in the d10-depleted mice compared to the control mice, when examined at 8 to 12 weeks of ages (Fig. 4A). A difference between the wk4-depleted and the control mice was not significant because of variability of the data. In contrast, the control and the wk12-depleted mice showed comparable levels of insulitis at 35 weeks of age.

In addition to insulitis, NOD mice also develop other inflammatory lesions such as thyroiditis and sialitis, which are usually observed in the later phase of life [28]. Interestingly, approximately a half of the d10-depleted mice developed thyroiditis (4/9) and sialitis (4/9), whereas the thyroid and salivary glands both remained intact in the wk4-depleted and the control mice (Fig. 4B). Furthermore, the wk12-depleted mice showed the higher degrees of thyroiditis compared to the control mice at 35 weeks of age. Sialitis developed in most of mice at 35 weeks of age: no effect of anti-CD25 antibody was observed (Fig. 4C). Representative histology of the islets, thyroid glands and salivary glands is shown in Fig. 5A.

Characterization of immune cells infiltrated into the islets was compared between control mice and those treated with anti-CD25 antibody at 4 weeks of age by immunehistochemistry.

As shown in Fig. 5B, the percentages of $CD19^+$ B, $CD4^+$ T and $CD8^+$ T cells are essentially same between 2 groups.

Discussion

This study was designed to compare the outcomes of Treg-depletion by anti-CD25 antibody on development of various autoimmune diseases in NOD mice of different ages. Our data obtained with direct injection of anti-CD25 antibody into NOD mice demonstrate that Treg-depletion at 10 days of age accelerated development of all three diseases we examined (insulitis/diabetes, thyroiditis and sialitis); Treg-depletion at 4 weeks of age accelerated only diabetes but not thyroiditis or sialitis; and Treg-depletion at 12 weeks of age hastened only development of thyroiditis and exhibited little influence on diabetes or sialitis. Thus these results clearly indicate that the spectrum of affected organs depends on the timing of Treg-depletion in NOD mice. These different results cannot be explained by the distinct kinetics of the ratios of Tregs/ $CD4^+$ T cells following anti-CD25 antibody injection in 3 groups.

Our data on Treg-depletion by anti-CD25 antibody at 10 days of age do not fit those by anti-IL-2 antibody starting at the same age [22]. Although acceleration of development of diabetes, thyroiditis and sialitis was commonly observed in Treg-depletions by anti-CD25 and anti-IL-2 antibodies, neuropathy developed only in the latter. Similar inconsistent data were also reported in BALB/c mice. Anti-IL-2 antibody induced gastritis in almost all mice [22], but anti-CD25 antibody rarely induced gastritis [29], when antibodies were given at 10 days of age. Thus anti-IL-2 antibody may have additional, Treg-independent effects on immune system as compared to anti-CD25 antibody.

Furthermore, in contrast to postnatal Treg-depletion, elimination of Tregs by FoxP3 gene disruption from the fetal periods causes life-threatening, systemic autoimmune reactions in NOD mice [23]. It can therefore be speculated that Treg-depletion in the fetal periods induces more severe autoimmune reactions than that in postnatal periods, although the methods used to

deplete Tregs were different.

Acceleration of insulitis and diabetes development by Treg-depletion in 10 day- and 4 week-, but not 12 week-, old mice indicate that Treg-sensitive, diabetogenic Teffs exist from the neonatal periods of life and their activities are being held in check by the co-existing Tregs at least up to 4 weeks of age. Thus suppressor function of Tregs is intact up to 4 weeks of age. However, aging gradually tips balance between Teffs and Tregs toward Tregs, and Teffs become dominant over Tregs at 12 weeks of age. By contrast, the data on thyroiditis and sialitis are very complicated. As for diabetes, acceleration of thyroiditis and sialitis in 10 day-old mice indicate that thyroiditogenic and sialitogenic Teffs exist in neonates and are sensitive to suppressor activity of Tregs. However, the numbers and/or activities of thyroiditogenic and sialitogenic Teffs appear to dramatically decline before 4 weeks of age because Treg-depletion had no effect on thyroiditis and sialitis at 4 weeks of age and Tregs are functionally intact at this time point (see above). These decreased numbers and/or activities of Teffs appear to recover at 12 weeks of age, because sialitis and thyroiditis developed in control mice and/or the wk12-depleted mice. Increased incidence of thyroiditis not sialitis by anti-CD25 antibody suggests that thyroiditogenic Teffs are still sensitive, but sialitogenic Teffs become resistant, to Tregs at this time point. That sialitis develops earlier than thyroiditis in NOD mice [30] may be consistent with the observation that sialitogenic Teffs become resistant to Tregs earlier than thyroiditogenic Teffs. In this regard, the timing of tipping Teff/Treg balance toward Teffs may vary in different organ/autoantigen-specific T cells. Thus spontaneous development of autoimmune diseases in NOD mice may not simply be explained by a decrease in numbers/function of Tregs. However, we will need further studies on the dynamics of Teff/Treg balance for each target organs/autoantigens.

Regarding humoral immunity, earlier appearance and higher titers of IAA were observed in the d10-depleted mice, but not the wk4-depleted mice. However, both groups of mice showed acceleration of diabetes. We have also previously found that diabetes-free strain NOR mice spontaneously produce IAA without developing diabetes [31]. Thus the levels of IAA are not

necessarily correlated with diabetes development.

In conclusion, the spectrum of affected organs depends on the timing and means of Treg-depletion in NOD mice. It is clear that aging gradually tips balance between Teffs and Tregs toward Teff-dominance for diabetes, but this balance for thyroiditis and sialitis likely alters more intricately.

Table 1. The percentages of $CD19^+$ B, $CD4^+$ T and $CD8^+$ T cells in the islets of ~12 week-old wk4-treated and ~35 week-old untreated mice.

	The percentages (/SPCs)		
Mice	$CD19^+$	$CD4^+$	$CD8^+$
~12 week-old, wk4-treated mice	31.8±14.1	55.7±14.9	12.4±5.3
~35 week-old, untreated mice	37.0±3.8	46.7±3.1	16.3±4.0

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Figure legends

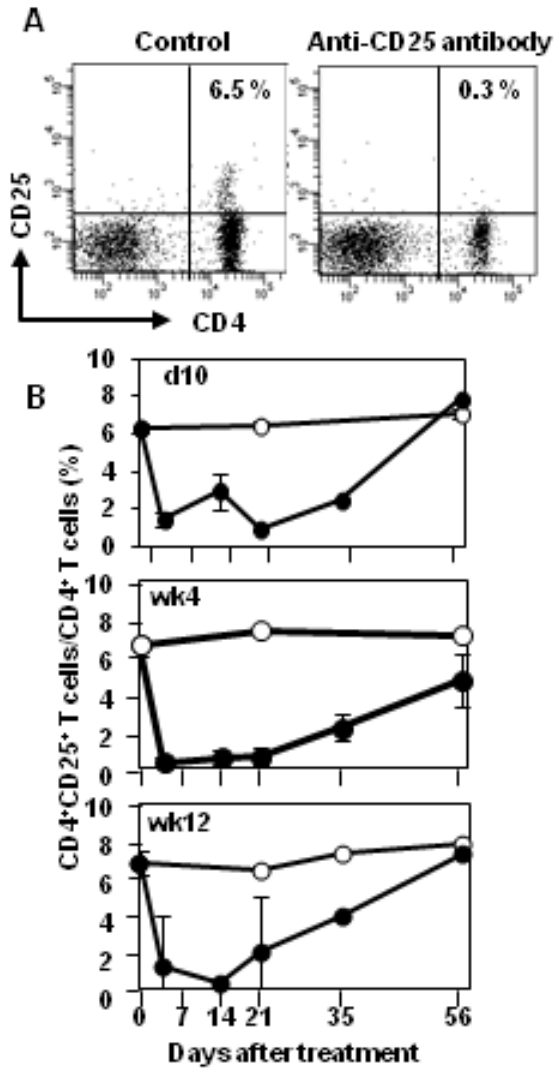

Figure 1. Flow cytometric analysis of CD4 and CD25 expression on splenocytes from the control and anti-CD25 antibody-treated NOD mice. (*A*) Representative flow cytometric data. Mice were treated with PBS (left) or 500 μg PC61 (right), and 4 days later CD4 and CD25 expression was analyzed as described in the *Materials and methods*. (*B*) Time course of the effect of PC61. Anti-CD25 antibody was injected into 10 day-, 4 week- and 12 week-old mice, and CD4 and CD25 expression was analyzed 4, 14, 21, 35 and 56 days later. Open circles, control; closed circles, anti-CD25 antibody-treated. Data are means ± S.D. (n = 3 in each group).

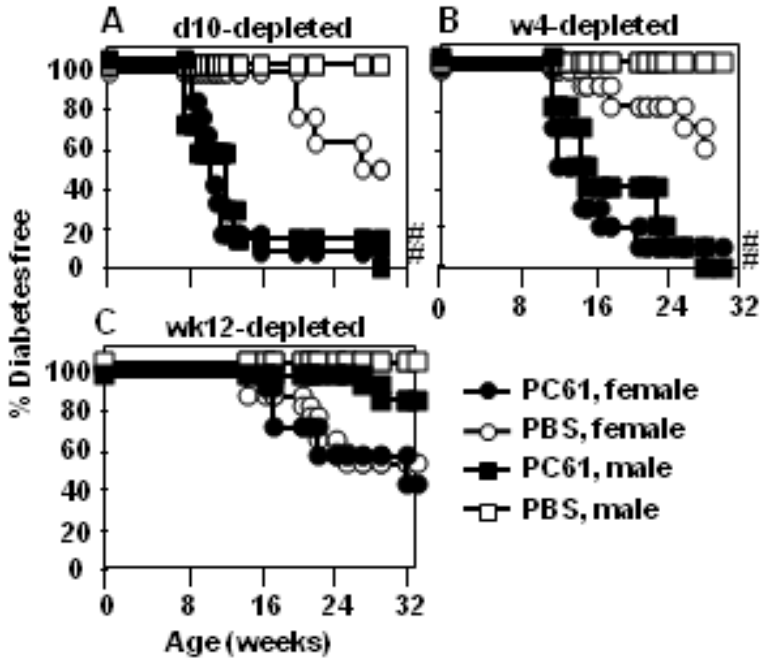

Figure 2. Diabetes free ratios in the control and anti-CD25 antibody-treated NOD mice. Life table analysis for the development of diabetes following injection of PC61 or PBS at 10 days (*A*), 4 weeks (*B*), or 12 weeks (*C*) of ages was shown (n = 8 - 13 in each group). The experiments were repeated at least twice with the essentially same results. #, $p < 0.00001$ (by log rank test).

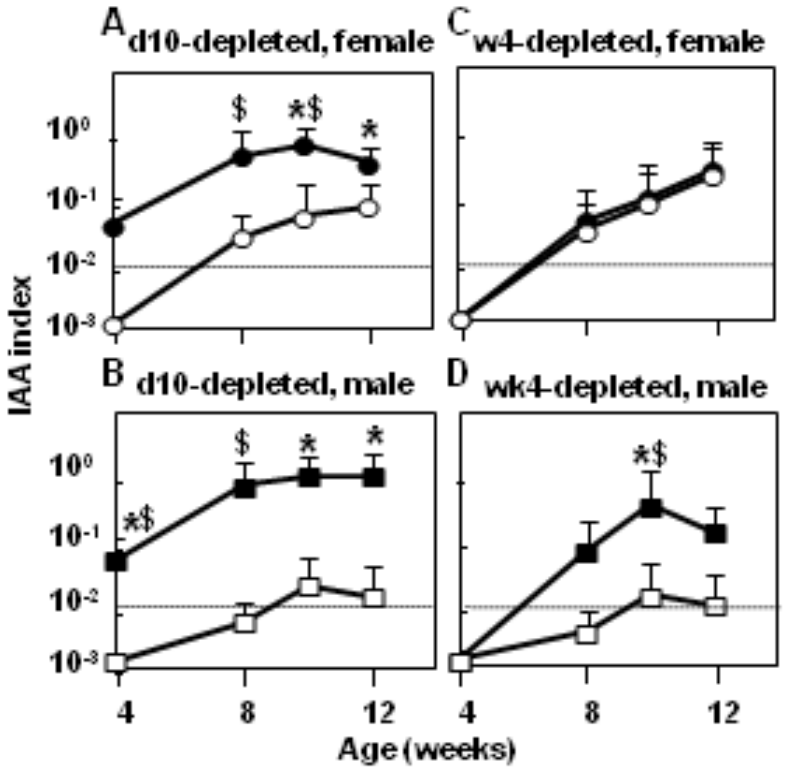

Figure 3. The IAA index in the control and anti-CD25 antibody-treated NOD mice. The IAA index following administration of anti-CD25 antibody or PBS at 10 days (*A* & *B*) or 4 weeks (*C* & *D*) of ages was shown. Data are means ± S.D. (n = 8 - 13 in each group). The dashed lines, the borderline for positive IAA; *, $p < 0.05$ (by *t* test), $, $p < 0.05$ (by chi square test).

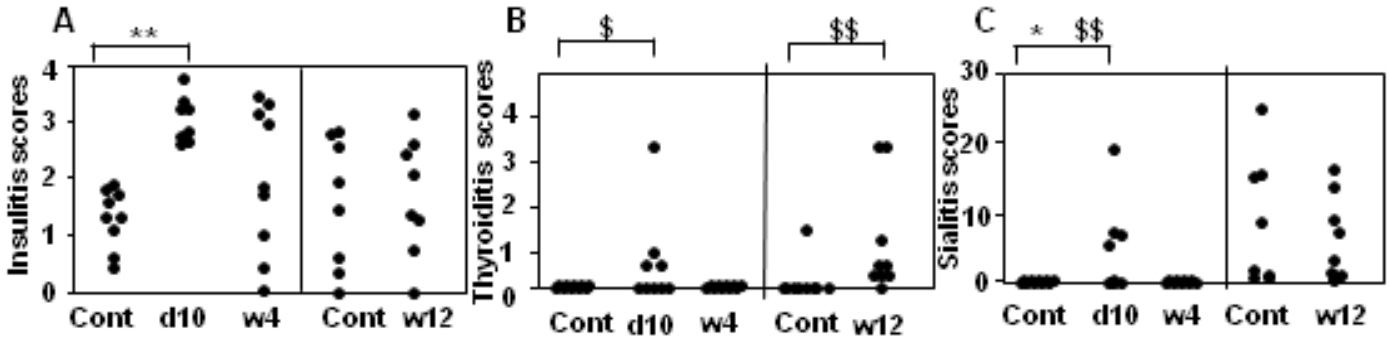

Figure 4. Infiltration scores of islets, thyroid glands and salivary glands in the control and anti-CD25 antibody-treated NOD mice. The tissues were obtained at 12 weeks (the d10- and wk4-depleted mice) or 35 weeks of age (the wk12-depleted mice), stained with H & E and examined as described in the *Materials and methods*. Each dot represents the infiltration score in individual mouse. *, $p < 0.05$ (by *t* test); $ and $$, $p < 0.05$ and 0.01, respectively (by chi square test).

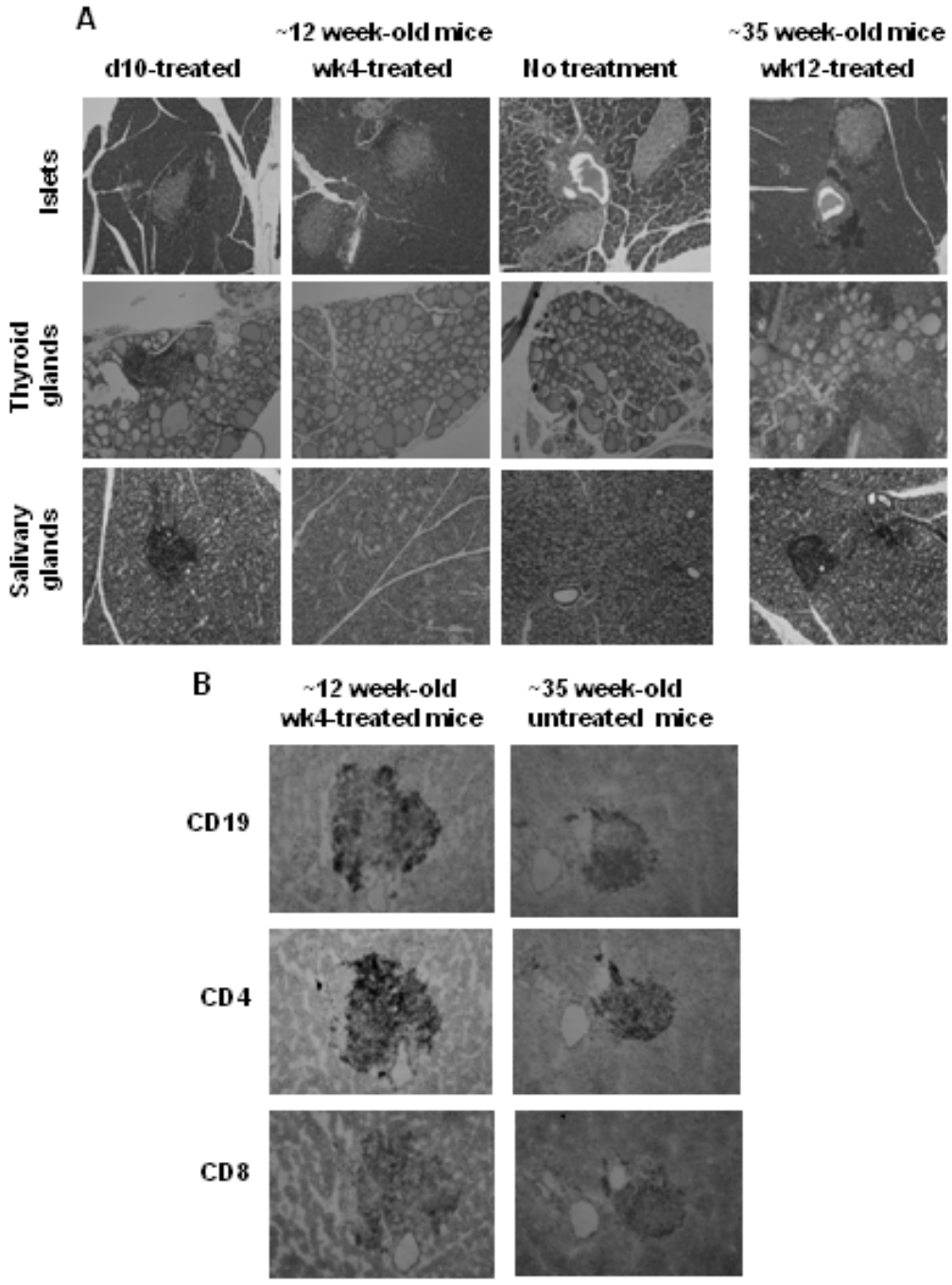

Figure 5. Histology of islets, thyroid glands and salivary glands in the control and anti-CD25 antibody-treated NOD mice. *(A)* Representative H & E staining of the islets, thyroid glands and salivary glands in ~12 week-old, d10-, wk4- and wk12-depleted and ~35 week-old, control mice. (*B*) Immunohistochemistry for $CD19^+$ B, $CD4^+$ T and $CD8^+$ T cells in islets of ~35 week-old, untreated and ~12 week-old, wk4-treated mice. Magnifications; x100.