Immune Disorders and Sex Differences in Spontaneously Diabetic Torii Rats, Type 2 Diabetic Model

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Short title

Immune disorders and sex differences in diabetic SDT rats

Summary

Type 2 diabetes (T2D) is believed to be a non-autoimmune metabolic disorder. However, there are increasing reports that some T2D patients have immune abnormalities. In addition, it is known that there are sex differences in the onset of diabetes and immune responses in humans. Spontaneously Diabetic Torii (SDT) rats, a non-obese T2D model, also have sex differences in the onset of diabetes, but the involvement of immune abnormalities in diabetes is unknown. In this study, we investigated immune abnormalities in SDT rats. Immune cell subset analysis was performed in male and female SDT rats and control Sprague-Dawley (SD) rats at 5, 11, and 17 weeks of age. Male and female SDT rats had swelling of the spleen and lymph nodes and a higher number of T cells and B cells in the blood, spleen, and lymph nodes than SD rats. Only male SDT rats developed diabetes at 17 weeks of age, and the number of classical and non-classical monocytes in the blood and spleen of male SDT rats was higher than that in male SD rats and female SDT rats that did not develop diabetes. Most of these findings were observed before the onset of diabetes (~11 weeks of age), suggesting that classical and non-classical monocytes may contribute to the development of diabetes in male SDT rats. In conclusion, SDT rats may be a useful T2D model involved in immune abnormalities, and further research will help elucidate the pathophysiology of T2D with immune abnormalities and develop new therapeutic agents.

Key words

Diabetes, SDT rat, Immune disorder, Sex difference, Monocyte

Introduction

Type 2 diabetes (T2D) is a polygenic disorder characterized by insulin deficiency and insulin resistance. High-calorie intake and sedentary lifestyles have resulted in an increased number of patients with this disorder worldwide. Type 1 diabetes (T1D) is considered a cell-mediated autoimmune disease, and T2D to be a non-autoimmune metabolic disorder. However, some patients with T2D have been identified with autoimmune abnormalities, such as islet autoantibodies and islet-reactive T cells associated with severe β-cell dysfunction [1-3]. Although the prevalence of islet autoimmunity in T2D patients is unknown, it has been estimated to be approximately 30% using islet autoantibodies as a biomarker [1,4]. The involvement of autoimmunity in T2D may be more significant than it is considered. Besides autoimmunity, the involvement of immunity and inflammation is often discussed in relation to obesity, but there are several uncertainties, and therapeutic interventions targeting them have not been realized.

In humans and diabetic animal models, there is a sex difference in the frequency of onset of diabetes. In humans, the prevalence of diabetes among women is lower than among men until 70 years of age, and the prevalence among women is higher after 70 years [5]. In preclinical studies, T2D model rats, such as Zucker diabetic fatty rats [6], WBN/Kob rats [7], and OLETF rats [8], have a higher prevalence of diabetes in male than that in female.

Spontaneously Diabetic Torii (SDT) rats are a non-obese T2D model that displays

hypoinsulinemia followed by severe hyperglycemia after ~15 weeks of age and diabetic complications such as retinopathy and nephropathy at ~40 weeks of age [9,10]. SDT rats also exhibit glucose intolerance prior to the onset of diabetes [11,12], with pancreatic β-cell injury observed in the pre-diabetes stage [13]. Male SDT rats have a higher number of lymphocytes, monocytes, and neutrophils in the blood than male Sprague-Dawley (SD) rats. The immunomodulator FTY720 inhibits the onset of diabetes in male SDT rats, suggesting that immune abnormalities are involved in the development of diabetes [14,15]. In addition, there is a sex difference in the onset of diabetes in SDT rats and male SDT rats have a 100% cumulative incidence of diabetes at 40 weeks of age, while female SDT rats have 0% [16]. There are several uncertainties in SDT rats regarding immune abnormalities and sex differences.

In this study, we examined immune disorders and sex differences in the onset of diabetes in SDT rats using multicolor flow cytometric analysis. The diabetic etiology in SDT rats was also examined to see if this is a useful model for the development of new therapeutic agents for T2D patients with immune abnormalities.

Methods

Animals

Male/female SDT rats and SD rats (CLEA Japan, Tokyo, Japan) aged 5, 11, and 17 weeks were used in the present study. All animal procedures and protocols complied with

the guidelines for animal experimentation set by the Ethics Committee for Animal Use at JT and Niigata University. Rats were maintained in a temperature-controlled room with 23 ± 3 °C temperature on a 12 h/12 h light-dark cycle with *ad libitum* access to a standard diet (CRF-1; Oriental Yeast, Tokyo, Japan) and water.

Body weight and biochemical parameters

Body weight and biochemical parameters were evaluated at 5, 11, and 17 weeks of age. Blood samples were collected from the tail vein under non-fasting conditions. Plasma glucose levels were measured using commercial kits (Roche Diagnostics, Basel, Switzerland) and an automatic analyzer (Hitachi 3500; Hitachi High-Technologies, Tokyo, Japan). Commercial ELISA kits were used to measure plasma insulin levels (Rat Insulin ELISA Kit; Morinaga Institute of Biological Science, Yokohama, Japan).

Flow cytometry

Blood samples were collected from the tail vein of rats at 5, 11, and 17 weeks of age, and red blood cells were lysed using VersaLyse™ Lysing solution (Beckman Coulter, Brea, CA, USA). Necropsy was performed at 5, 11, and 17 weeks of age. All animals were sacrificed via exsanguination under isoflurane anesthesia. The spleen and inguinal lymph nodes of rats were immediately removed, and their weights were measured. Spleen and lymph nodes were dissociated with gentleMACS™ C tubes and gentleMACS™

Dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany) following the manufacturer's instructions. All tissues were strained using a 40 μm Cell strainer (Corning, NY, USA). Single-cell suspensions from blood, spleen, and lymph nodes were stained as follows: cells were blocked with anti-CD32 antibody (BD Biosciences, San Jose, CA, USA) to prevent Fc-mediated non-specific binding; cells were then stained with antibodies in Brilliant Stain Buffer (BD Biosciences) at 4°C for 20 min, followed by further washing and fixation with Fixation Buffer (BD Biosciences) at 4°C for 30 min. The monoclonal antibodies and dyes used were: anti-CD45 (clone OX1, eFluor 450), anti-CD4 (clone OX35, SuperBright 600), anti-His48 (clone HIS48, FITC), anti-CD161 (clone 10/78, PerCP-eFluor 710), anti-CD43 (clone W3/13HLK, PE), anti-CD45R (clone HIS24, PE-Cy7), anti-CD8a (clone OX8, Alexa Fluor 700), and dead cell stain (Fixable Viability Dye, eFluor 780) (Thermo Fisher Scientific, Waltham, MA, USA), and anti-CD3 (clone 1F4, APC) (BD Biosciences). Cells were analyzed using an Attune™ NxT Acoustic Focusing Cytometer (Thermo Fisher Scientific). Flow cytometric compensation was performed using single-stained cells. For the identification of positive and negative populations, the fluorescence minus one ("FMO") principle was utilized to account for background antibody fluorescence. The gating strategy was based on a previous study [17]. The number of total leukocytes, CD3+T cells, CD4+T cells, CD8+T cells, B cells, natural killer (NK) cells, neutrophils, CD43-low and His48-high (classical) monocytes, and CD43-high and His48- intermediate/low (non-classical) monocytes was analyzed.

The number of cells in each population was calculated using CountBright™ Absolute Counting Beads (Thermo Fisher Scientific).

Statistical analysis

Data are expressed as mean ± standard deviation. The following statistical analyses were performed to derive the differences between the mean values: homogeneity of variance was evaluated by the F-test followed by the Student's *t*-test or Aspin-Welch's *t*-test for homoscedastic data or heteroscedastic data, respectively. Statistical analyses were performed between male SD rats and male SDT rats, female SD rats and female SDT rats, and male SDT rats and female SDT rats, and the *P* value was adjusted using the Bonferroni procedure. All statistical analyses were performed using GraphPad Prism® 6.07 (GraphPad Software, San Diego, CA, USA). Differences were considered significant at $P < .05$.

Results

Differences of body weight and weights of the spleen and lymph nodes

Body weight, absolute and relative weights of the spleen and inguinal lymph nodes are shown in Fig. 1. The body weight of SDT rats was lower than that of SD rats in male, but not in female. The body weight of male SDT rats was higher than that of female SDT rats (Fig. 1A). The absolute weights of the spleen and lymph nodes are shown in Fig. 1B and

C, and the relative weights are shown in Fig. 1D and E because the body weights were different between the groups. The relative weights of the spleen and the lymph nodes in SDT rats were higher than those in SD rats in either sex. In SDT rats, the relative spleen weight in male was slightly lower than that in female, but there was no sex difference in the relative lymph nodes weight.

Differences of biochemical parameters

The non-fasting plasma glucose and insulin levels are shown in Fig. 2. The glucose level in male SDT rats was higher than that in male SD rats and female SDT rats at 17 weeks of age (male SD rats, 228 ± 40 mg/dl; male SDT rats, 463 ± 145 mg/dl; female SDT rats, 152 ± 16 mg/dl; male SD vs. male SDT, $p<0.05$; male SDT vs. female SDT, $p<0.01$) (Fig. 2A). The insulin level in SDT rats tended to be lower than that in SD rats. However, there was the large dispersion of insulin concentrations, because of the non-fasting condition or the hemolytic plasma taken from the tail vein affecting measurement by ELISA.

Differences of the major leukocyte populations of blood

The major leukocyte populations of blood is shown in Fig. 3 and 4. The gating strategy in the blood as a representative is shown in Fig. 3, and the same strategy was applied to the spleen and lymph nodes. In SDT rats, the number of total leukocytes, CD3+T cells,

CD4+T cells, and CD8+T cells was higher than that in SD rats in either sex, and there were no differences between male and female SDT rats. On the other hand, the number of B cells, classical and non-classical monocytes was higher than that in SD rats in either sex, but the number of these cells in male SDT rats was higher than that in female SDT rats. For neutrophils, there was the same tendency as classical monocytes, but was not a significant. The number of NK cells was higher in male SDT rats than in female SDT rats under some conditions, but there was no characteristic change overall.

Differences of the major leukocyte populations of spleen

The major leukocyte population in the spleen is shown in Fig. 5. In SDT rats, the number of CD3+T cells, CD4+T cells, and CD8+T cells was higher than that in SD rats in either sex, and there were no differences between male and female SDT rats. On the other hand, the number of total leukocytes, B cells, neutrophils, classical and non-classical monocytes was higher than that in SD rats in either sex, but the number of these cells in male SDT rats was higher than that in female SDT rats. The number of NK cells was higher in male SDT rats than in female SDT rats.

Differences of the major leukocyte populations of lymph nodes

The major leukocyte population in the inguinal lymph nodes is shown in Fig. 6. The number of neutrophils, classical and non-classical monocytes in lymph nodes was not

analyzed because of the small sample size. In SDT rats, the number of total leukocytes, CD3+T cells, CD4+T cells, CD8+T cells, B cells, and NK cells was higher than that in SD rats in either sex, and the number of these cells in male SDT rats was higher than that in female SDT rats at 5 weeks of age, but lower at 17 weeks of age.

Discussion

It has been reported that the number of lymphocytes, monocytes, and neutrophils in the blood of male SDT rats is higher than that in male SD rats [14,15]. However, there are no reports on circulating immune cell subsets in female SDT rats and on subsets in the spleen and lymph nodes in male and female SDT rats. The results obtained in this study are consistent with those of previous reports, showing that the number of circulating immune cell subsets, such as lymphocytes, monocytes, and neutrophils, was higher in male SDT rats than in male SD rats. Furthermore, male and female SDT rats had early swelling of the spleen and lymph nodes relative to male and female SD rats and a higher number of T cells and B cells in the blood, spleen, and lymph nodes. Only male SDT rats developed diabetes (>250 mg/dl in non-fasting plasma glucose level) at 17 weeks of age, and the number of B cells, neutrophils, and classical/non-classical monocytes in the blood and spleen of male SDT rats was higher than that in male SD rats and female SDT rats that did not develop diabetes. Moreover, many of these findings were observed before the onset of diabetes (~11 weeks of age).

The most characteristic finding in male SDT rats related to the onset of diabetes in this study was a higher number of classical and non-classical monocytes, B cells and neutrophils in the blood and spleen. We considered neutrophils less significant because of our previous reports that FTY720, an immunomodulator affecting lymphocyte homing, suppressed the onset of diabetes by reducing circulating lymphocytes and monocytes without affecting the number of neutrophils in male SDT rats [15]. The number of B cells in male SDT rats was higher in the blood and spleen but lower in the lymph nodes than in female SDT rats; therefore, it is difficult to interpret their relation with the onset of diabetes. The clinical and preclinical information for neutrophils and B cells related to T2D is limited, and it is difficult to consider the currently available information. Further information and detailed examinations are required. Therefore, we analyzed the relationship between monocytes and T2D. Monocytes are the immune cells involved in local and systemic inflammatory responses in the early phase, and when they migrate to peripheral tissues, they differentiate into macrophages and dendritic cells [18]. In peripheral blood, two subsets of monocytes, “classical” and “non-classical,” have been identified in humans and rodents. In humans, these are characterized by differential expression of CD14 and CD16 [19], in rats by expression of CD43 [20]. In rats, high- and low-CD43 monocytes are considered to be analogous to low- (non-classical) and high-Ly6C (classical) murine monocytes, respectively [21]. Classical monocytes are critical for the initial inflammatory response, and non-classical monocytes have been widely

viewed as anti-inflammatory as they maintain vascular homeostasis, such as recognition and removal of pathogens [22]. However, their involvement in T2D is unclear.

Male SDT rats showed infiltration of inflammatory cells such as lymphocytes and macrophages in and around the pancreatic islets at 10 to 20 weeks of age, but not at 4 weeks of age [11,16]. Treatment of SDT rats with Cl2MDP-liposomes reduced the number of monocytes in the blood and infiltrated macrophages in the islets, and inhibited islet fibrosis [14], suggesting that macrophages are involved in pancreatic islet injury in SDT rats. Higher number of blood and spleen monocytes in male SDT rats we found in this study can differentiate into macrophage in tissue and can promote inflammation. On the other hand, SDT rats are the model for the diabetic retinopathy and nephropathy, and these disorders involve an inflammatory response via macrophage infiltration [23,24], and these organs including the pancreas, may be damaged by similar mechanisms.

Why was the number of monocytes lower and did not develop the onset of diabetes in female SDT rats relative to male SDT rats, even though there were immune abnormalities such as a higher weight of spleen and lymph nodes and a higher number of lymphocytes in the blood, spleen, and lymph nodes compared to male and female SD rats, and a higher number of monocytes compared to female SD rats? This sex difference may be partly attributed to estrogen, which inhibits the development of diabetes in female SDT rats [25]. In db/db mice, which are other T2D models, estrogen-treated mice did not develop hyperinsulinemia, hyperglycemia, or islet atrophy [26]. In addition, WBN/Kob rats [27]

and OLETF rats [28] do not develop diabetes in females like SDT rats, even though development of pancreatic lesions and increased incidence of diabetes after ovariectomy have been reported. Estrogen has regulatory effects on the immune system in addition to regulating reproductive function through the conventionally known nuclear receptors ERα and ERβ, and GPER1 (GPR30), a recently discovered GPCR on the cell membrane [29]. A typical estrogen, 17β-Estradiol (E2), and the receptors ERα and GPER1 directly modulate monocyte functions, such as the expression of adhesion molecules, proinflammatory cytokines, and chemokines [30-33]. From these reports, endogenous estrogen may act on monocytes/macrophages to show anti-inflammatory effects via ERα and GPER1 in female SDT rats, but further experimental verification is needed.

However, some reports suggest that androgen may contribute to an increased risk of developing monocyte-mediated pathologies [34], and it is difficult to deny the possibility that androgen enhances monocyte-mediated pathology in male SDT rats. Further research is needed because the effects of androgen on the immune system are less known than the effects of estrogen. In addition, it is an undeniable possibility that estrogen acts on immune subsets other than monocytes/macrophages and affects the onset of diabetes. Estrogen regulates insulin resistance in peripheral tissues such as adipose tissue, skeletal muscle, and liver, and these tissues respond to insulin appropriately, resulting in effective glucose uptake [35]. Thus, the improvement of insulin resistance in peripheral tissues by endogenous estrogen may have suppressed the onset of diabetes in female SDT rats.

Female SDT rats did not develop diabetes at 16 and 25 weeks of age but were reported to have glucose intolerance as in male SDT rats [36]. In this study, common immune abnormalities such as a higher weight of spleen and lymph nodes and a higher number of lymphocytes (T cells in particular) in the blood, spleen, and lymph nodes were observed in male and female SDT rats compared to male and female SD rats. T cells play a dominant role in promoting and sustaining inflammatory processes and insulin resistance by inducing proinflammatory cytokines in metabolic organs, such as the adipose tissue, liver, muscle, and pancreas [37]. Hence, female SDT rats may be useful in studying the involvement of immune disorders in insulin resistance and impaired glucose tolerance (IGT), and administration of FTY720 to female SDT rats may reveal the association between lymphocytes such as T cells and IGT.

There are immunological similarities between human T2D and SDT rats. In human T2D patients, there are more leukocytes, lymphocytes (especially T cells, activated CD4+ T cells, and activated CD8+T cells), granulocytes, and monocytes in the blood than healthy subjects [38,39]. Postmenopausal women develop visceral obesity and insulin resistance and are at an increased risk for T2D [40], and estrogen replacement therapy reduces the risk [41,42]. It has been reported that postmenopausal women have a higher number of blood monocytes, which decline following estrogen replacement therapy [43]. Based on the above results, several characteristics are consistent with the characteristics of SDT rats found in this study, such as immune disorders are involved in the pathology

of human T2D, and sex hormones such as estrogen may regulate monocytes and contribute to the onset of diabetes.

In conclusion, by analyzing immune cell subsets and sex differences in SDT rats, we found that male SDT rats may be a beneficial model of T2D involved in especially monocyte/macrophage-mediated immune disorders, and female SDT rats may be a useful model of studying the association between IGT and lymphocytes. Further research will help elucidate the pathophysiology of T2D with immune abnormalities and develop new therapeutic agents.

Conflict of Interest

Kazuma Kobayashi, Tomohiko Sasase, Tatsuya Maekawa, Yuichi Shinozaki, and Ryuhei Sano are employees of Japan Tobacco Inc. Takahisa Yamada and Takeshi Ohta have no conflict of interest.

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Figure Legends

Fig. 1 Body weights and absolute/relative weights of the spleen and lymph nodes at 5, 11, and 17 weeks of age.

Body weights (A), absolute weights of the spleen (B), absolute weights of the lymph nodes (C), relative weights of the spleen (D), relative weights of the lymph nodes (E) at 5, 11, and 17 weeks of age. Data represent mean ± standard deviation (n = 6). **P* < .05, ***P* < .01, ****P* < .001, ns; not significant.

Fig. 2 Non-fasting blood glucose and insulin levels at 5, 11, and 17 weeks of age.

The levels of non-fasting blood glucose (A) and insulin (B) at 5, 11, and 17 weeks of age. Data represent mean ± standard deviation (n = 6). **P* < .05, ***P* < .01, ns; not significant.

Fig. 3 The gating strategy of major leukocyte populations.

The gating strategy of major leukocyte populations in whole blood as a representative, and the same strategy was applied to the spleen and lymph nodes.

Fig. 4 Major leukocyte populations in the blood at 5, 11, and 17 weeks of age.

The number of total leukocytes (A), CD3+T cells (B), CD4+T cells (C), CD8+T cells (D), B cells (E), NK cells (F), neutrophils (G), classical monocytes (H), and non-classical

monocytes (I) in the blood at 5, 11, and 17 weeks of age. Data represent mean ± standard deviation (n = 6). *$P < .05$, **$P < .01$, ***$P < .001$, ns; not significant.

Fig. 5 Major leukocyte populations in spleen at 5, 11, and 17 weeks of age.

The number of total leukocytes (A), CD3+T cells (B), CD4+T cells (C), CD8+T cells (D), B cells (E), NK cells (F), neutrophils (G), classical monocytes (H) and non-classical monocytes (I) in the spleen at 5, 11, and 17 weeks of age. Data represent mean ± standard deviation (n = 6). *$P < .05$, **$P < .01$, ***$P < .001$, ns; not significant.

Fig. 6 Major leukocyte populations in lymph node at 5, 11, and 17 weeks of age.

The number of total leukocytes (A), CD3+T cells (B), CD4+T cells (C), CD8+T cells (D), B cells (E), NK cells (F) at 5, 11, and 17 weeks of age. Data represent mean ± standard deviation (n = 6). *$P < .05$, **$P < .01$, ***$P < .001$, ns; not significant

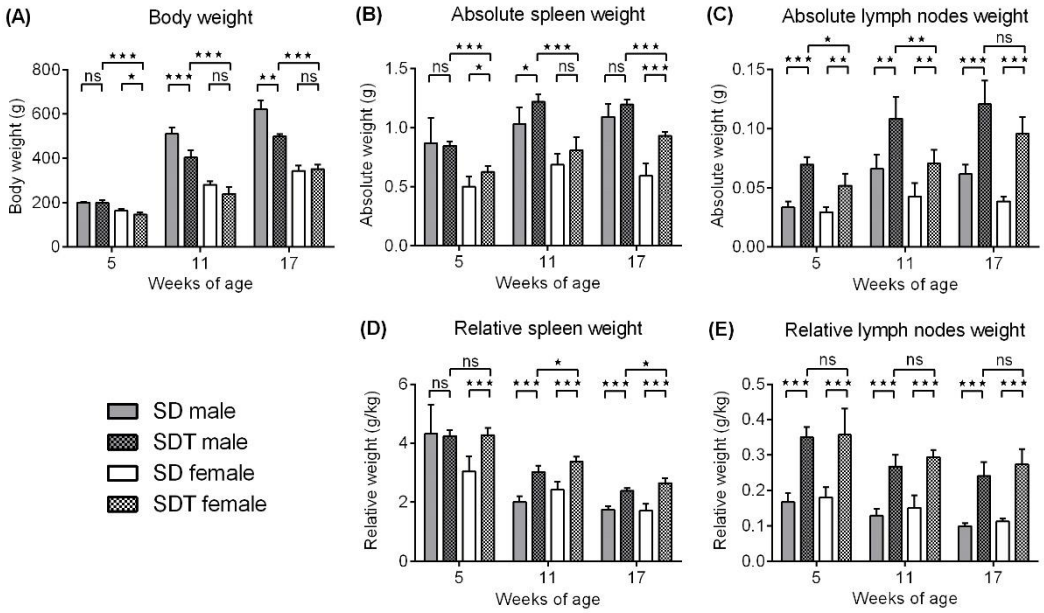

(A)
Body weight
Body weight (g)
Weeks of age
(B)
Absolute spleen weight
Absolute weight (g)
Weeks of age
(C)
Absolute lymph nodes weight
Absolute weight (g)
Weeks of age
(D)
Relative spleen weight
Relative weight (g/kg)
Weeks of age
(E)
Relative lymph nodes weight
Relative weight (g/kg)
Weeks of age
SD male
SDT male
SD female
SDT female

Fig. 1


SD male
SDT male
SD female
SDT female
(A)
Glucose
Glucose (mg/dL)
800
600
400
200
0
ns
ns
ns
ns
ns
ns
*
**
**
5
11
17
Weeks of age
(B)
Insulin
Insulin (ng/mL)
15
10
5
0
ns
ns
ns
ns
ns
*
ns
ns
5
11
17
Weeks of age

1

2 **Fig. 2**

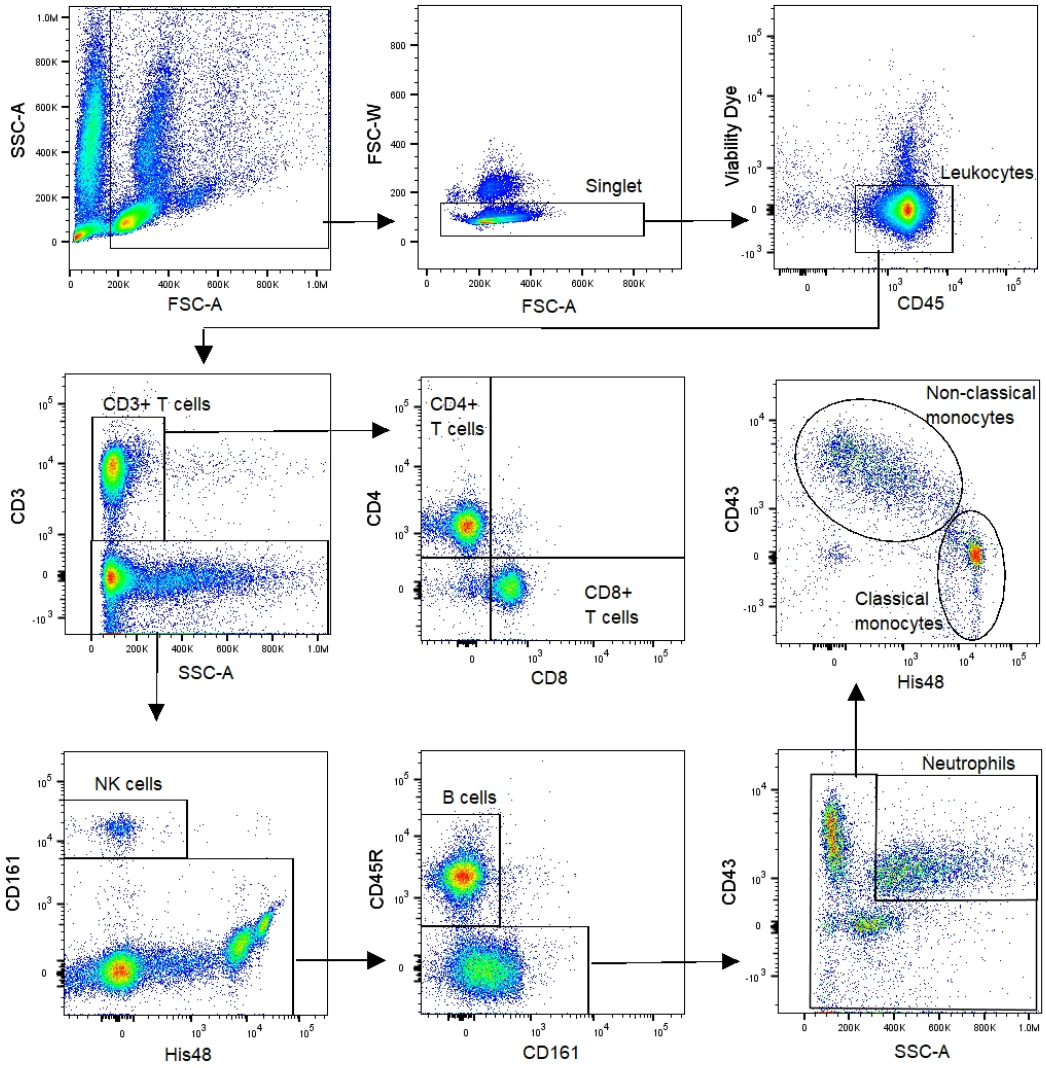
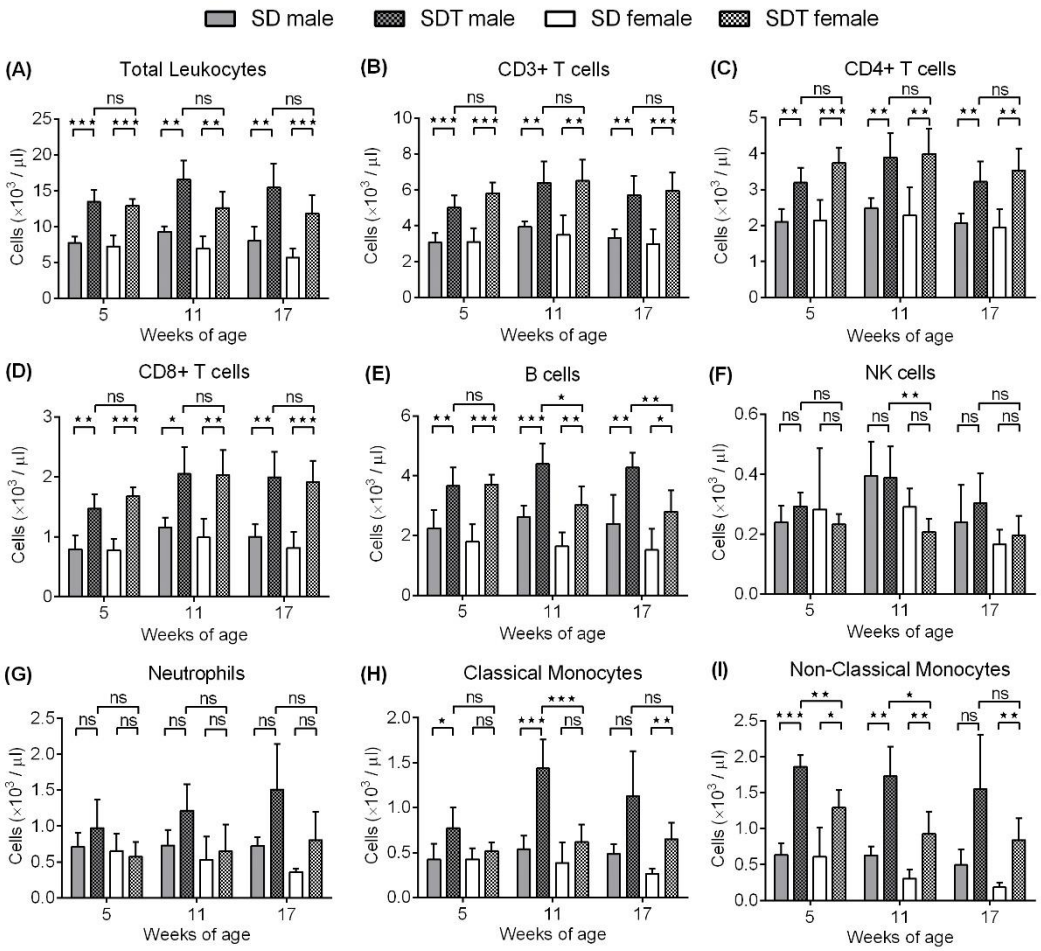

SSC-A
FSC-A
FSC-W
Singlet
Viability Dye
Leukocytes
CD45
CD3+ T cells
CD3
SSC-A
CD4+
T cells
CD4
CD8+
T cells
CD8
CD43
Non-classical
monocytes
Classical
monocytes
His48
NK cells
CD161
His48
B cells
CD45R
CD161
Neutrophils
CD43
SSC-A

Fig. 3


SD male
SDT male
SD female
SDT female
(A) Total Leukocytes
(B) CD3+ T cells
(C) CD4+ T cells
(D) CD8+ T cells
(E) B cells
(F) NK cells
(G) Neutrophils
(H) Classical Monocytes
(I) Non-Classical Monocytes
Cells (×10³ / µl)
Weeks of age
5
11
17
ns

1

2 **Fig. 4**

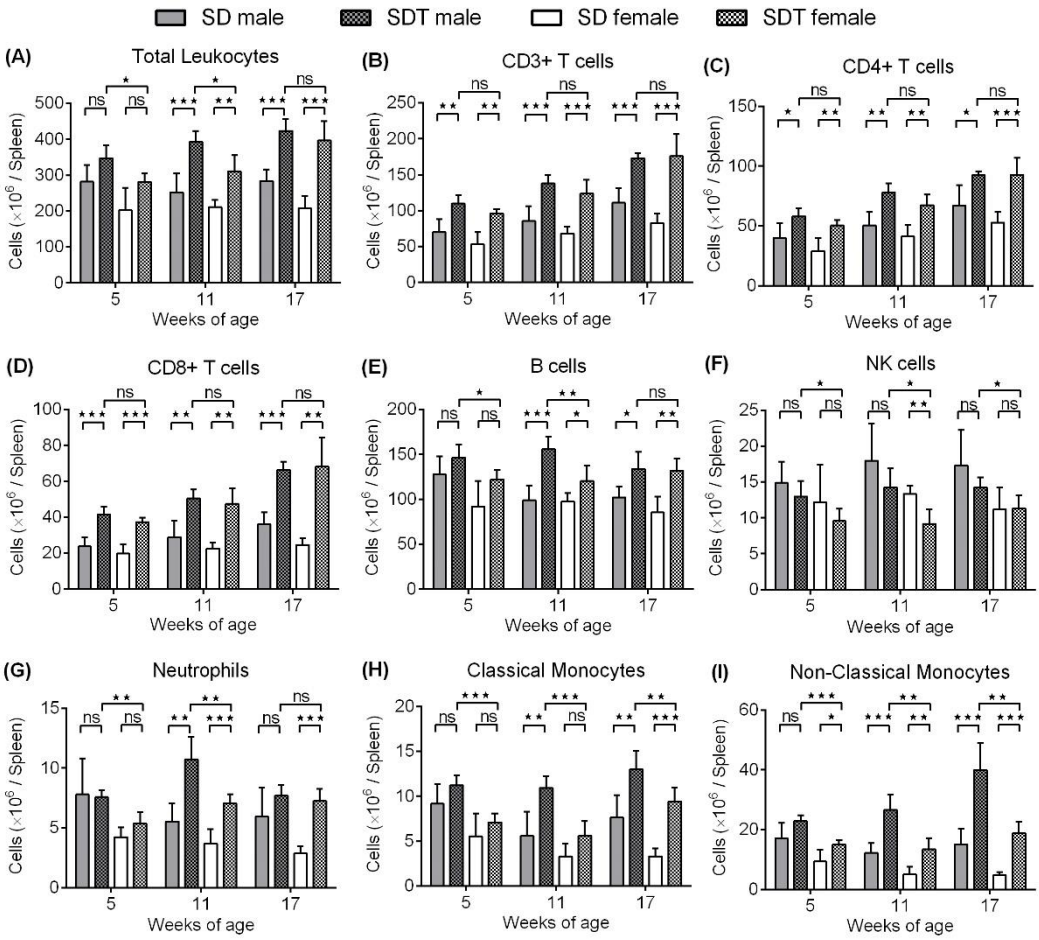

SD male
SDT male
SD female
SDT female
(A) Total Leukocytes
(B) CD3+ T cells
(C) CD4+ T cells
(D) CD8+ T cells
(E) B cells
(F) NK cells
(G) Neutrophils
(H) Classical Monocytes
(I) Non-Classical Monocytes
Cells (×10^6 / Spleen)
Weeks of age
5
11
17
ns

Fig. 5

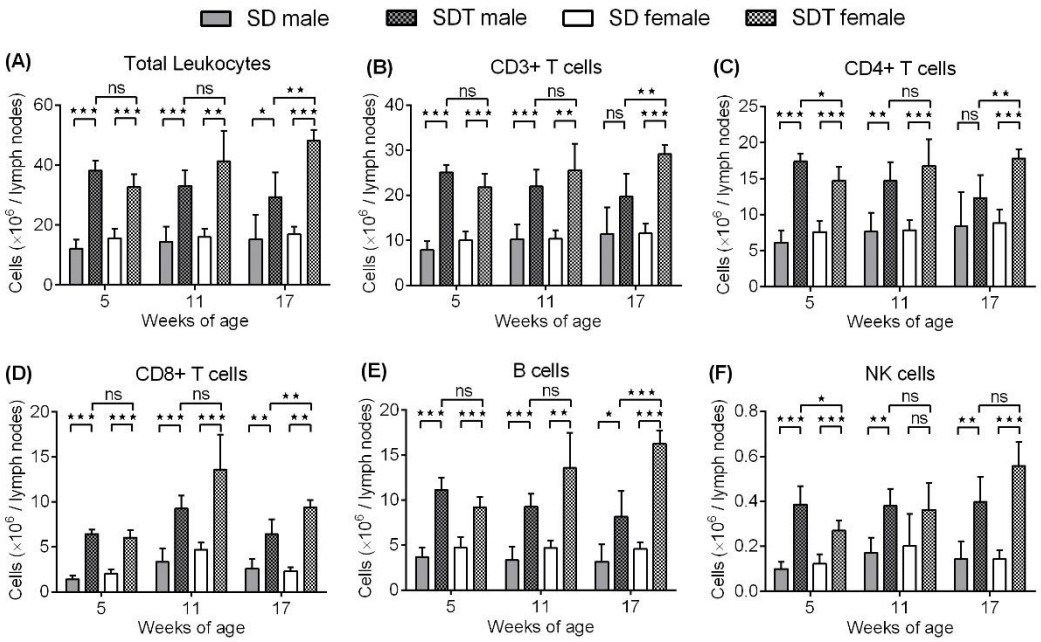

SD male
SDT male
SD female
SDT female
(A)
Total Leukocytes
(B)
CD3+ T cells
(C)
CD4+ T cells
(D)
CD8+ T cells
(E)
B cells
(F)
NK cells
Cells (×10^6 / lymph nodes)
Weeks of age
5
11
17
ns

Fig. 6