Original

Features of T-cell subset composition in a D-galactose-induced senescence mouse model

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Abstract: Long-term administration of D-galactose induces oxidative stress and accelerates normal age-related changes. Hence, the D-galactose-treated rodent model has been widely used for aging research. In this study, we examined the immunological characteristics, especially CD4$^+$ T-cell subset composition, of D-galactose-induced aging model mice to evaluate the model's utility in immunosenescence studies. The spleens of aging model mice subjected to repeated subcutaneous injections of D-galactose exhibited significant increases in T cells with the memory phenotype (CD62L^{low} CD44^{high}) and individual T-cell subsets (Th1, Th2, Th17 and T$_{reg}$). Furthermore, cells with the phenotype of T follicular helper (Tfh) cells were spontaneously increased. The features of T-cell subset composition in D-galactose-treated mice were in close agreement with those observed in normal aged mice and appeared to mimic the currently known normal aging processes associated with T-cell homeostasis. Our results suggest that D-galactose-induced aging models would be useful for immunosenescence studies focusing on T-cell homeostasis and give valuable insight into age-related immune system dysregulation.
Key words: aging model, D-galactose, immunosenescence, T-cell subsets

Introduction

D-galactose is a reducing sugar that induces a non-enzymatic saccharification reaction both *in vivo* and *in vitro*, leading to the formation of advanced glycation end-products (AGEs). Accumulation of AGEs in tissues induces oxidative stress through the production of excessive reactive oxygen species (ROS) and can accelerate the age-related changes [1–3]. On the basis of these mechanisms, the D-galactose-treated rodent model has been widely used for aging research. Previous studies have shown that chronic injection of D-galactose induces age-related changes such as neurodegeneration, osteoporosis and age-related hearing loss in mice or rats [4–6].

Immunosenescence, age-related changes in immune function, is characterized by a reduction in the acquired immune response to foreign antigens, while also inducing excessive inflammation and autoimmune response. These phenomena are involved in the onset and pathogenesis of age-related diseases such as metabolic diseases, cancer and autoimmune diseases. Age-related changes in immune function, such as reduced lymphocyte mitogenesis and IL-2 activity, have also been shown in D-galactose-treated mice [7]. In addition, it has been reported that structural changes in the thymus similar to aging are found in D-galactose-treated mice [8]. As the immune organ, the thymus plays a central role in T-cell differentiation and maturation and its age-related involution reduces the production of T cells. Therefore, it is speculated that D-galactose-treated mice might exhibit changes in T-cell function and composition that are

similar to aged mice.

Naïve CD4+ T cells are activated upon antigen presenting cell (APC) stimulation and differentiate into various types of CD4+ T-cell subsets, such as Th1, Th2, Th17 and regulatory T (T$_{reg}$) cells. While T-cell subset composition is regulated by various factors in a balanced manner, aging has a pronounced effect on the number of cells in each T-cell subset [9–11]. In addition to the CD4+ T-cell subset, T follicular helper (Tfh) cells are reported to be a distinctive T-cell subset that plays an important role in B cell proliferation, selection and maturation in germinal centers (GC) of lymphoid follicles [12–14]. It has been strongly suggested that changes to the number and function of Tfh cells, which directly interact with B cells and control antibody production, would be involved in the decline of antibody production associated with normal aging [15].

To date, CD4+ T-cell subset composition and function in D-galactose-treated mice has not been reported, and the characteristics of the T-cell-associated immune system in this mouse model remain unexamined. In this study, we investigated the CD4+ T-cell subset composition in the spleen of D-galactose-treated mice to evaluate the utility of the model in immunosenescence studies focused on T cells. Furthermore, we found an accumulation of T cells having similar phenotypes with Tfh cells in this mouse model and evaluated their association with aging.

Materials and Methods

Animals and treatments

All experiments using mice were performed in accordance with the institutional guidelines of Fukushima Medical University for the care and use of animals. C57BL/6J female mice were purchased from CLEA Japan (Tokyo, Japan) and maintained in a specific pathogen-free animal facility at Fukushima Medical University. After a one-week acclimation, the mice were used to establish D-galactose-induced aging model. Three-month-old mice were randomly divided into vehicle (n=5) and aging model groups (n=5). The aging model was induced by subcutaneous injection of 100 mg/kg/day D-galactose, dissolved in 0.9% normal saline, for 3 months. The mice in the vehicle group received subcutaneous injections of equivalent volumes of 0.9% normal saline according to the same schedule. After the last injection, the mice were maintained in a normal breeding environment for 3 months. In addition, 14-month-old C57BL/6J female mice were purchased and housed untreated for 10 months for use as a normal aging group. All mice were sacrificed after the last treat-

ment, and the thymus and spleen of each mouse was collected.

Hematoxylin and eosin (H&E) staining

Thymus and spleen samples were fixed with a 10% neutral buffered formalin solution, paraffin-embedded, sectioned (3 μm), and subsequently stained with H&E. H&E stained sections were analyzed using a light microscope (Olympus, Tokyo, Japan).

Antibodies

Fluorescent dye-conjugated anti-mouse mAbs were used for flow cytometry, including PE-Cy7-anti-CD3 (145-2C11), PerCP-Cy5.5-anti-CD4 (RM4-5), Brilliant-Violet-421-anti-CD44 (IM7), APC/Cy7-anti-CD62L (MEL-14), PE-anti-BCL-6 (K112-91), PE-anti-CD153 (RM153) (BD Biosciences, San Jose, CA, USA), Pacific blue-anti-CD4 (GK1.5), APC-anti-PD-1 (29F.1A12), FITC-anti-CXCR5 (L138D7), Brilliant Violet 421-anti CXCR5 (L138D7), PE-anti-ICOS (15F9), PE-anti-B220 (RA3-6B2) (BioLegend, San Diego, CA, USA), PerCP-Cy5.5-anti-IFN-γ (XMG1.2), PE-anti-IL-4 (11B11), APC-anti-IL-17A (17B7), PE-anti-IL-21 (mhalx21), APC-anti-FOXP3 (FJK-16s) (eBioscience, San Diego, CA, USA).

Flow cytometric analysis (FACS)

Total mouse spleen cells for FACS analysis were prepared by lysing red blood cells with RBC Lysis Buffer (BioLegend). Subsequently, cell staining with specific surface markers (CD3, CD4, PD-1, CXCR5, CD153, ICOS) for flow cytometry were performed. To analyze the expression of cytokines (IFN-γ, IL-4, IL-17 and IL-21), intracellular staining was performed using Intracellular Fixation & Permeabilization Buffer (eBioscience) after 6 h stimulation with Cell Stimulation Cocktail (Invitrogen, Carlsbad, CA, USA), as described in the manufacturer's protocol. Furthermore, transcription factors (BCL6 and FOXP3) were detected by intracellular staining according to the protocol of the Foxp3/Transcription Factor Staining Buffer Set (eBioscience). The cells were analyzed using a FACSCanto II (BD Biosciences). In each experiment, specimens were analyzed for singlet events with doublet discrimination. All data were evaluated using FACSDiva software (BD Biosciences) and Flowjo software (Tree star Inc, Ashland, OR, USA).

Cell sorting

CD4+ T cells were purified from mouse total spleen cells using a Mouse CD4 T-cell Isolation Kit (BioLegend) as described in the manufacturer's protocol. Sub-

sequently, the cells were stained with specific antibodies for flow cytometry, and sorted using a FACSAria (BD Biosciences). The purity of FACS-sorted cells was defined as more than 95%.

Cell culture and quantification of IgG and IL-4 production

Sorted Tfh cells (CD3+CD4+PD-1+CXCR5+; 7.5×10^4 cells) from D-galactose-induced mice, aged mice, and mice on day 7 after immunization by intraperitoneal administration of 200 μl of 10% sheep red blood cells (SRBCs) were co-cultured with sorted B cells from non-treated 8-week-old mice (CD3−B220+; 1×10^5 cells) in media containing anti-mouse CD28 mAb (2 μg/ml; BD Biosciences) on a 96-well plate-bound with anti-mouse CD3 mAb (145-2C11; BD Biosciences). After 4 days, the levels of IgG and IL-4 in the culture supernatant were measured using an IgG mouse ELISA kit (Abcam, Cambridge, MA, USA) and Mouse IL-4 sandwich ELISA kit (Proteintech, Wuhan, China), respectively, following the manufacturer's protocols. RPMI 1640 medium (Invitrogen) supplemented with 10% FCS, 50 μg/ml streptomycin, and 100 U/ml penicillin was used in all experiments, and all experiments were performed at 37°C in a humidified atmosphere with 5% carbon dioxide.

Statistics

Results were analyzed using SPSS 24 software and expressed as means ± SE. The student's *t*-test was used to compare experimental groups to control values, with $P<0.05$ considered significant.

Results

Histological changes in the thymus in D-galactose-treated mice

The thymus is directly involved in T-cell development and its functions gradually declines with age. In our experiments, no significant changes in cellularity of spleen were observed, while absolute thymocyte counts of D-galactose-treated mice were significantly decreased in comparison with those of young control mice, as in the results between young control and normal aged mice (Fig. 1A). Typical thymic tissues consist of cortical and medullary regions that meet at the corticomedullary junction (CMJ), which is the entry and exit point for developing thymocytes [16]. It has been reported that the CMJ is poorly defined in normal aged mice and similar histology is found in D-galactose-treated mice [8]. Similar to the previous reports mentioned above, the D-galactose-treated mice and normal aged mice (2-year-old control mice) used in this study have a poorly defined CMJ,

while the CMJ of PBS-treated control mice was clearly defined (Fig. 1B). Thus, it was confirmed that D-galactose-treated mice exhibit thymic histological changes that are thought to be the primary cause of immune senescence, and may reflect the immune aging process associated with thymus atrophy.

Naïve/memory T-cell compartments in D-galactose-treated mice

The effects of aging on the T-cell compartment are defined by a reduction in thymic T-cell production, followed by a decrease in naïve T cells and a relative increase of memory T cells [17, 18]. As reported previously, the percentages of naïve CD4+ T cells (CD62Lhigh CD44low) were markedly decreased in the spleen of normal aged mice, while T cells with the memory phenotype (CD62Llow CD44high) were significantly increased in comparison with those of young control mice (Fig. 2). Similarly, the proportion of memory T cells in D-galactose-treated mice was significantly higher than in PBS-treated control mice (Fig. 2). The composition of naïve/memory T cells in D-galactose-treated mice was consistent with those observed in normal aged mice in this study, and is in accordance with previously reported age related changes in T-cell composition.

Altered distribution of CD4+ T-cell subsets in D-galactose-treated mice

Several reports have shown that the composition of the CD4+ T-cell subset and subset-specific cytokines change with aging [9–11]. Therefore, we analyzed CD4+ T-cell subsets (IFN-γ-producing Th1 cells, IL-4-producing Th2 cells, IL-17-producing Th17 cells and FOXP3+ T_{reg} T cells) in spleen to compare the subset composition of D-galactose-treated mice with that of normal aged mice. In our experiments, significant accumulation of Th1, Th2, Th17 and T_{reg} cells was observed in normal aged mice (Fig. 3). Similarly, the proportion of each CD4+ T-cell subset in D-galactose-treated mice was significantly higher than in PBS-treated control mice. Thus, alterations of CD4+ T-cell subset proportions in D-galactose-treated mice were generally in accordance with alterations observed in normal aged mice, suggesting that the D-galactose-treatment model accurately reflects changes in T-cell subset composition during the normal aging process.

Induction of Tfh-like cells in D-galactose-treated mice

Tfh cells are reported to be a novel T-cell subset that plays an important role in B cell proliferation, selection and maturation in GCs of lymphoid follicles [12–14].
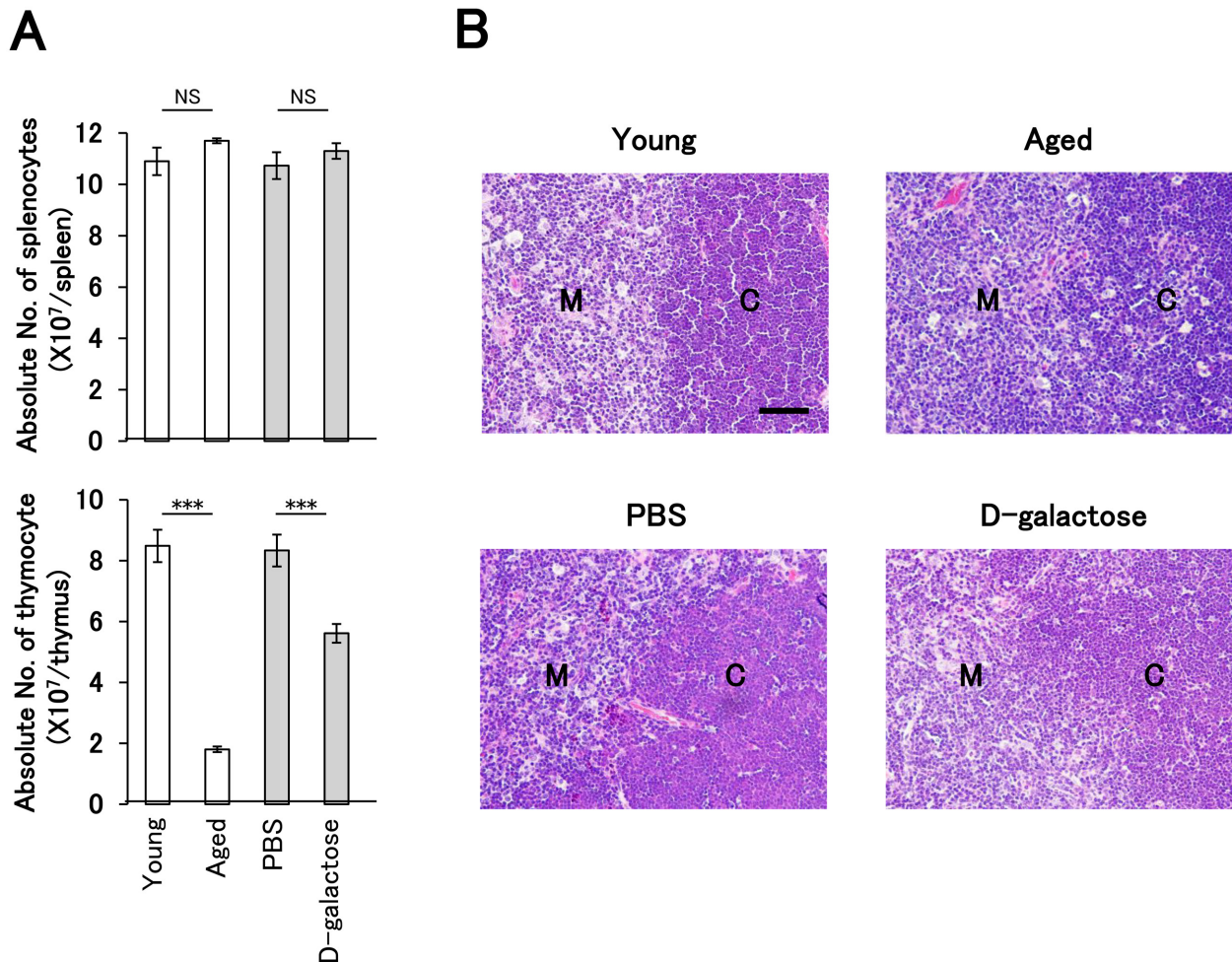
Fig. 1. Histological changes in the thymus of normal aged and D-galactose-treated mice. (A) Absolute splenocyte and thymocyte numbers. Results are expressed as the means ± SD of five determinations of each group (n=5). ***P<0.001 with Student's t-test. (B) Morphological changes in thymus of young control mice (upper left), normal aged (14-month-old) mice (upper right), PBS-treated control mice (lower left) and D-galactose-treated mice (lower right). Formalin-fixed paraffin-embedded thymus sections were stained with H&E. Images are from a single experiment and representative of each group (n=5). "M" and "C" indicate medulla and cortex, respectively. Original magnification: ×200 (scale bar, 50 μm).

Since the number of Tfh cells is reported to change during aging and may contribute to the decline of T-cell-dependent antibody responses [15], we investigated whether the proportion of Tfh cells was altered in D-galactose-treated mice. Although Tfh cells are usually generated in response to foreign antigen stimulation, cells exhibiting phenotypes similar to Tfh cells (CD3$^+$ CD4$^+$ CXCR5$^+$ PD-1$^+$) were significantly increased in the absence of foreign antigen in normal aged mouse spleen (Fig. 4A). The increases of these "Tfh-like cells" were also observed at the latest six months old in age-matched nontreated-control and PBS control mice. In D-galactose-treated mice, the increases of these cells were significantly enhanced compared to age-matched nontreated-control and PBS-treated control mice (Fig. 4B). In addition, the cells producing a Tfh-specific cytokine, IL-21, were significantly increased in normal aged and D-galactose-treated mice spleen and those cells

expressed IL-21 at the same level with that in SRBC-induced Tfh (Fig. 4C). Furthermore, these Tfh-like cells also showed clear expression of BCL-6 and ICOS and were almost indistinguishable from Tfh cells on the basis of transcription factor and surface antigens (Fig. 4D).

On the other hand, the Tfh-like cell population from aged mice and D-galactose-treated mice showed increased expression of PD-1 and contained more CD153 positive cells compared to SRBC-induced Tfh (Figs. 4E and F). Alterations of these markers have been described as aging-related characteristics of T cells including Tfh cells in normal aged mice [9–11, 15], thus suggesting that D-galactose-treated mice reflect aging-related changes in the induction of Tfh-like cells and their phenotypes.
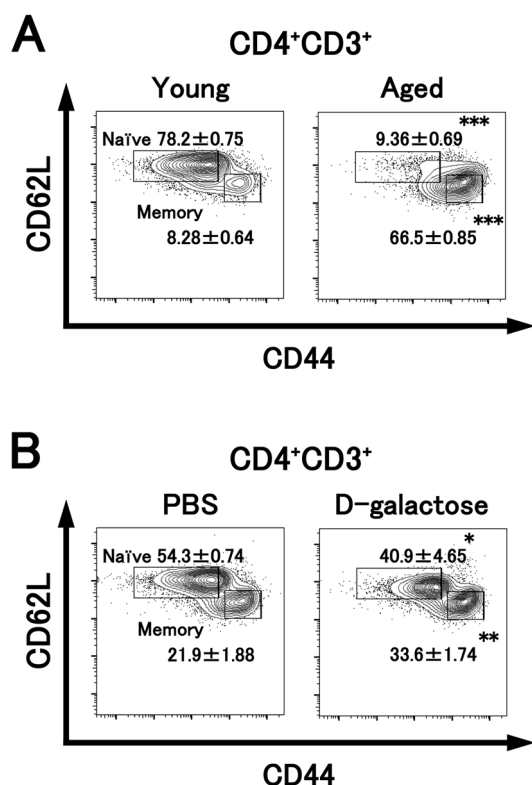
Fig. 2. Naïve/memory T-cell compartments in D-galactose-treated mice. (A) Young control mice (left) and normal aged (14-month-old) mice (right). (B) PBS-treated control mice (left) and D-galactose-treated mice (right). Percentages of naïve T cells (CD3$^+$CD4$^+$CD44$^-$CD62L$^+$) and memory T cells (CD3$^+$CD4$^+$CD44$^+$CD62L$^-$) within splenic total CD4$^+$ T cells are shown. The identity of each population was evaluated by FACS analysis. The values in the plots represent the percent of cells within the CD3$^+$CD4$^+$ cell population as mean ± SD of five determinations for each group (n=5). *$P<0.05$, **$P<0.01$, ***$P<0.001$ with Student's *t*-test.

Capacity of Tfh-like cells from D-galactose-treated mice to help B cells

Since aging-related changes in Tfh-like cells were observed in normal aged and D-galactose-treated mice, we examined whether these cells retain the function of aiding B cell IgG production. To investigate this, we performed mixed cell co-culture experiments using Tfh-like cells from D-galactose-treated mice, aged mice or SRBC-treated control mice with B cells from non-treated young control mice. The concentration of IgG in the supernatant from co-cultures with B-cells and Tfh-like cells from aged mice was significantly lower than in co-cultures with B-cells and Tfh-like cells from SRBC-treated control mice. Similarly, the concentration of IgG in the supernatant from co-cultures with B-cells and Tfh-like cells from D-galactose-treated mice also show a significantly lower levels in comparison with cells Tfh-like cells from SRBC-treated control mice (Fig. 5A).

Similar results were observed also in IL-4 production, which is the functional marker of Tfh and play an important role for IgG production by B cells. The concentration of IL-4 in the supernatant from co-cultures with B-cells and Tfh-like cells from aged mice and D-galactose-treated mice were significantly lower than that from co-cultures with B-cells and Tfh-like cells from SRBC-treated control mice (Fig. 5B). These results suggest that aging-related Tfh-like cells have a reduced ability to help B cells produce antibodies, an effect that was reproduced in Tfh-like cells from D-galactose-treated mice.

Discussion

D-galactose administration to mice induces oxidative stress through AGE accumulation in vivo, and elicits various age-related histomorphological changes [4–6, 15]. It has been reported that the AGE related mechanism affects the thymus, resulting in the induction of structural and functional changes to the immune system that are similar to aging [7, 8]. In this study, D-galactose treatment induced degeneration of the thymus cortex and affected the distribution and function of T cells.

Along with age related thymus involution, thymic naïve T-cell output declines [17] and memory T cells become dominant in the peripheral T-cell pool during aging [18]. In addition, T-cell depletion causes residual naïve T cells to proliferate for homeostatic maintenance of T-cell numbers. In the process of "homeostatic proliferation", naïve T cells differentiate into cells with a memory-like phenotype [19]. This mechanism is involved in the increase in T cells with a memory phenotype in normal aged mice observed in this study. Similar changes in the memory T-cell compartment were observed in D-galactose-treated mice, suggesting that the age-related mechanisms underlying the alterations in the T-cell compartments are reproduced in this aging model.

Similarities in the composition of the T-cell compartment in D-galactose-treated and normal aged mice were also observed in four CD4$^+$ T-cell subset, Th1, Th2, Th17 and T$_{reg}$. The proportions of the CD4$^+$ T-cell subsets were significantly increased in both groups. Moreover, the proportion of Th17 and T$_{reg}$ cells has been previously reported to increase in aged mice [10, 11, 20–23]. The age-related accumulation of Th17 cells seems to be caused by augmentation of Th17 differentiation attributable to decreased IL-2 production, which is a well-known immunosenescence symptom that has also been reported in D-galactose-treated mice [7, 11]. The increase in Th17 cells observed in D-galactose-treated mice is likely to be partially attributable to an IL-2 based senescence mechanism. On the other hand, the accumulation of T$_{reg}$
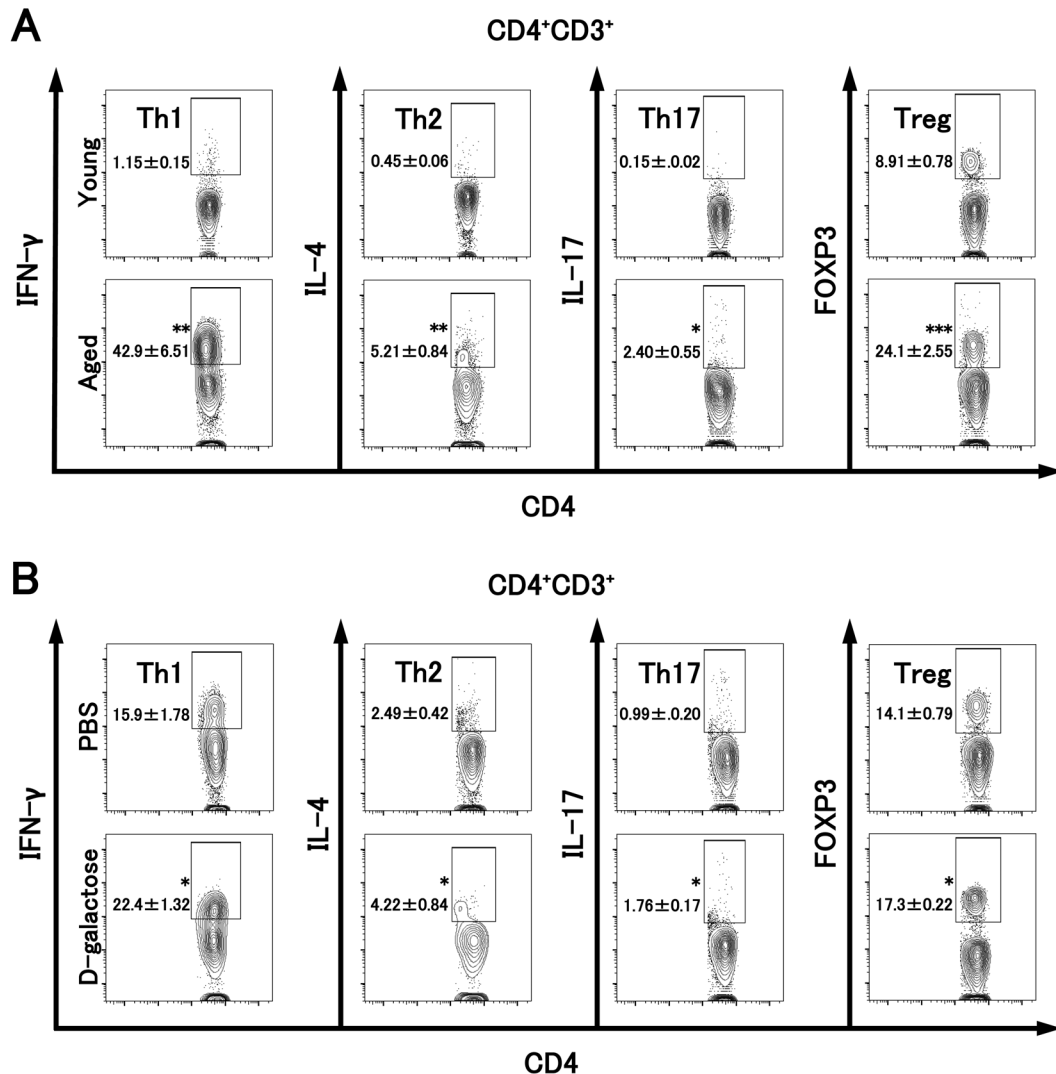
Fig. 3. Altered distribution of CD4$^+$ T-cell subsets in D-galactose-treated mice. (A) Young control mice (upper panels) and normal aged (14-month-old) mice (lower panels). (B) PBS-treated control mice (upper panels) and D-galactose-treated mice (lower panels). Percentages of Th subsets including Th1 cells (CD3$^+$CD4$^+$IFN-γ^+), Th2 cells (CD3$^+$CD4$^+$IL-4$^+$), Th17 cells (CD3$^+$CD4$^+$IL-17$^+$) and T$_{reg}$ cells (CD3$^+$CD4$^+$FOXP3$^+$) within splenic total CD4$^+$ T cells are shown. The identity of each population was evaluated by FACS analysis. The values in the plots represent the percent of cells within the CD3$^+$CD4$^+$ cell population as mean ± SD of five determinations of each group (n=5). * $P<0.05$, **$P<0.01$, ***$P<0.001$ with Student's t-test.

cells with aging has been reported by several studies and is expected to play an important role in increased susceptibility to emerging infections and neoplasms [10, 22–24]. However, a detailed mechanism for this phenomenon remains unknown. A previous study revealed that T$_{reg}$ cells can be generated from CD44$^+$ (effector/ memory) T cells, and the increased T$_{reg}$ cells might originate from the expanded pool of CD44$^+$ T cells associated with aging [25]. The increase of T cells with a memory phenotype occurring in parallel with accumulation of T$_{reg}$ cells was also observed in D-galactose-treated mice, suggesting that similar mechanisms affect the regulation of T$_{reg}$ cells in normal aged mice and D-galactose-treated mice.

Tfh cells are induced by foreign antigen stimulation and establish humoral immunity by helping B cells produce antigen specific antibodies. It has been reported that Tfh cells from aged mice are different from those in young mice, where higher expression of programmed cell death protein-1 (PD-1), a negative regulator of immune responses, and a reduced capacity for antigen-specific stimulation of antibody production are observed in aged mice [26, 27]. Interestingly, Tfh phenotype cells were increased in the spleen of D-galactose-treated and normal aged mice in the absence of foreign antigen stimulation. The spontaneous age-related Tfh-like cell populations observed in our experiment showed higher expression of PD-1 and a lower capacity to stimulate
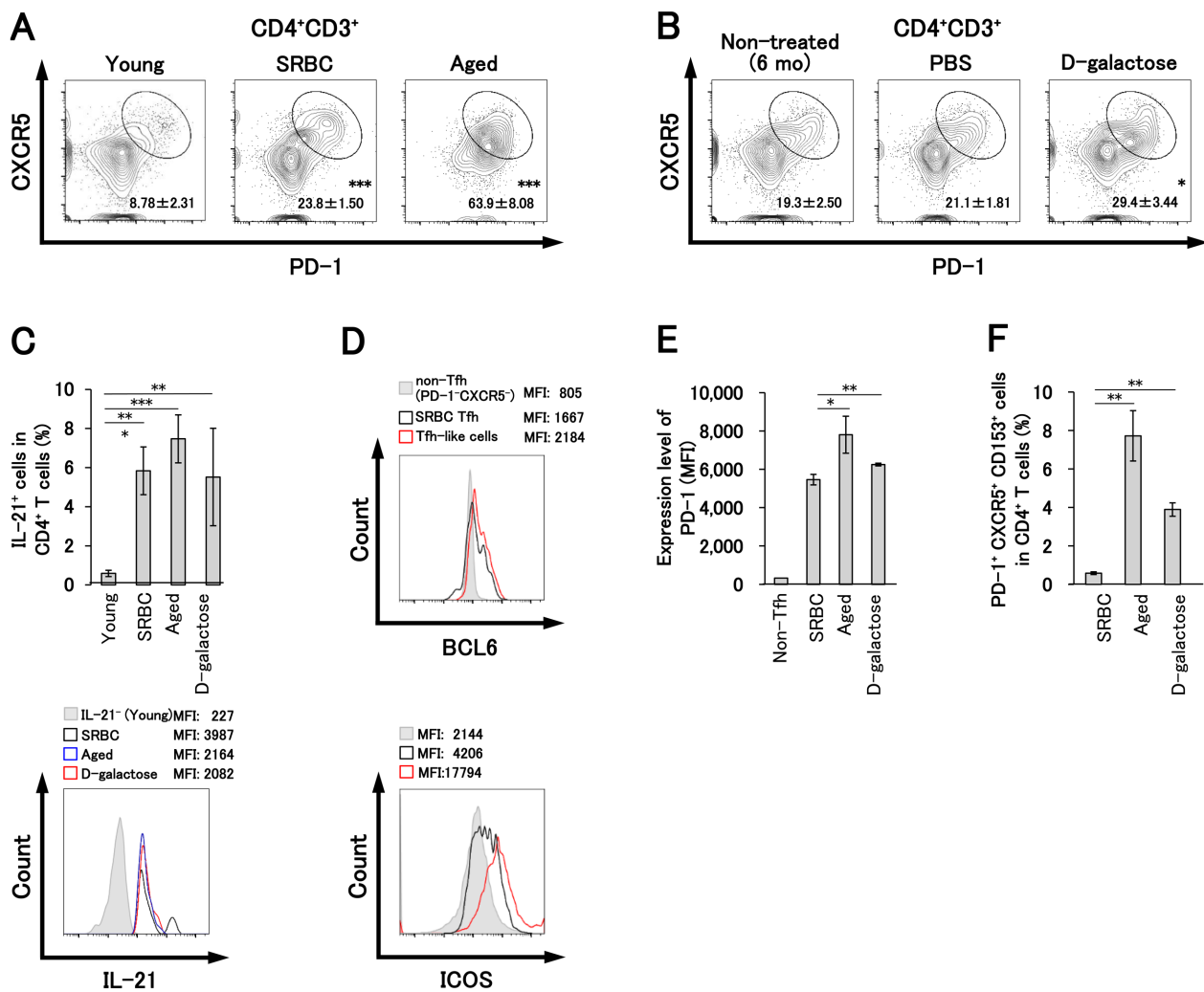
Fig. 4. Induction of Tfh-like cells in D-galactose-treated mice. (A) Percentage of cells with the Tfh phenotype (CD3^{+}CD4^{+}CXCR5^{+}PD-1^{+}) within splenic total CD4^{+} T cells from young control (non-treated) (left), SRBC-immunized (middle) and normal aged mice (right). (B) Tfh-like cells in splenic total CD4^{+} T cells from age-matched (6 months old) nontreated-control (left), PBS-treated control mice (middle) and D-galactose-treated mice (right). The values in the plots represent the percent of cells within the CD3^{+}CD4^{+} cell population. (C) Production of IL-21 in splenic total CD4^{+} T cells from SRBC-immunized, normal aged and D-galactose-treated mice. Percentages of IL-21 producing cells in total CD4^{+} T cells are shown in upper panel and IL-21 expression levels in them are shown in lower panel. The filled histogram represents IL-21 non-producing CD4^{+} T cells from young control mice (D) Expression of BCL-6 (upper) and ICOS (lower) in SRBC-induced Tfh cells and Tfh-like cells observed in (A) and (B). The filled histogram represents non-Tfh cells (CD3^{+}CD4^{+}CXCR5^{−}PD-1^{−}). (E) PD-1 expression in SRBC-induced Tfh cells and Tfh-like cells from aged mice and D-galactose-treated mice observed in (A) and (B). Mean fluorescence intensities (MFI) of PD-1 assessed by FACS are shown. (F) Percentages of CD153 positive cells (CD3^{+}CD4^{+}CXCR5^{+}PD-1^{+}CD153^{+}) per total CD4^{+} T-cell population in spleens of each group. The identity of each population was evaluated by FACS analysis. The FACS plots are from a single representative experiment for each group (n=5) (A–D). Results are expressed as the means ± SD of five determinations of each group (n=5). *$P<0.05$, **$P<0.01$, ***$P<0.001$ with Student's *t*-test.

IgG production in B cells as compared to SRBC-induced Tfh cells from young mice. Thus, these Tfh-like cells exhibit characteristics similar to antigen inducible Tfh cells from aged mice, except that they are generated spontaneously without foreign antigen stimulation. Shimatani *et al.* have reported a unique PD-1^{+} T follicular (TF) cell population that spontaneously increases in normal aged mice, terming them senescence associated T (SA-T) cells [28]. These cells develop spontaneous GCs in the spleen of normal aged mice and exhibit phe-notypes similar to Tfh cells in expression of Tfh markers (CXCR5 and BCL6). Furthermore, SA-T cells consist of two subpopulations, CD153^{+} and CD153^{−} cells, and CD153^{+} cells exhibit lower level of IL-4 production [29]. Tfh-like cells observed in D-galactose-treated and normal aged mice used in this study contain both CD153^{+} and CD153^{−} cells and produce less amount of IL-4, suggesting that these Tfh-like cells are likely to be identical to SA-T cells. Furthermore, experiments involving thymectomy in young mice and fetal thymus transplantation
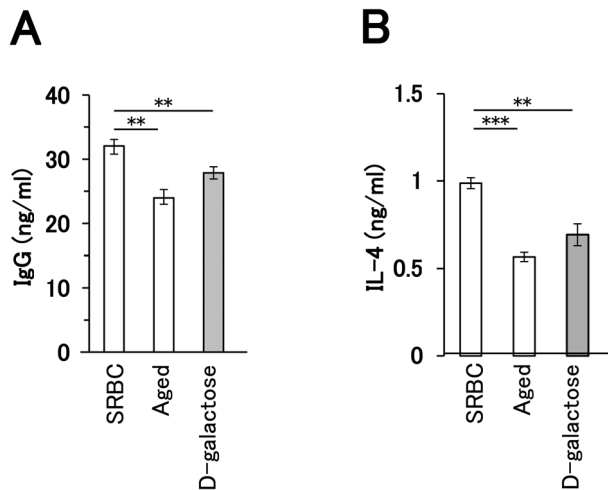
Fig. 5. Capacity of Tfh cells from D-galactose-treated mice to help B cells. Sorted Tfh-like cells from D-galactose-induced mice, aged mice, and mice on day 7 after immunization with SRBC were co-cultured with sorted B cells from non-treated 8-week-old mice (CD3⁻B220⁺) in media containing anti-mouse CD28 mAb on a 96-well plate bound with anti-mouse CD3 mAb. After 4 days, the levels of IgG (A) and IL-4 (B) in the culture supernatant were measured by ELISA. Data are expressed as the means ± SD of results pooled from three independent experiments, each performed with three mouse spleens per experiment. **$P<0.01$, ***$P<0.001$ with Student's t-test.

in aged mice indicate that accumulation of SA-T cells is thought to be caused by reduced T-cell generation due to age-dependent thymic involution and subsequent homeostatic proliferation of naïve CD4⁺ T cells [30]. It is likely that the observed increase in SA-T cells in D-galactose-treated mice is a consequence of a lymphopenic condition due to D-galactose-induced thymus degeneration.

In conclusion, our study demonstrated that D-galactose administration mimics features of T-cell composition alterations associated with normal aging. These senescence-like changes are thought to be attributable to the depletion of T cells caused by degeneration of the thymus cortex and subsequent homeostatic proliferation. The D-galactose-treated mouse model appears to be useful for immunosenescence studies, especially those focusing on the effects of aging on T-cell differentiation and regulation.

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