High dietary intake of vitamin C suppresses age-related thymic atrophy and contributes to the maintenance of immune cells in vitamin C-deficient senescence marker protein-30 knockout mice

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Abstract
Vitamin C (VC) is an essential nutrient for humans and certain other animals. It has antioxidant properties and has been reported to ameliorate oxidative damage to lipids, DNA and proteins. However, the effects of VC on immune function are poorly understood, especially the influence of long-term high-dose VC intake on the number and function of immune cells. In the present study, to evaluate the immune effects of VC, VC-deficient senescence marker protein-30 knockout (SMP30KO) mice were fed a diet containing the recommended level of VC (20 mg/kg per d; 0·02 % VC) or a high level of VC (200 mg/kg per d; 0·2 % VC) for 1 year. The plasma VC concentration of the 0·02 % group was the same as that of age-matched C57BL/6 mice after 1 year of feeding; however, plasma VC concentration and thymus weight were significantly higher in the 0·2 % VC group than in the 0·02 % VC group. The total counts of leucocytes, lymphocytes, granulocytes and monocytes in the peripheral blood, as well as the number of splenocytes and thymocytes, were all significantly higher in the 0·2 % VC group than in the 0·02 % VC group. In addition, the number of naive T cells in peripheral blood lymphocytes, the number of memory T-cell populations in splenocytes, and the number of cluster of differentiation (CD)4+CD8− or CD4−CD8+ or CD4+CD8+ T cells in thymocytes were all markedly higher in the 0·2 % VC group than in the 0·02 % VC group after 1 year of dietary treatment. These results suggest that a long-term high-dose intake of VC is effective in the maintenance of immune cells, partly through the suppression of age-related thymic involution in VC-deficient SMP30KO mice.

Key words: Vitamin C; Senescence marker protein-30 knockout mice; Thymus; Naive T cells

Ageing is a complex process that affects a variety of physiological functions. It is widely accepted that the ageing process attenuates the immune response and results in greater susceptibility to infection or an increased risk of cancer(1). The thymus is a central lymphoid organ responsible for the production of naive T cells, which migrate to the peripheral lymphoid tissues and play a vital role in mediating both cellular and humoral immunity. Age-related involution of the thymus is characterised by a decrease in its size, weight and cellularity, along with structural and functional impairment, culminating in a decrease of cells entering the peripheral T-cell pool. Chronic involution of the thymus is thought to be one of the major factors contributing to the decline of immune function with increasing age(2,5).

Vitamin C (VC) is an essential micronutrient that is required for normal metabolism in humans. It is essential for the biosynthesis of collagen and L-carnitine(4,5), and is also known to be an antioxidant that protects against oxidative stress. The current RDA of VC is 75–90 mg/d in the USA or 100 mg/d in Germany, Switzerland, Austria and Japan(6). Recently, dietary intake of VC at five to twenty times the RDA has been reported to decrease lipid peroxidation(7,8), oxidative DNA damage in lymphocytes(9), total cholesterol(10), arterial stiffness(11) and endothelial damage(12), as well as prevent the common cold(13). It has also been reported that VC intake improves some immune cell functions(14–16); however, the effects of long-term high-dose VC consumption on the number and function of immune cells are poorly understood.

Senescence marker protein-30 (SMP30) is a 34 kDa protein that was originally identified in the rat liver, and its expression has been reported to decrease with age in both sexes(17). SMP30 is also known as regucalcin (RGN) or gluconolactonase (GNL), and it acts as a lactone-hydrolysing enzyme in VC biosynthesis(18). Recently, Ishigami et al.(19) established...
SMP30/GNL knockout (SMP30KO) mice and demonstrated that these mice display symptoms of scurvy such as fractures and a rachitic rosary when fed a VC-deficient diet, and die within 135 d\(^{20}\). Thus, SMP30KO mice are thought to be a more useful animal model for investigating the physiological functions of VC than widely used VC-deficient animals such as osteogenic disorder Shionogi (ODS) rats or guinea pigs.

In the present study, to investigate the effects of long-term high-dose VC intake on age-related changes in immune status, we estimated the daily dietary VC requirement of SMP30KO mice and fed these mice normal- or high-VC diets for 1 year, after which we measured the weights of immune organs and the numbers of immune cells in the peripheral blood, spleen and thymus.

Materials and methods

Animals

SMP30KO mice were generated from C57BL/6 mice by gene targeting, as described previously\(^{19}\). Specific pathogen-free male C57BL/6 (wild-type; WT) mice were purchased from Charles River Japan and were allowed to acclimatise for 7 d before the experiments. Throughout the experiments, mice were housed individually and maintained under specific pathogen-free conditions at our laboratory in a controlled environment (room temperature 23 ± 1 °C, relative humidity 55 ± 5 % and photoperiod 12 h light–12 h dark cycle). All experiments were performed with 6- to 7-week-old male SMP30KO mice and 7-week-old male WT mice in accordance with the guidelines of the Animal Care and Use Committee of the House Wellness Foods Corporation.

Experimental design

In a preliminary study, SMP30KO mice were fed a purified diet containing 0·02 % VC (20 mg/kg per d) for 5 months. Then, the plasma ascorbic acid concentration of SMP30KO mice was compared with that of age-matched WT mice to investigate whether our estimated RDA of VC was appropriate. The RDA was estimated by scaling the human dose down to an equivalent dose normalised to the body surface area. In the subsequent main study, SMP30KO mice were randomly allocated to two groups and fed a purified diet (Oriental Yeast Company) containing 0·02 % VC (n 10) or 0·2 % VC (n 11) for 1 year. The composition of the diet was based on the American Institute of Nutrition (AIN)-93M diet\(^{21}\), as detailed in Table 1. The sample size was determined from the results of a previous study on the effect of melatonin on thymic atrophy\(^{22}\). An estimated sample size of sixteen mice was targeted 25 % restoration of thymus weight, a statistical power of 80 % and a type 1 error of 5 %. We initially allocated ten to eleven mice to each group to allow for an estimated 20 % dropout rate over the study period. WT C57BL/6 mice (WT group, n 5) were fed a commercial diet (CE-2; CLEA Japan, Inc.) for 1 year and used as aged control mice. Mice were given the aforementioned diets and drinking-water without VC \textit{ad libitum} throughout the study. After receiving the respective diets for 1 year, the body weight, organ weights and plasma ascorbic acid levels were measured, and peripheral blood leucocytes, splenocytes and thymocytes were prepared for further analyses. Mice were anaesthetised with diethyl ether, and blood samples were taken from the inferior vena cava before mice were killed by exsanguination.

<table>
<thead>
<tr>
<th>Table 1. Composition of the basal diet(^{\dagger})</th>
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<tbody>
<tr>
<td><strong>Ingredients</strong></td>
</tr>
<tr>
<td>Maize starch</td>
</tr>
<tr>
<td>α-Maize starch</td>
</tr>
<tr>
<td>Casein</td>
</tr>
<tr>
<td>Sucrose</td>
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<tr>
<td>Cellulose powder</td>
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<tr>
<td>Soyabean oil</td>
</tr>
<tr>
<td>Mineral mix (AIN-93M-MX)</td>
</tr>
<tr>
<td>Vitamin mix (AIN-93-VX)</td>
</tr>
<tr>
<td>Choline bitartrate</td>
</tr>
<tr>
<td>L-Cystine</td>
</tr>
<tr>
<td>tert-Butylhydroquinone (mg)</td>
</tr>
<tr>
<td>Ascorbic acid</td>
</tr>
</tbody>
</table>

VC, vitamin C; AIN, American Institute of Nutrition.

\(^{\dagger}\) The basal diet was based on the AIN-93M diet, which was supplemented with either 0·2 % or 2·0 % ascorbic acid.

Measurement of plasma ascorbic acid levels

The plasma level of ascorbic acid was measured by HPLC with electrochemical detection\(^{23,24}\). The plasma sample was vigorously mixed with an equal volume of 10 % (w/v) metaphosphoric acid containing 0·2 mM-EDTA-2Na, and was then centrifuged at 8000 \(\times\) g for 10 min at 4 °C. The supernatant was collected and stored at −80 °C for only 1 week. Before HPLC analysis, the supernatant (10 µl) was vigorously mixed with 40 µl of 10 % (w/v) TCA, and was then centrifuged at 5200 \(\times\) g for 10 s at room temperature. The supernatant (10 µl) thus obtained was diluted with 40 µl of the mobile phase and was then injected into a semi-micro HPLC system (Nanospace SI-2; Shiseido). Chromatographic separation was carried out on a Capcell Pak C18 MG (3 × 75 mm, 3 µm; Shiseido) using an isocratic mobile phase (0·1 M-potassium phosphate buffer, pH 2·0). The flow rate was 400 µl/min, the injection volume was 5 µl, and the column oven temperature was 40 °C. The electrochemical detector (Model 3016; Shiseido) was operated in the amperometric mode at an oxidation potential of +700 mV.

Preparation of leucocytes, splenocytes and thymocytes

In one set of experiments, blood samples were incubated with cold lysis solution (150 mM-NH\(_4\)Cl, 10 mM-NaHCO\(_3\) and 1·27 mM-EDTA) for 5 min at room temperature. After centrifugation at 250 \(\times\) g for 5 min at 4 °C, the lysed erythrocytes were removed. The pellet of leucocytes was washed with...
PBS containing 2% fetal bovine serum (FBS) and resuspended in PBS containing 2% FBS. The spleen or thymus gland was initially minced with scissors and then mashed with the plunger of a syringe, after which the cell suspension was centrifuged at 250 g for 5 min at 4°C. For thymus samples, after removing the supernatant, the pellet of thymocytes was washed and resuspended in PBS containing 2% FBS. For spleen samples, after removing the supernatant, erythrocytes were lysed by incubation for 5 min at 4°C in lysis solution (17 mM-NH₄Cl and 140 mM-Tris–HCl, pH 8.0). Then, an excess of Hank’s solution containing 10% FBS was added and the suspension was centrifuged at 250 g for 5 min at 4°C. Subsequently, the pellet of splenocytes was washed and suspended in PBS containing 2% FBS. Single-cell suspensions of leucocytes, splenocytes and thymocytes were obtained by passing each suspension through a 70 μm nylon cell strainer (Nippon Becton-Dickinson Company Limited), and were used for cell counting.

**Cell counting**

Erythrocytes, total splenocytes and total thymocytes were counted with an automated cell counter (CDA-500; Sysmex). The absolute count of each population of leucocytes was analysed by flow cytometry (EPICS XL ADC) using EXPO 32 ADC software (Beckman Coulter, Inc.). In brief, the number of leucocytes was measured using Flow-Count™ (Beckman Coulter, Inc.), and the percentages of lymphocytes, granulocytes and monocytes in the gated population of leucocytes were determined. Then, the absolute count of each cell subpopulation was calculated by multiplying the total number of leucocytes by the percentage of each cell type.

**Antibodies**

Antibodies used in the present study were as follows: anti-cluster of differentiation (CD)3-fluorescein isothiocyanate (FITC) (clone 145-2C11; Santa Cruz Biotechnology, Inc.); anti-CD4–phycoerythrin (PE) (clone RM4-5; eBioscience, Inc.); anti-CD8–peridinin chlorophyll protein complex (PerCP) (clone 53-6.7; Biolegend); anti-CD4–FITC (clone RM4-5; BD Pharmingen); anti-CD8–FITC (clone 53-6.7; Beckman Coulter, Inc.); anti-CD44–PE (clone KM201; Beckman Coulter, Inc.); anti-CD45RB–PerCP/cyanine5.5 (clone C363-16A; Biolegend); anti-CD16/CD32 (clone 93; Biolegend).

**Flow cytometry**

Cells were pre-incubated with anti-CD16/CD32 antibodies to block Fc receptors and were then stained with each monoclonal antibody, according to the manufacturer’s instructions. After staining, the cells were analysed on a flow cytometer. Thymocytes were stained with anti-CD4–PE and anti-CD8–PerCP antibodies. Double-negative or double-positive thymocytes were defined as CD4<sup>+</sup>CD8<sup>+</sup> or CD4<sup>+</sup>CD8<sup>+</sup> thymocytes, respectively, while CD4<sup>+</sup> or CD8<sup>+</sup> single-positive (SP) thymocytes were defined as CD4<sup>+</sup>CD8<sup>+</sup> or CD4<sup>+</sup>CD8<sup>+</sup> thymocytes, respectively. Leucocytes or splenocytes were stained with anti-CD3–FITC antibodies and with anti-CD4–PE or anti-CD8–PerCP antibodies. In some experiments, these cells were also stained with anti-CD4–FITC or anti-CD8–FITC antibodies, followed by anti-CD44–PE and anti-CD45RB–PerCP/cyanine5.5 antibodies. Among the SP T cells, CD4<sup>+</sup>CD8<sup>+</sup>CD45RB<sup>high</sup> cells were defined as naïve T cells, and CD4<sup>+</sup>CD8<sup>+</sup>CD45RB<sup>low</sup> cells were defined as memory T cells. The absolute count of each cell subpopulation was calculated by multiplying the total number of thymocytes, splenocytes or lymphocytes by the percentage of each subpopulation.

**Statistical analysis**

Differences between the 0.02% VC group and the 0.2% VC group were assessed by unpaired Student’s t test using Statcel 2 software (OMS Publishing). Data are presented as means and standard deviations. A P value <0.05 was considered as statistically significant.

**Results**

*Estimating the dietary vitamin C requirement of senescence marker protein-30 knockout mice*

Because the RDA for VC is 100 mg/d in Japan<sup>(6)</sup>, we estimated that the daily dietary VC requirement of SMP30KO mice was 20 mg/kg per d based on normalisation using the body surface area. To evaluate whether this estimate was appropriate, SMP30KO mice were fed a purified diet containing 0.02%

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| Table 2. Effects of high dietary intake of vitamin C (VC) on body weight, thymus and spleen weights, and plasma ascorbic acid concentration in senescence marker protein-30 knockout mice (Mean values and standard deviations) |
|---------------------------------|-----------------|-----------------|-----------------|
|                                 | 0.02% VC group (n = 10) | 0.2% VC group (n = 11) | WT† |
|                                 | Mean | SD  | Mean | SD  | Mean | SD  |
| Body weight (g)                 | 43.0 | 5.6 | 45.2 | 5.1 | 46.4 | 8.7 |
| Spleen weight (mg)              | 106.3 | 20.9 | 118.1 | 14.3 | 78.3 | 8.1 |
| Thymus weight (mg)              | 13.8 | 3.0 | 28.3<sup>**</sup> | 8.6 | 16.1 | 6.6 |
| Plasma ascorbic acid concentration (mg/l) | 6.2 | 1.2 | 13.7<sup>**</sup> | 1.7 | 7.1 | 1.1 |

WT, wild type.

Mean value was significantly different from that of the 0.02% VC group: **P<0.01 (unpaired Student’s t test).

† Data obtained from age-matched WT mice are shown as reference (n = 5).
VC (20 mg/kg per d) for 5 months and the plasma ascorbic acid level of SMP30KO mice was compared with that of age-matched C57BL/6 (WT) mice. The result revealed that the plasma ascorbic acid concentration of SMP30KO mice was almost the same as that of age-matched WT mice (6.6 (SD 1.7) and 6.8 (SD 0.8) mg/l, n 7 and n 5, respectively) after 5 months on a diet containing 0.02% VC (20 mg/kg per d). This demonstrated that the daily dietary VC requirement of SMP30KO mice was 20 mg/kg per d.

Effects of high vitamin C intake on body weight, organ weights and plasma ascorbic acid level in senescence marker protein-30 knockout mice

To examine the influence of long-term high-dose VC intake on age-related changes in body weight, spleen and thymus weights, and plasma ascorbic acid level, twenty-one SMP30KO mice (mean body weight 21.4 (SD 0.4) g) were randomly divided into two groups and were fed a diet containing 0.02% VC (20 mg/kg per d) for 5 months and the plasma ascorbic acid level of SMP30KO mice was compared with that of age-matched WT mice. The result revealed that the plasma ascorbic acid concentration of SMP30KO mice was almost the same as that of age-matched WT mice (6.6 (SD 1.7) and 6.8 (SD 0.8) mg/l, n 7 and n 5, respectively) after 5 months on a diet containing 0.02% VC (20 mg/kg per d). This demonstrated that the daily dietary VC requirement of SMP30KO mice was 20 mg/kg per d.

Table 3. Effects of high dietary vitamin C (VC) intake on the number of peripheral blood cells, splenocytes and thymocytes in senescence marker protein-30 knockout mice

<table>
<thead>
<tr>
<th></th>
<th>0.02% VC group (n 10)</th>
<th>0.2% VC group (n 11)</th>
<th>WT†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean SD</td>
<td>Mean SD</td>
<td>Mean SD</td>
</tr>
<tr>
<td>Erythrocytes (×10¹²/ml)</td>
<td>8.03 ± 1.04</td>
<td>8.65 ± 1.18</td>
<td>8.57 ± 0.90</td>
</tr>
<tr>
<td>Leucocytes (×10⁹/ml)</td>
<td>2.33 ± 1.08</td>
<td>3.84* ± 1.66</td>
<td>1.88 ± 0.61</td>
</tr>
<tr>
<td>Lymphocytes (×10⁹/ml)</td>
<td>1.80 ± 0.90</td>
<td>3.00* ± 1.30</td>
<td>1.40 ± 0.50</td>
</tr>
<tr>
<td>Granulocytes (×10⁹/ml)</td>
<td>2.30 ± 1.33</td>
<td>4.24** ± 1.70</td>
<td>2.95 ± 0.57</td>
</tr>
<tr>
<td>Monocytes (×10⁶/ml)</td>
<td>0.71 ± 0.34</td>
<td>1.21* ± 0.59</td>
<td>0.53 ± 0.17</td>
</tr>
<tr>
<td>Splenocytes (×10⁹)</td>
<td>13.4 ± 1.9</td>
<td>16.8** ± 1.8</td>
<td>11.2 ± 2.3</td>
</tr>
<tr>
<td>Thymocytes (×10⁶)</td>
<td>2.09 ± 0.75</td>
<td>3.80* ± 1.86</td>
<td>1.91 ± 0.82</td>
</tr>
</tbody>
</table>

WT, wild type.

Mean value was significantly different from that of the 0.02% VC group: *P<0.05, **P<0.01 (unpaired Student’s t test).
† Data obtained from age-matched WT mice are shown as reference (n 5).

It is well known that thymic involution occurs with ageing, leading to a decrease in the number of naive T cells in the peripheral blood. Therefore, we assessed T-cell populations after 1 year on the respective experimental diets. Among peripheral blood lymphocytes, the number of CD44lowCD45RBhigh cells in both CD4+ and CD8+ T-cell populations (naive CD4+ and CD8+ T cells, respectively) was significantly higher in the 0.2% VC group than in the 0.02% VC group. Among thymocytes, the number of CD44lowCD45RBhigh cells in both CD4+ and CD8+ T cells (memory CD4+ and CD8+ T cells, respectively) was also significantly higher in the 0.2% VC group than in the 0.02% VC group. The total number of splenocytes and thymocytes was also markedly higher in the 0.2% VC group than in the 0.02% VC group. Immune cell numbers in the 0.02% VC group were approximately the same as those in the WT group (Table 3).

Effects of high vitamin C intake on the subpopulations of leucocytes, splenocytes and thymocytes

Discussion

In the present study, long-term high dietary intake of VC (at ten times the RDA) inhibited age-related thymic atrophy and maintained immune cells such as peripheral blood leucocytes, thymocytes and splenocytes in VC-deficient SMP30KO mice. In addition, the number of naive T cells in the peripheral blood and the memory T-cell populations of splenocytes was significantly higher in the 0.2% VC group than in the 0.02% VC group. Among thymocytes, the number of double-positive cells and CD4+ or CD8+ SP cells was significantly higher in the 0.2% VC group than in the 0.02% VC group. These findings suggest that high dietary intake of VC over a long period contributes to sustaining immune cells, and could be effective in improving immune function in...
elderly people by preventing a decline in the number of immune cells.

SMP30KO mice cannot synthesise VC due to genetic disruption of the Rgn gene that encodes gluconolactonase, so this is a useful animal model for investigating the actions of VC. The daily dietary VC requirement of SMP30KO mice was estimated to be 20 mg/kg per d based on normalisation using the body surface area, and mice were fed a diet containing this VC level (20 mg/kg per d; 0·02 %) or a high VC level (200 mg/kg per d; 0·2 %). Plasma ascorbic acid concentration (Table 2) of fibronectin, laminin and collagen by trabecular meshwork cells(30), these components of the thymic extracellular matrix may be sustained in SMP30KO mice by long-term high dietary VC intake, and this may be one mechanism by which thymic atrophy was inhibited in the present study.

Thiobarbituric acid-reactive substances are a marker of oxidative stress that increase in the thymus with age, followed by the acceleration of thymic involution and the loss of thymocytes(31–33). Melatonin is a strong antioxidant, and either long-term (8 months) or short-term (2 months) melatonin consumption prevents age-dependent thymic atrophy and attenuates apoptotic thymocyte death caused by free radicals in aged BALB/c and C57BL/6 mice(22,34). It is possible that the high dose of VC used in the present study had a strong antioxidant effect, inhibiting both age-dependent involution of the thymus and the resulting loss of thymocytes. There is a significant age-associated increase in apoptosis that affects the double-negative cell population of thymocytes, partly due to the elevated expression of the cell death gene caspase-3 in aged mice(35,36). VC has been shown to inhibit thymocyte apoptosis by up-regulating the expression of the anti-apoptotic protein B-cell lymphoma-2(37–39). We found that the total number of thymocytes and the number of double-positive cells and CD4+ or CD8+ SP thymocyte subpopulations were markedly higher in the 0·2 % VC group than in the 0·02 % VC group after 1 year of dietary treatment, possibly reflecting the inhibitory effect of high-dose dietary VC intake on the apoptosis of thymocytes through the induction of B-cell lymphoma-2. In contrast, recent studies have shown that the SMP30 protein has a suppressive effect on apoptosis induced by various factors(40), although it is unclear how much of the SMP30 protein is involved in inhibiting age-related thymic involution and the apoptosis of thymocytes. Because the thymus weight and plasma VC level of SMP30KO mice fed the diet containing 0·02 % VC were.

### Table 4. Effects of high dietary vitamin C (VC) intake on the T-cell subpopulations of peripheral blood lymphocytes, thymocytes and splenocytes in senescence marker protein-30 knockout mice

<table>
<thead>
<tr>
<th>T-cell subpopulations of peripheral blood lymphocytes</th>
<th>0·02 % VC group (n 10)</th>
<th>0·2 % VC group (n 11)</th>
<th>WT†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean SD</td>
<td>Mean SD</td>
<td>Mean SD</td>
<td></td>
</tr>
<tr>
<td>CD4+ thymocytes (× 10⁷)</td>
<td>9·51  7·54</td>
<td>14·3  5·93</td>
<td>7·96  5·45</td>
</tr>
<tr>
<td>+ CD4+CD45RB+ cells (naive cells, × 10⁵/ml)</td>
<td>4·27  3·27</td>
<td>7·19*  2·59</td>
<td>2·43  0·66</td>
</tr>
<tr>
<td>+ CD4+CD45RBlow cells (memory cells, × 10⁵/ml)</td>
<td>4·89  4·82</td>
<td>6·38  3·19</td>
<td>2·71  0·90</td>
</tr>
<tr>
<td>CD3+ CD4+ T cells (× 10⁷)</td>
<td>7·10  4·17</td>
<td>10·6  5·43</td>
<td>7·95  5·45</td>
</tr>
<tr>
<td>+ CD4+CD45RB+ cells (naive cells, × 10⁵/ml)</td>
<td>1·03  0·58</td>
<td>1·81*  0·85</td>
<td>1·23  0·85</td>
</tr>
<tr>
<td>+ CD4+CD45RBlow cells (memory cells, × 10⁵/ml)</td>
<td>5·68  3·73</td>
<td>8·20  4·60</td>
<td>5·64  4·45</td>
</tr>
</tbody>
</table>

Mean value was significantly different from that of the 0·02 % VC group: *P<0·05 (unpaired Student’s t test).

† Data obtained from age-matched WT mice are shown as reference (n 5).
almost the same as those found in age-matched WT mice that had a sufficient amount of the SMP30 protein (Table 2), the inhibitory effect of SMP30 on thymic atrophy might be smaller than that of VC.

We also demonstrated that the number of peripheral blood lymphocytes, particularly naive CD4<sup>+</sup> and CD8<sup>+</sup> T cells, that have the ability to fight new infections, was significantly higher in the 0·2% VC group than in the 0·02% VC group (Table 4). An age-related deterioration of thymic production is thought to be one of the major factors leading to a decline in the number of naive T cells in the peripheral blood<sup>41</sup>. The long-term high dietary intake of VC may have ameliorated this decline of thymic cellular output, providing a stable supply of naive T cells in aged mice. We do not know why memory T cells were sustained at a high level among splenocytes in the 0·2% VC group; however, this may have been related to the maintained thymic production of naive T cells.

The number of peripheral blood granulocytes or monocytes, which are recruited from the bone marrow to the periphery without passing through the thymus, was also higher in the 0·2% VC group than in the 0·02% VC group. Haematopoietic stem cells in the bone marrow generate multiple haematopoietic lineages through a series of intermediate progenitors. These progenitor cells include common lymphoid progenitors that can generate B cells, T cells or natural killer (NK) cells, and common myeloid progenitors that can generate erythrocytes, platelets, granulocytes or monocytes<sup>41,42</sup>. Mesenchymal stem cells are a population of pluripotent cells within the bone marrow microenvironment, which participate in the regulation of the self-renewal and differentiation of haematopoietic stem cells<sup>43</sup>. Several studies have indicated that VC can induce proliferation and differentiation of bone marrow-derived mesenchymal stem cells in a dose-dependent manner<sup>44</sup>. Therefore, it seems that high dietary VC intake improves age-related dysfunction of the bone marrow microenvironment, thereby permitting the maintenance of a wide variety of immune cells, not only T cells that are produced in the bone marrow and matured within the thymus, but also granulocytes and monocytes that are released directly into the peripheral blood from the bone marrow. It is well established that ageing affects acquired or innate immune functions as well as the number of immune cells<sup>45</sup>. To clarify the effect that VC has on various immune functions, further studies employing SMP30KO mice will be needed.

In conclusion, we demonstrated that high dietary intake of VC inhibited age-related thymic involution and maintained thymic output, resulting in stable peripheral immune cell counts in aged SMP30KO mice. The results from the present study suggest that long-term high dietary intake of VC may be effective in the maintenance of immune cells in elderly people.

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The author’s contributions are as follows: R. U., Y. H. and S. M. designed the study; R. U. conducted the study; R. U. and Y. H. analysed the data; Y. Y. and A. I. participated in the interpretation of the results; R. U. wrote the manuscript; S. M. had primary responsibility for the final content of the manuscript. All authors read and approved the final manuscript.

The authors declare that they have no conflict of interest.

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