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RESTRICTED EXPRESSION OF NEW GUANINE NUCLEOTIDE EXCHANGE FACTOR ZIZIMIN2 IN AGED ACQUIRED IMMUNE SYSTEM

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ABSTRACT

The activity of various biological functions, such as nervous, endocrine and immune systems including acquired immunity, is known to decline along with aging. To elucidate the molecular mechanism of this phenomenon, we here compared the number of thymocytes, splenocytes, and bone marrow lymphocytes in young and aged mice and found the age-related functional fragility of the immune system. However, the molecular mechanisms or even the key molecules remain elusive. Therefore, we further focused on a candidate for immunosenesence-related molecules, Zizimin2, which we have recently isolated and identified as a novel guanine nucleotide exchange factor that is highly expressed in murine splenic germinal center B cells after immunization with a T cell-dependent antigen. Here, we showed that endogenous Zizimin2 protein as well as mRNA expression levels in immune organs are strictly suppressed in aged mice. We further observed that the serum antigen specific antibody response is hampered in aged mice compared to that in young animals. Moreover, the Zizimin2 mRNA expression level was not activated after immunization in aged mice. Taken together, these data suggested that Zizimin2 is associated with the reduction of immune response in acquired immunity along with aging.

Key Words: Zizimin2, Guanine nucleotide exchange factor, Immunosenescence, Aging

INTRODUCTION

Dysfunction of the acquired immune response is associated with aging, causing an inefficient surveillance mechanism against various harmful microorganisms, cancer or even self-antigens. Consequently, the incidence of morbidity and mortality is increased in the elderly. In acquired immunity, this fragility is strongly linked with deficiencies of intrinsic B cells in T cell-dependent (TD) immune response, such as antibody establishment, responses or maintenance. However, the detailed mechanisms associated with such age-related deficiencies remain unclear, while age-dependent decline of T cell function has been well characterized.

Zizimin2 was identified and cloned as a factor expressing in murine splenic germinal center

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(GC) B lymphocytes after immunization of TD antigen.[2] Zizimin2, denoted Dock11, was reported as one of the human Dock180 super family proteins.[3] The following structural analysis has demonstrated that Zizimin2 is a member of the Dock-D subfamily among dock families of guanine nucleotide exchange factors (GEFs). As a putative functional homologue, Zizimin1, also called Dock9, contains specific GEF activity towards the small G protein cell division cycle 42 homologue (Cdc42), one of the well-known Rho family members[7-9] and involves important physiological functions such as cell migration,[4] cytoskeletal reorganization and dendrite growth.[5] Zizimin2 is expressed in lymphocytes and immune organs such as spleen, thymus and lymph nodes.[2] In a previous study, we also reported that Zizimin2 binds and activates nucleotide-free Cdc42 via its CZH2 [CDM (ced-5/DOCK180/myoblast city)-Zizimin homology 2] domain[6] and mediates positive feedback on the active form, GTP-bound Cdc42, *in vitro*.[2]

In the present study, we first compared lymphocyte populations in immune organs between young and aged mice. Contrary to our expectations, we observed little difference or even a slight increase in the number of splenic and bone marrow lymphocytes in aged mice as compared to that in young mice.

We further examined B cell developmental stages by using fluorescence-activated cell sorting (FACS). The fraction of late Pre-B cells was decreased in 24-month-old aged mice as compared to 6~8-week-old young mice. Moreover, the protein and mRNA expression levels of Zizimin2 were also down-regulated in the immune tissues from aged mice as compared to young animals. In addition, the immune response and Zizimin2 expression after TNP-KLH were reduced in aged mice. Although the molecular function of Zizimin2 in the acquired immunity must be clarified further, these data indicated that Zizimin2 may be involved in B cell development and senescence on acquired immunity.

MATERIALS AND METHODS

Animals and cell preparation

Male C57BL/6 mice aged 6~12-weeks and 24-months were used. Spleen, bone marrow and thymus were prepared from those mice and homogenized with 100 μm nylon cell strainer and syringe piston in 5 ml of RPMI medium. The homogenates were centrifuged at 120 x g for 5 minutes at 4°C. For spleen and bone marrow cells, 1 ml of RBC lysis buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM EDTA, pH 7.3) was added and incubated for 1 minute on ice. The lysed cells from the spleen or bone marrow and thymocytes were washed two times with 10 ml of FACS buffer (phosphate-buffered saline (PBS) containing 0.5 % fetal bovine serum and 0.1 % NaN_3).

FACS analysis

To compare the percentages of each population in the B cell development *in vivo* between young and aged animals, lymphocytes from spleen and bone marrow in 6~12-week-old (young) or 24-month-old (aged) mice were collected. The cells from bone marrow were stained with the following antibodies; CD24-Biotin (dilution factor= 1:3000, 13-0242-81, eBioscience, San Diego, CA, USA), CD43-FITC (1:500, 121205, BioLegend, San Diego, CA, USA), BP1-PE (1:80, 108307, BioLegend), B220-APC (1:160, 103211, BioLegend), IgD-PE (1:500, 405705, BioLegend), IgM-PECy7 (1:400, 406513, BioLegend) and the cells from spleen were stained with the following antibodies; IgM-PECy7 (1:400, 406513, BioLegend), IgD-FITC (1:50, 553439, BD, Franklin Lakes, NJ, USA), CD21-PE (1:80, 123409, BioLegend), and CD23-Biotin (1:100, 101603, BioLegend). The appropriate secondary reagents were used for the samples [Streptavidin-

PECy7 (1:1000, 557598, BD) for bone marrow and Streptavidin-APC (1:1000, 17-4317-82, eBioscience) for spleen]. Dead cells were excluded from the analysis using 7AAD (420404, BioLegend). Data were recorded with Gallios (Beckman Coulter, Indianapolis, IN, USA) and analyzed with Kaluza (ver. 1.2, Beckman Coulter).

Western blotting

For protein preparations, various mouse tissues were collected, and homogenized in ice-cold homogenizing buffer [50 mM Tris pH 7.5, 1% NP-40, 0.5% Na-deoxycholate, 0.05 % SDS, 1 mM EDTA, 150 mM NaCl with protease inhibitors (Roche, Mannheim, Germany)]. The lysates were prepared from supernatants after centrifugation at 15,000 x g for 20 minutes at 4°C, and each protein concentration in the supernatant was quantified by BCA assay (Pierce, Rockford, IL, USA). Prepared proteins (30 μg) dissolved in SDS sample loading buffer were boiled for 5 minutes, subjected to 6 % SDS-PAGE, and transferred to polyvinylidene fluovide membrane. After blocking in Tris-Buffered Saline Tween-20 (TBST) (25 mM Tris pH7.4, 0.15 M NaCl, 0.1% Tween-20) with 5 % nonfat milk for 24 hours at 4°C, the membrane was probed with hybridoma culture supernatant (1:1) or anti-α-tubulin antibody (1:2000) in TBST containing 5 % non-fat milk powder for 2 hours, washed three times with TBST, then incubated with peroxidase-conjugated goat anti-rat IgG (1:2000) or peroxidase-conjugated goat anti-mouse IgG for 2 hours at room temperature (RT). After several washes with TBST, the specific proteins on the membrane were visualized with ECL (ECL plus western blotting Detection System, GE Healthcare, Piscataway, NJ, USA).

Quantitative real-time PCR

Total RNA was extracted from mice tissues by using TRI-Reagent (Invitrogen, Carlsbad, CA, USA) and treated with DNaseI (Invitrogen). cDNA was synthesized using RevaTra Ace Kit (TOYOBO, Osaka, Japan) with Oligo-dT primer, following the manufacturer's instructions. Quantitative real-time PCR was performed with SYBR Green Real-time PCR Master Mix (TOYOBO), following the manufacturer's instructions. Zizimin2 or GAPDH cDNA fragment was amplified with the following primers; Zizimin2 control primer [5'-TTG CCT TTT ATG GCC AGT CT-3' (sense) and 5'-GAG CGA ATT TTG GAT CAA GC-3' (anti-sense)] and GAPDH control primer [5'-AAT GGT GAA GGT CGG TGT G-3' (sense); 5'-GAA GAT GGT GAT GGG CTT CC-3' (anti-sense)]. GAPDH was used for the internal control.

Immunization of mice

For priming, Trinitrophenyl Keyhole Limpet Haemocyanin (TNP-KLH) was injected intra-peritoneally to mice as 100 μg/body in 100 μl of Alum aqueous solution (PIERCE, Rockford, IL, USA) and the following boosting injection was performed at 30 μg/body 37 days after the priming. The blood was collected just before the immunization (day 0), 9 days (day 9) and 40 days (day 40) after priming for determining the antibody titer in the serum by enzyme-linked immunosorbent assay (ELISA).

ELISA

ELISA was performed as described previously[10]. Briefly, as an antigen, TNP-OVA (50 μg/well) was coated and for detection, biotin conjugated goat anti-mouse secondary antibodies (1:2000, Santa Cruz Biotech., Santa Cruz, CA, USA) was incubated for 1 hour at 37°C, followed by incubation with alkaline phosphatase conjugated streptavidin (1:2000, Invitrogen) and the substrate solution (Bio-Rad, Hercules, CA, USA). The absorbance was measured at 405 nm by microplate reader. Titers were calculated as relative values.

RESULTS

Profiling of B cell development in immunosenescence

It has been well documented that aging causes defects on cell proliferation or cell survival, affecting the various physiological functions including the immune system. In the course of dysfunction of protection against infectious diseases, defects in T cell function have been intensively characterized. However, little evidence has been obtained on B cell development[10-12]. Therefore, we here focused on the age-related deterioration in B cell development. We first calculated the lymphocyte numbers of bone marrow and spleen fractions to examine the magnitude of the transitional and mature B cell compartments, respectively. As demonstrated in Table 1, the number of lymphocytes from bone marrow and spleen from aged mice showed a slight increase as compared to young mice, but no significant difference was detected. On the other hand, a remarkable decrease of thymocytes in aged mice was confirmed.

To gain more insight into which developmental stage or sub-compartment of B cell was influenced by aging, we next examined various populations in B cell development in bone marrow and spleen by flow cytometry. Interestingly, the fraction of immature differentiating B cells such as IgD^- IgM^- cells in $B220^+$ $CD43^-$ population (late pre-B cells) was decreased in aged mice as compared to young mice (Hardy's D in Fig. 1A and C). On the other hand, IgD^+ IgM^+ cells in $B220^+$ $CD43^-$ population (mature B cells) were increased in the bone marrow in aged mice (Hardy's F in Fig. 1A and C). We further characterized splenocytes and found that the fraction of marginal zone B cells ($CD21^+$ IgM^+ cells in $CD23^-$ population) were decreased in aged mice as compared to young mice (MZ in Fig. 1B and C).

Restricted expression of Aging-related GEF, Zizimin2 in immunosenescence

In a previous study, we reported the identification and characterization of a novel GEF Zizimin2 highly expressed in lymphocytes, especially in B cells by quantitative real-time PCR[2], suggesting that Zizimin2 possesses a putative role in proliferation and B cell differentiation. Moreover, Zizimin2 mRNA is reportedly expressed in the tissues, which are involved in acquired immunity, spleen, thymus and lymph node[2]. In addition, a reduced population of immature B cells (late pre-B cells) were elucidated in Figure 1A. Our next question is the expression levels of Zizimin2 protein in the tissues. The expression levels of Zizimin2 protein were examined by immunoblot. As shown in Figure 2A, Zizimin2 protein expression was highly restricted in the immune tissues, which implies that Zizimin2 has a role in immunity. Interestingly, the expression levels of Zizimin2 protein were decreased remarkably in aged mice as compared to that in young animals (Fig. 2A). In order to verify that Zizimin2 expression is indeed transcriptionally decreased in these lymphoid tissues in an age-dependent manner, the mRNA expressions were analyzed by quantitative real-time PCR. As shown in Fig. 2B, the expression levels of Zizimin2 mRNA in aged mice were less than a half of that in young mice. These results therefore suggested a

Table 1 The number of cells in spleen, bone marrow, and thymus from 6~8-week-old or 24-month-old mice.

	Young	Aged
Spleen	$2.3 \times 10^7 \pm 0.8$	$3.2 \times 10^7 \pm 0.6$
Bone-marrow [a)]	$2.0 \times 10^7 \pm 0.3$	$2.1 \times 10^7 \pm 0.6$
Thymus	$5.1 \times 10^7 \pm 2.9$	$1.3 \times 10^7 \pm 0.2$

Each value represents mean ± S.D. for three mice.

[a)] Bone-marrow cells were collected from both femurs.

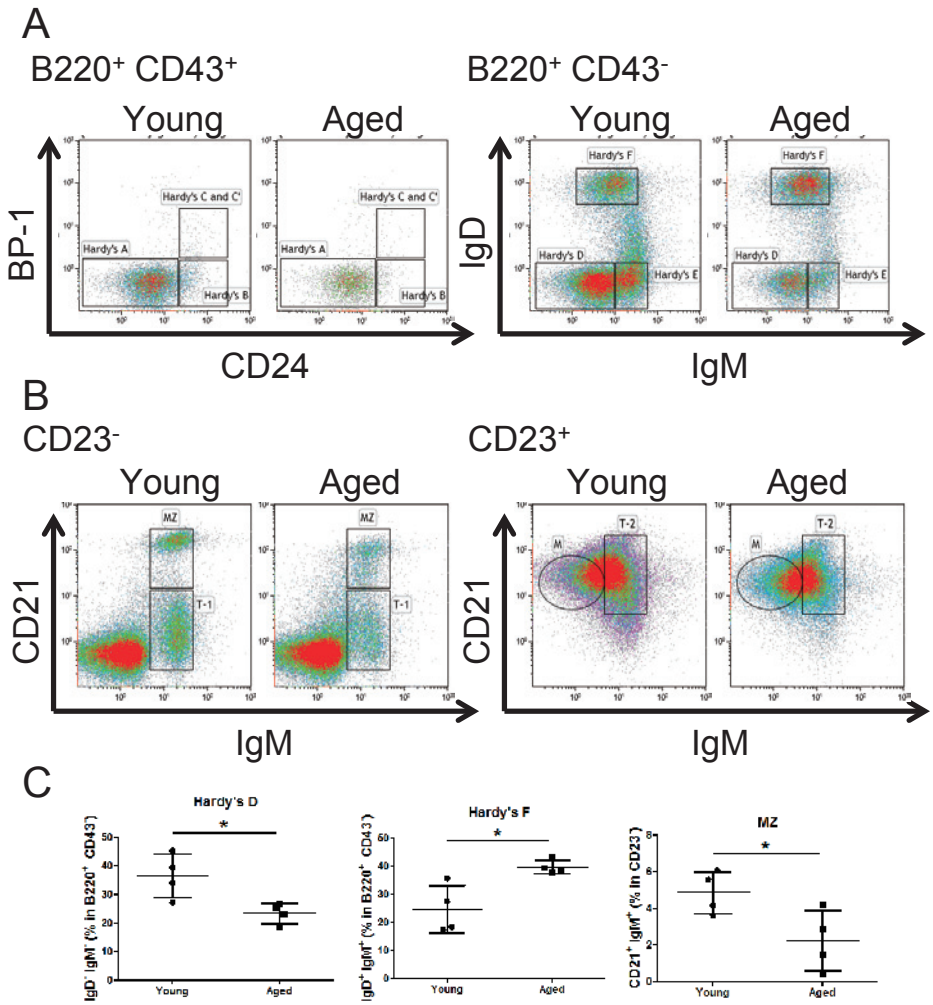

Fig. 1 Flow cytometric analysis of B cell development in bone marrow and spleen.

A. Surface CD24-BP1 and IgM-IgD profiles of bone marrow B cells.
Bone marrow cells were analyzed for CD24, BP-1, IgM and IgD after gating for B220$^+$ CD43$^+$ (left panel) or B220$^+$ CD43$^-$ (right panel) population. The percentage of the late pre-B cells (Hardy's D; IgM$^-$ IgD$^-$ cells in B220$^+$ CD43$^-$ population) was decreased in aged mice compared to that in young animals. On the other hand, the percentage of the mature B cells (Hardy's F; IgM$^+$ IgD$^+$ in B220$^+$ CD43$^-$ population) was increased in aged mice. These results are representative of four independent experiments. Hardy's A: Progenitor B cells. Hardy's B, C and C': Early Pro-B cells. Hardy's D: Late Pre-B cells. Hardy's E: Newly formed B cells. Hardy's F: Mature B cells.

B. Surface IgM-CD21 profiles of spleen B cells.
Spleen cells were analyzed for IgM and CD21 after gating for CD23$^-$ (left panel) or CD23$^+$ (right panel) population. The percentage of mature marginal zone B cells (MZ; IgM$^+$ CD21$^+$ in CD23$^-$ population) was decreased in aged mice as compared to that in young animals. These results are representative of four independent experiments. T-1: Immature B cells. T-2: Immature B cells. M: Mature B cells. MZ: Mature marginal zone B cells.

C. Statistical analysis for the flow cytometric analysis
Percentages of each population indicated in the figures were statistically analyzed for four mice per group. Average (long lines) ± SD (short lines) are shown. Each closed circle indicates data from young mouse and each closed rectangle indicates data from aged mouse. * $P < 0.05$

possible involvement of Zizimin2 function in senescence in acquired immunity.

To address further a physiological function of Zizimin2 in the immune system, especially in relation to immunosenescence, we investigated the possible correlation between aging and immune response to a TD antigen. In order to quantify the amount of TNP-specific antibody in the serum from young or aged mice before and after immunization, ELISA was carried out with TNP-chicken ovalbumin (OVA) - coated plates. As shown in Fig. 3A, although young mice exhibited

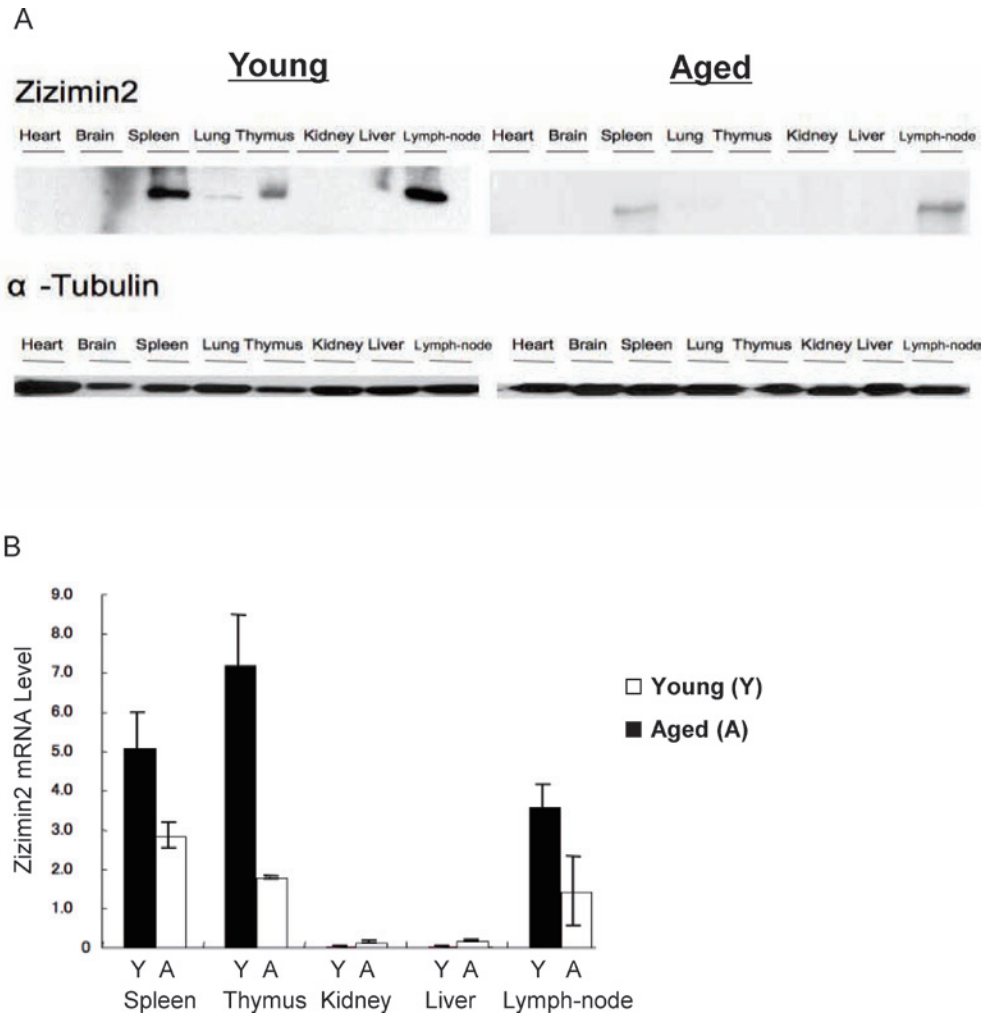

Fig. 2 Expression of Zizimin2 in young and aged mouse.

A. Expression of Zizimin2 protein in young and aged mouse.
Lysates from the tissues indicated in this figure were prepared. After protein concentration was quantified, an equal amount of protein was subjected to immunoblot analysis. α-Tubulin was used as a loading control.

B. Expression of Zizimin2 mRNA in young and aged mice.
cDNA from the tissues designated in this figure was subjected to quantitative real-time PCR using gene specific primer set for Zizimin2 or GAPDH. Relative expression levels were calculated by delta-delta CT method using GAPDH as a internal control gene. These results are representative of at least three independent experiments.

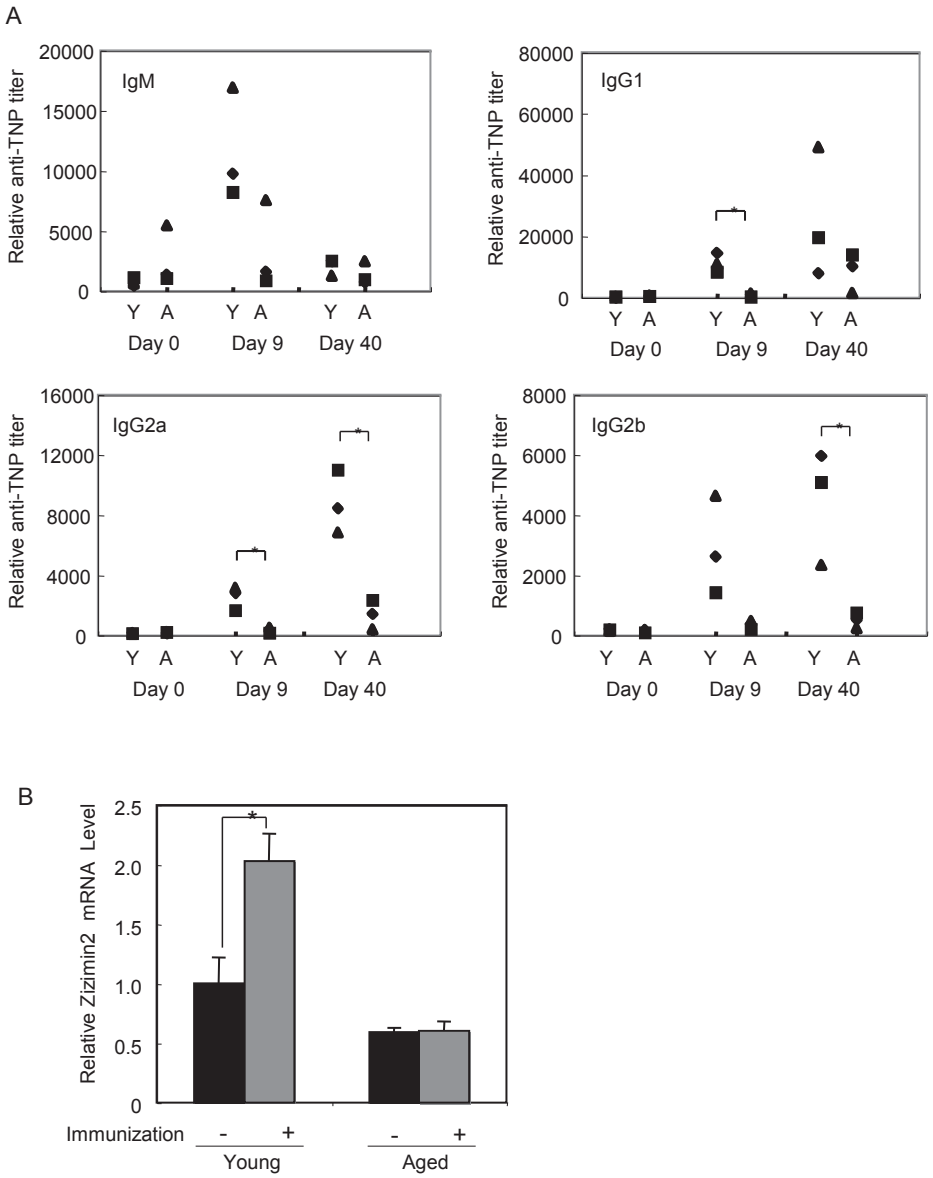

Fig. 3 Immuno-response in young and aged mice.

A. Immunological response in serum after immunization with TNP-KLH
Young (Y; 3-month-old, n=3) or aged mice (A: 24-month-old, n=3) were immunized with 100 µg/body of TNP-KLH in 100 µl of Alum with intraperitoneally (ip) and boosted with 30 µg/body of TNP-OVA in 30 µl of Alum with ip 37 days after immunization. Serum was prepared from young or aged mice at three time points, Day 0: time at immunization, Day 9, 9 days after immunization; Day 40, 40 days after immunization. ELISA was carried out using TNP-OVA as an antigen and secondary antibodies specific to IgM, IgG1, IgG2a or IgG2b. Relative amounts of TNP-specific antibodies were quantified. * $P < 0.05$

B. Zizimin2 mRNA expression levels after immunization with TNP-KLH
Young or aged mice were immunized as described above. cDNA was prepared with RNA from the splenic B cells from young or aged mice. Quantitative real-time PCR carried out and relative Zizimin2 expression levels (n=3) were calculated as described in Fig. 2B. * $P < 0.05$

normal response, aged mice reacted with only a little response after a boost immunization in addition to a primary response. Moreover, Zizimin2 mRNA expression in splenic B cells was increased significantly after immunization in young mice, but not in aged mice (Fig. 3B). Taken together, these results would imply that there is a correlation between Zizimin2 expression and responses in acquired immunity.

DISCUSSION

With advancing age, various biological functions become fragile and dysfunctional. Immunological responses, such as lymphocyte activation and antibody or cytokine secretion, are also gradually impaired and reach 'immunosenescence', although its molecular mechanisms have not yet been elucidated in detail.[11-13] We here first investigated the quantitative difference of lymphocytes in spleen and bone marrow. We observed little alteration in the numbers (Table 1), suggesting that more precise analysis will be required for developmental stage-specific impairment of the immune systems along with aging. Because we are interested in Zizimin2 gene, which is highly expressed in murine splenic germinal center B lymphocytes after immunization of TD antigen, we further analyzed B cell development from progenitor B cells in bone marrow to splenic mature B cells. As shown in Fig. 1A-C, not mature B but immature B cell (late pre-B cell) fraction was conspicuously decreased in bone marrow from aged mice. This result might coincide with the increase of Hardy's F population in bone marrow of aged mice because the two populations (Hardy's D and F) were sub-populations from the same mother population ($B220^+$ $CD43^-$). Therefore, late pre-B cell development was possibly hampered along with aging in bone marrow. In the spleen, the marginal zone B cells was decreased in aged mice. The marginal zone B cells are known as mature B cells developed from T2 cells in spleen through TLR activation without BCR activation. Thus, this result might imply impairment of B cell development also in spleen through TLR activation along with aging.

Because the lack of immature B cell population in bone marrow would result in a reduced population of antigen-specific antibody-producing mature B cells and possibly be associated with the expression levels of Zizimin2, we next focused on Zizimin2 expression and secondary immune responses in the aged mice. Consequently, the results demonstrated restricted Zizimin2 protein and mRNA expression levels in the immune tissues (including spleen) from aged mice (Fig. 2). Moreover, we confirmed that the immune response against TNP-KLH and the mRNA expression levels of Zizimin2 after immunization were greatly reduced in aged mice as compared to those in young mice (Fig. 3). The reduction of the immune response might be associated with lower Zizimin2 expression levels in the immune tissues in aged mice. If this is the case, the function of Zizimin2 protein might be related to the number of B cells which produce antibody against specific antigens.

Taken together, the above findings would suggest an association between downregulation of Zizimin2 gene expression and immunosenescence along with aging. The further *in vivo* evidence from Zizimin2 knock-out mice may shed light on the function of Zizimin2 *per se* and Zizimin2-mediated immunosenescence.

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