Altered Allogeneic Immune Responses in Middle-Aged Mice

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It is well known that leukocyte composition, T cell phenotypes and immune function change in aged mice and humans. However, limited and conflicting results on the age-related immune changes in middle-aged mice were reported. Identification of the characteristics of allogeneic immune responses in aging mice may offer important information for transplantation immunology. The major age-related changes in the immune cell phenotypes and function of 12 months old mice include: 1) the significantly decreased $CD4^+$ cell population in the peripheral blood, the major peripheral $CD4^+$ cells is $CD45RB^{low}CD62L^{low}$ memory phenotype; 2) the *in vitro* responses to alloantigens and Con A of splenocytes markedly reduced; 3) the *in vivo* secondary humoral immune responses to alloantigens significantly declined; 4) the age-related alteration in the thymus mainly occurred in CD4/CD8 double positive (DP) stage; and 5) increased $CD80^+$ and MHC class II^+ cell population in spleens. Thus, the major age-related immune changes in 12 months old mice occurred in $CD4^+$ T cells in the periphery and DP stage in the thymus, which may subsequently lead to the decreased allogeneic immune responses and the different sensitivity to immunosuppressive drugs and treatments. Further studies on the characteristics of allogeneic immunity in aging individuals may help to determine the appropriated treatment for transplant aging individuals. *Cellular & Molecular Immunology*. 2004;1(6):440-446.

Key Words: allogene, middle-aged, transplantation

Introduction

A progressive decline in the integrity of the immune system is one of the physiologic changes during mammalian aging. The aging-associated immunity alterations occur in every component of the immune system, including T, B, NK, monocytes, dendritic cells, macrophages, granulocytes, and erythrocytes (1-4). In aged individuals, T cells shift from naïve to memory phenotypes and from Th1 to Th2 cytokine production, increase the proportion of T cells expressing NK markers or receptors, and produce more proinflammatory cytokines (5-7). Numerous studies have demonstrated a deficiency in the ability of splenocytes from aged mice to respond to antigens in the respects of antibody products, cell proliferation, and generation of cytotoxic cells (8-11).

Mice that were older than 18-24 months were usually recognized as aged mice (2, 12). Most of studies on aging-related immunity changes were performed in mice older than 20 months. However, it is difficult using mice that are more than 2 years old to study transplantation immunology and its tolerance, as the short survival time does not allow following long term graft acceptance. Thus, in the present study, in order to get the basic information about the effect of aging on transplantation immunology in middle-aged mouse models, we investigated the changes of the immune system and the allogeneic immunity of mice that were 12 months old. Our studies have shown that the T cell phenotypes, thymocyte development and anti-allo-antigen immune responses significantly changed in mice that were 12 months old. These results indicate that 12 months old mice may be used as aging recipients to study transplantation immunology and tolerance induction.

Materials and Methods

Animals

C57BL/6 (B6) (H-2^b), and BALB/c (H-2^d) mice were purchased from the Frederick Cancer Research Facility, Frederick, MD. All mice were maintained in a specific pathogen-free facility, and were housed in microisolator cages containing autoclaved feed, water and bedding.

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Abbreviations: DN, double negative; DP, double positive; FCM, flow cytometry; FITC, fluorescein isothiocyanate; MFI, median fluorescence intensity; MLR, mixed lymphocyte reaction; PE, phycoerythrin; PI, propidium iodide.

Animal care was in accordance with the American Association for the Accreditation of Laboratory Animal Care and institutional guidelines.

Monoclonal antibodies (mAbs) and flow cytometry (FCM)
The following mAbs were used: phycoerythrin (PE)-conjugated rat anti-mouse CD4 mAb (RM 4-5, IgG2a), PE-conjugated rat anti-mouse CD8 mAb (53-6.7, IgG2a), fluorescein isothiocyanate (FITC)-labeled hamster anti-mouse TCRβ chain mAb (H57-597, IgG), FITC-labeled rat anti-mouse CD45RB mAb (16A, IgG2a), FITC-labeled rat anti-mouse CD62L (L-selectin) mAb (MEL-14, IgG2a), FITC labeled rat anti-mouse IL-2 receptor α chain (CD25) mAb (7D4, IgM), FITC-labeled hamster anti-mouse CD69 mAb (H1.2F3, IgG), FITC-conjugated rat anti-mouse CD8 mAb (53-6.7, IgG2a), biotinylated mouse anti-mouse MHC class I, H-2K^b (AF6-88.5, IgG2a), FITC-labeled hamster anti-mouse CD80 mAb (16-10A1, IgG), FITC-rat anti-mouse CD86 mAb mAb (GL1, IgG2a), FITC-conjugated rat anti-mouse CD11b (Mac-1α chain) mAb (M1/70, IgG2b), FITC-conjugated rat anti-mouse IgG1 mAb (A85-1, IgG1), FITC-conjugated mouse anti-mouse NK1.1 (Ly-55) mAb (PK136, IgG2a), FITC-conjugated rat anti-mouse IgG2a mAb (R19-15, IgG1), FITC-conjugated rat anti-mouse IgG2b mAb (R12-3, IgG2a), FITC-conjugated rat anti-mouse IgG3 mAb (R40-82, IgG2a), PE-conjugated rat IgG2a, and FITC-labeled mIgG2b were used as non-staining control Abs. All mAbs were purchased from PharMingen (San Diego, CA).

Mouse spleen cell suspensions were prepared and RBCs were lysed with ammonium chloride potassium as described. Cells were stained with PE-labeled anti-CD4 or anti-CD8 mAb versus FITC-labeled anti-TCR or control mAb. Some cells were stained with FITC-anti-mouse CD80, anti-CD86, anti-NK1.1, anti-Mac-1 or anti-MHC H-2D^b mAb. Cells were analyzed by single-color or two-color FCM using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). Non-viable cells were excluded in two-color FCM using the vital nucleic acid stain propidium iodide (PI). The percentage of cells staining with particular reagents was determined by subtracting the percentage of cells staining nonspecifically with the negative control mAbs from staining in the same region with the control mAbs.

Detection of anti-donor antibody levels in sera by FCM
To detect anti-donor antibody productions by using FCM, 1 × 10^6 donor B6 splenocytes were stained with 10 μl of mouse serum for 30 min at 4°C, washed, then incubated with each of FITC-conjugated rat anti-mouse IgG1, IgG2a, IgG2b, or IgG3 mAbs at 4°C for 30 min. 10,000 cells for each sample were analyzed by using a FACScan flow cytometer. Non-viable cells were excluded using the vital nucleic acid stain propidium iodide. The levels of anti-donor Abs in sera were determined by subtracting the median fluorescence intensity (MFI) of cells staining nonspecifically with naïve mouse serum from MFI staining with mouse serum samples.

Mixed lymphocyte reactions (MLR)
Murine splenocytes were prepared using sterile technique as described before (13). Red blood cells were lysed using

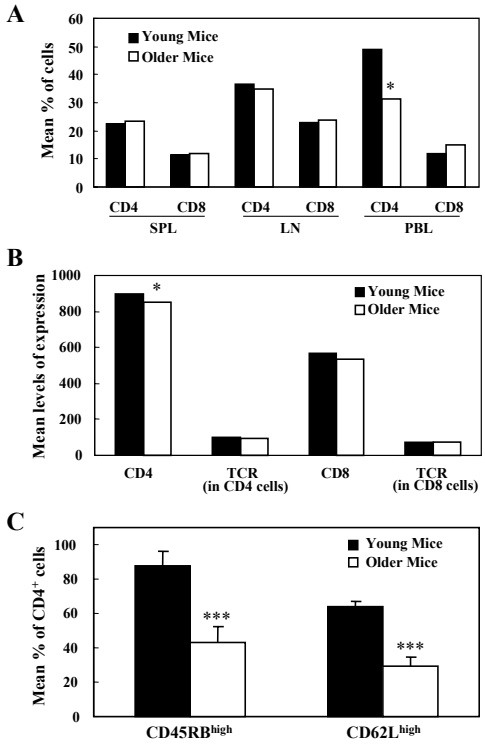

Figure 1. The immune cell phenotypes in the periphery of young and old mice. (A) The mean percentages of CD4$^+$ or CD8$^+$ T cells in the spleens, lymph nodes and peripheral blood of 12 and 2 months old mice. Signal cell suspension harvested from the spleens, lymph nodes and peripheral blood lymphocytes were stained with PE-anti-CD4 or PE-anti-CD8 mAb plus FITC-anti-TCR mAb. Cells (10^4) for each sample were assayed by FCM. (B) The levels of CD4, CD8, TCR expression on T cells of young and old mice. Splenocytes were stained with PE-anti-CD4 or PE-anti-CD8 mAb plus FITC-anti-TCR mAb, and detected by FCM. The results present the mean value of the mean fluorescence intensity of each staining. (C) The phenotype of CD4 cells in spleens of young and old mice. Splenocytes were stained with PE-anti-CD4 plus FITC-anti-CD45RB or CD62L mAb, 5,000 gated CD4$^+$ cells were detected by FCM. Results present the mean ± SD. *$p < 0.05$, ***$p < 0.001$ compared with the control group. At least three mice in each group were analyzed.

Ack Lysis Buffer (GibcoBRL, Grand Island, NY). Cells were suspended in RPMI 1640 medium supplemented with 10% (vol/vol) fetal calf serum (GibcoBRL, Grand Island, NY), 2 mM L-glutamine, 0.1 mM non-essential amino acids (GibcoBRL, Grand Island, NY), 1 mM sodium pyruvate, 10 U/ml penicillin and 10 μg/ml streptomycin, 1% HEPES buffer (GibcoBRL, Grand Island, NY), and 10 μM 2-mercaptoethanol. Triplicate wells containing 4 × 10^5 responders with 4 × 10^5 stimulators (30 Gy irradiated) in a total volume of 0.2 ml of medium were incubated at 37°C in 5% CO_2. Duplicate plates were pulsed with 0.5 μCi of ^{3}H-labeled thymidine (NEN^{TM} Life Science Products, Inc., Boston, MA) per well on days 3 and 4 and, after 18 hours' further incubation, were harvested with a cell harvester (Skatron Instruments, Lier, Norway). Samples were assayed in a Liquid Scintillation Analyzer, Tri-Carb 2100TR (Packard, Meriden, CT). Stimulation index (S.I.)

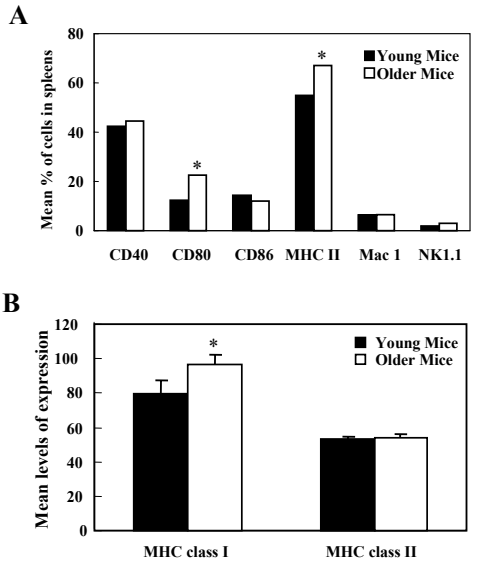

Figure 2. The expression of CD40, CD80, CD86, Mac-1, NK1.1, and MHC molecules on the splenocytes of young and old mice. (A) The percentages of $CD40^+$, $CD80^+$, $CD86^+$, $Mac\text{-}1^+$, $NK1.1^+$, and MHC class II^+ cells in the spleens of 12 and 2 months old mice. (B) The levels of MHC expression on splenocytes of 12 and 2 months old mice. Splenocytes were stained with FITC-anti-CD40, CD80, CD86, Mac-1, NK1.1, MHC class I or class II mAbs, respectively. Ten thousand viable cells were assayed by FCM. $*p < 0.05$ compared with the control group. At least three mice in each group were analyzed.

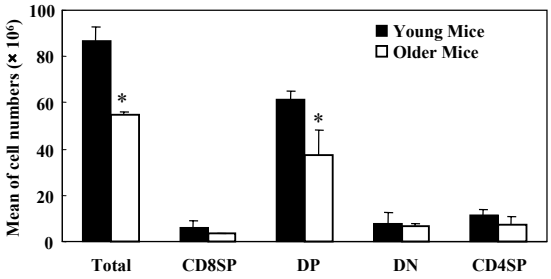

Figure 3. Markedly decreased DP thymocyte subset in the thymuses of 12 months old mice. Thymocytes were stained with PE-anti-CD4 and FITC-anti-CD8 mAbs. Two color FCM was performed. The cell numbers of each subset was calculated by the total cell numbers of thymocytes times the percentage of each thymocyte subset. Results present the mean ± SD. $*p < 0.05$ compared with the control group. At least three mice in each group were analyzed.

was calculated as follows: S.I. = cpm of responders in wells with allogeneic or xenogeneic stimulators / cpm of same responders in wells with medium or syngeneic stimulator cells.

Skin grafting

Graft beds were prepared on the posterolateral thorax of recipient BALB/c mice under Ketamine/Xylazine anesthesia (Fort Dodge, Iowa; Bayer, Shawnee Mission, Kansas, respectively). Full thickness B6 tail skin was grafted onto the lateral thoracic wall with 5-0 silk sutures and bandaids as described (14). Skin grafts were evaluated daily from day 7 onward.

Statistical analysis

All data are reported as the mean ± SD. Student's *t* test for comparison of means was used to compare groups. A *p* value less than 0.05 was considered to be statistically significant.

Results

Phenotype changes of peripheral immune cells in aging mice

It has been reported that there are significant changes in the levels and phenotypes of different immune cells in mice that are more than 18 months old (15-17). In order to determine whether immune system starts changing as early as 12 month old in mice, we first compared the cell levels and phenotypes of immune cells in different tissues of mice that were 2 month- or 12 month-old. As shown in Figure 1, compared with young mice, significantly decreased $CD4^+$ T cell subpopulation in peripheral blood lymphocytes (PBL) was observed in mice that were 12 months old ($p < 0.05$, Figure 1A). The total cell numbers and percentages of $CD4^+$ or $CD8^+$ T cells in spleens did not show statistic differences between young and older mice (data not shown). However, the $CD4^+$ T cells in spleens of mice that were 12 months old expressed significantly lower levels of CD4 molecules on the cell surface ($p < 0.05$, Figure 1B). Significantly decreased percentages of $CD45RB^{high}CD62L^{high}$ cells in $CD4^+$ splenocytes were detected in older mice, compared with young mice ($p < 0.05$, Figure 1C), indicating $CD4^+$ cells shift from naïve to memory phenotype in 12 months old mice.

In addition, the percentages of $CD80^+$ or MHC class II^+ cells in the spleens of aging mice showed markedly higher levels than control young mice ($p < 0.05$, Figure 2A). The percentages of $CD40^+$, $Mac1^+$ or $NK1.1^+$ cells in the spleens of aging mice were identical to that of young mice ($p > 0.05$). Interestingly, splenocytes from aging mice expressed higher levels of MHC class I molecules than young mice, whereas the expression of MHC class II did not change (Figure 2B). Thus, multiple changes of peripheral immune system occurred in mice that were 12 months old.

Declined total cell numbers of double positive thymocytes in aging mice

It has been reported that the thymus, served as central immune tissue and are critical for T cell development, decline with aging (18, 19). We found that total thymocyte numbers and CD4/CD8 double positive (DP) cell numbers significantly decreased in mice that were 12 months old compared with 2 months old mice ($p < 0.05$, Figure 3). No significant differences for the cell numbers of CD4/CD8 double negative (DN), CD4 or CD8 single positive (SP) thymocytes were observed between aging and young mice. These data suggest that the major changes of the thymuses in middle-aged mice occurred in DP thymocyte stage.

Decreased responses to Con A by splenocytes from aging

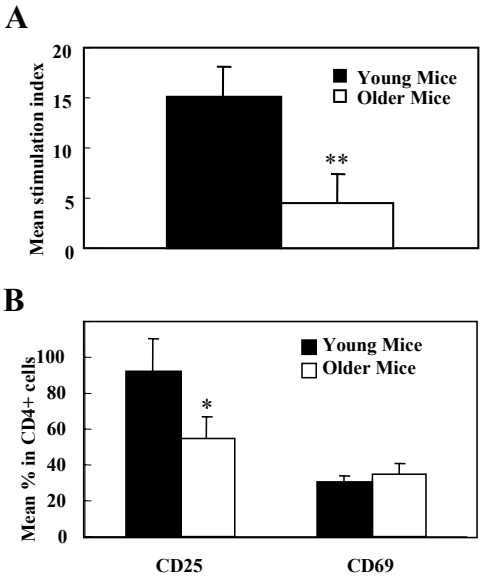

Figure 4. Significantly decreased proliferative reaction to Con A by splenocytes from 12 months old mice. Splenocytes from 12 and 2 months old mice were cultured with Con A for three days. The cell proliferation was determined by ^{3}H-thymidine incorporation. The expressions of cell activation markers were detected by FCM. Cells were stained with PE-anti-CD4 plus FITC-anti-CD25 or FITC-anti-CD69 mAb. Five thousand gated $CD4^+$ cells for each sample were assayed. Three mice in each group were analyzed. Results present the mean ± SD. *$p < 0.05$, **$p < 0.01$ compared with the control group.

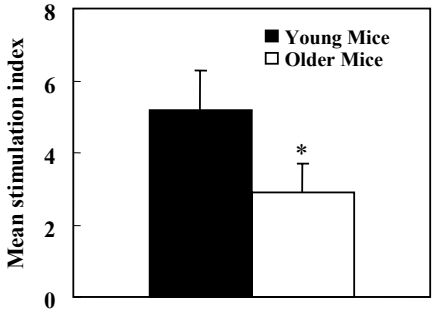

Figure 5. Significantly decreased proliferative reaction to allogeneic antigens by splenocytes from 12 months old mice. Splenocytes from 12 and 2 months old mice were cultured with lethally irradiated BALB/c splenocytes for three days. The cell proliferation was determined by ^{3}H-thymidine incorporation. Three mice in each group were analyzed. Results present the mean ± SD. *$p < 0.05$ compared with 2 months old mice. Similar results were observed when cells were cultured for 4 days.

mice

Splenocytes from both young and aging mice were cultured with Con A for 3 days, the cell proliferation and activation marker expression was evaluated. As shown in Figure 4, significantly lower proliferative reaction of splenocytes from aging mice was observed, compared with syngeneic young mice ($p < 0.01$, Figure 4A). After Con A stimulation *in vitro*, much less of $CD4^+$ splenocytes from aging mice expressed IL-2R α chain (CD25) was observed than that of young mice, whereas the early activation marker, CD69, did not show significantly difference between these two groups (Figure 4B).

Decreased proliferative reaction to allogeneic antigen of splenocytes from aging mice

After demonstrating the phenotype changes of immune cells in mice that were 12 months old, we studied the responses to allogeneic antigens of splenocytes from aging mice. As shown in Figure 5, compared with young mice (n = 3), markedly decreased proliferative reaction to allo-antigens of T cells from aging mice (n = 3) was observed ($p < 0.05$). Two separated experiments were performed, and similar results were detected. Thus, decreased cellular immunity to allogeneic antigens occurred in 12 months old mice.

Allogeneic skin graft rejection and the related humor immune responses of aging mice

To determine the allogeneic immune responses of aging mice, we evaluated the allogeneic skin graft rejection and the humoral immune responses. As shown in Figure 6, there was no statistic difference for the allogeneic BALB/c skin graft rejection in both young and aging mice. The levels of anti-donor BALB/c antibody products in sera of aging mice were similar as young mice 2 weeks post first skin grafting (Figure 7A). However, if the secondary BALB/c skin grafts were implanted 3 weeks post first skin grafting, the levels of anti-donor IgG2b and IgG3 anti-bodies in sera of aging mice were significantly lower than young mice at 1 week post the secondary skin grafting ($p < 0.05$, Figure 7B).

Discussion

Numerous studies have demonstrated a deficiency of immunity occur in aged mice that were older than 20 months. Although these mice are usually recognized as aged mice, the short survival of these mice, however, limited their application in studies in transplantation immunology. The purpose of the present studies were to determine the phenotypes and function of T cells in 12 months old mice in order to find an middle-aged mouse model which may be proper to perform studies on the transplantation immune tolerance induction. These studies indicated that 12 months old mice have a significant alteration of immunity with aging.

The advancing age is associated with significant alternations in the function of both human and murine T cells (1, 5). Significantly decreased immunity was observed in 12 months old B6 mice as marked by decreased responses to Con A and alloantigens *in vitro* (Figures 4 and 5). The significantly decreased proliferative reaction to mitogen Con A of T cells from 12 months old mice suggests that the signals in these T cells may alter with aging. It has been reported that healthy old mice exhibit multiple defects in the signal transduction cascade including calcium signal generation, phosphorylation of multiple substrates in responses initiated by protein kinase C activator PMA and by calcium ionophore ionomycin, Shc-tyrosine phosphorylation, and c-jun N-terminal kinase/

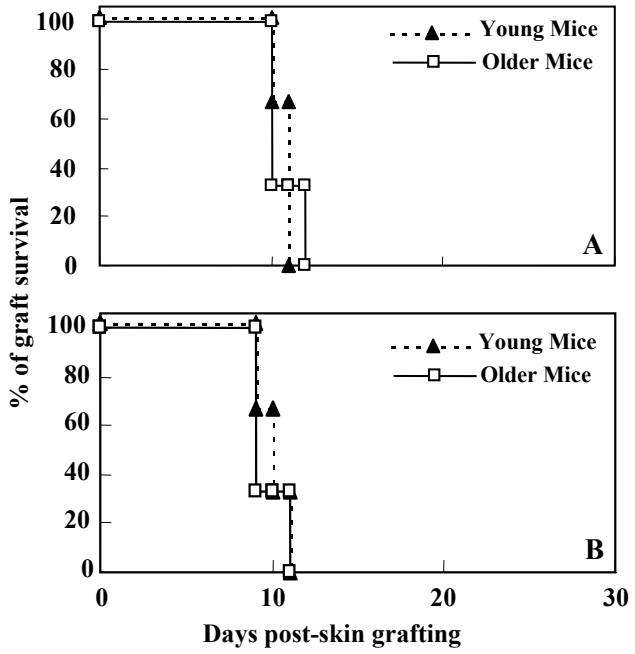

Figure 6. Unchanged allogeneic skin graft rejection in 12 and 2 months old mice. Allogeneic BALB/c tail skin tissues were grafted to young and old mice. Twelve and two months old mice were grafted with BALB/c tail skin twice at the interval of three weeks. (A) Allogeneic skin graft rejection in naïve 12 and 2 months old mice. (B) Allogeneic BALB/c skin graft rejection 12 and 2 months old mice that were immunized with first Balb/c skin grafts. No significant difference for the graft survival was observed between these two groups ($p > 0.05$). Three mice in each group were analyzed.

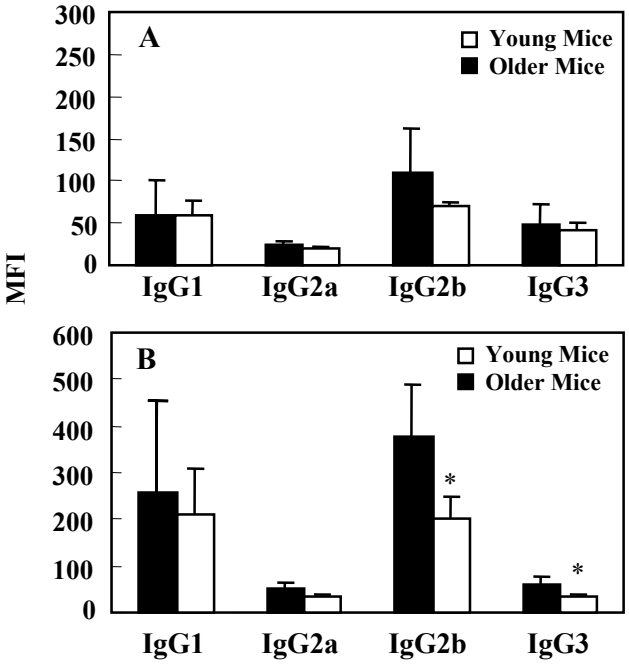

Figure 7. Decreased humoral secondary but not primary immune response of 12 months old mice. Twelve and two months old mice were grafted with BALB/c tail skin twice at the interval of three weeks. Sera samples were collected 2 weeks post-first skin grafting (A) and 1 week post the secondary skin grafting (B). Three mice in each group were analyzed. Results present the mean ± SD. *$p < 0.05$ compared with 2 months old mice.

stress-activated protein kinase pathway (20-25). Thus, the alteration of signal transduction pathways with aging may occur as early as 12 months in mice.

Aging leads to a decline in the ability to mount strong T responses to new antigens and to previously encountered recall antigens both in mice and in humans (26). However, 12 months old mice produced normal levels of T cell-dependent anti-alloantigen antibody after *in vivo* immunization as marked by the similar levels of anti-allogeneic antigen antibody products in sera of 12 months old mice as that in young mice after allogeneic skin grafting (Figure 7A). These results suggest that 12 months old mice have the normal primary immune responses to alloantigens. In contrast, 12 months old mice showed significantly lower levels of anti-alloantigen antibody products in sera after the secondary *in vivo* immunization compared with 2 months old mice (Figure 7B). These data indicate that 12 months old mice have decreased ability to produce memory cells and that recall immune responses are more sensitive to aging than primary immune responses.

It has been proposed that the altered responses of T cells from aged animals result from the accumulation of memory T cells. The percentages of memory T cells increase with aging as marked by increased expression of CD44, decreased expression of CD45RB and CD62L (15-17, 27). In accordance with these reports, 12 months old mice have higher levels of memory T cells in the periphery as indicated by the markedly decreased numbers of $CD45RB^{high}$ and $CD62L^{high}$ naïve T cells and the significantly higher numbers of $CD45RB^{low}$ and $CD62L^{low}$ T cells in the spleens of 12 months old mice compared with the young mice (Figure 1C). Normal cell numbers of CD4 and CD8 SP thymocytes in 12 months old mice (Figure 3) suggest that the T cell phenotype changes in this age may not be due to the poor thymic function as occurred in aged mice. These results indicate that the T cell phenotype alteration with aging is mainly due to the peripheral immune responses and increased memory cells but not due to the poor T cell outcome from the thymuses. This speculation was supported by the observation that the age-related shift from naïve to memory T cells is associated with a dramatic decrease in T cell Fas expression (28). The reduced Fas-mediated T cell apoptosis may cause memory T cells to accumulate (28, 29).

Whereas normal percentages and total cell numbers of CD4 or CD8 cells were observed in spleens and lymph nodes in 12 months old mice, significantly lower percentages of $CD4^+$ cells in the peripheral blood and the decreased expression of CD4 molecules on $CD4^+$ cells in spleens were observed. This observation was supported by a recent report that the major changes of peripheral blood leukocyte composition caused by aging is the reduced peripheral blood $CD4^+$ lymphocyte population (30). Therefore, a decrease in the proportion of peripheral blood $CD4^+$ T cell subset is a sensitive parameter for the aging-related immune system alteration.

In addition to the T cell alteration in 12 months old

mice, significantly higher levels of $CD80^+$ or MHC class II^+ cells and the expression of MHC class I molecules on splenocytes were observed in spleens of 12 months old mice. The levels of MHC class II molecule expressions on splenocytes in these mice were not significantly changed. In accordance to these results, it has been reported MHC class I protein levels increased significantly on both peripheral blood and spleen lymphocytes with aging (31). MHC class II protein levels did not show a significant change with aging (31, 32). However, in contrast to our results, the percentage of class II-expressing spleen lymphocytes decreased in old animals (31). MHC class II proteins are normally expressed on a limited number of cell types, including B cells, dendritic cells, as well as activated macrophages (33). The expression of MHC class II molecules on the cell surface is regulated by cytokines, mainly IFN-γ, primarily through transcriptional activation (2, 34, 35). Thus, the inconsistency results about the levels of MHC class II^+ cells in spleens may be due to the different age of the experimental mice.

The levels of $CD40^+$, $CD86^+$, $Mac1^+$ or $NK1.1^+$ cells were not impaired in spleens of these mice, indicating the population of macrophages, NK and NKT cells keep normal in middle-aged mice. The declines in splenic macrophage and NK cell function in aged mice (20-33 months old mice) have been previously reported (32, 36). Thus, the changes of these innate cells may occur in the later aging.

It is well known that the thymus involutes progressively throughout life, beginning at around the sexual maturation in human beings (37). In mice, the thymic capacity to induce T cell differentiation begins to decline earlier than the onset of thymic involution (38, 39). The accumulation of CD4/CD8 DN stage of thymocytes in 22-month-old mice was associated with a developmental block between the $CD25^-CD44^+$ and the $CD25^+CD44^+$ stages (39). However, although there were normal total cell numbers of CD4/CD8 DN thymocytes as well as CD4 or CD8 single positive cells in the thymuses of 12 months old mice, the significantly decreased total cell numbers of CD4/CD8 DP thymocytes was observed in the thymuses of 12 months old mice. This study indicates that the decline of thymus function with aging may happen first in the CD4/CD8 DP stage of thymocytes.

In summary, the present studies indicate that the phenotypes and function of immune cells occur in 12 months old mice, including: 1) The major age-related changes in the periphery of middle aged mice are the decreased $CD4^+$ cell population in the peripheral blood, the major phenotype of peripheral $CD4^+$ cells is memory cells in middle-aged mice; 2) the *in vitro* responses to alloantigens and mitogen Con A of splenocytes markedly reduced; 3) the *in vivo* secondary humoral immune responses to allogeneic antigens significantly declined; and 4) the age-related alteration in the thymus occurred in CD4/CD8 DP stage in middle-aged mice.

Acknowledgements

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