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Bcl-2-expression in Dys-regulated Distribution of T and B Lymphocytes in Aged Mice

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Abstract

The expression of Bcl-2 in peripheral blood lymphocytes in aged mice was assessed using flow cytometry and compared with young mice. The results showed that the frequencies of $CD3^+$, $CD4^+$ and $CD8^+$ cells and the frequencies of $CD3^+$, $CD4^+$ and $CD8^+$ cells expressing Bcl-2 were decreased in aged mice. Bcl-2 expression in T cells was positively correlated with frequencies of each sub-population of T cells but negatively correlated with age (old and young). In contrast, the frequencies of B cells ($CD19^+$) and Bcl-2^+ B cells in total lymphocytes as well as Bcl-2 positive rate in $CD19^+$ B cells were all increased in old mice. The mouse age, $CD19^+$ cell frequency and Bcl-2^+ B cell frequency were positively correlated each other. These results suggest that Bcl-2 expression on T and B cells could play an important role in the regulation of T and B cells by inhibiting apoptosis during aging.

Keywords: Aging; Lymphocytes; Apoptosis; Bcl-2; Immune regulation

Abbreviations

MID_{50}: Fifty percent mouse infectious dose; AICD: Activation-induced Cell Death; ICF: Intracellular Cytokine Flow cytometry

Introduction

Bcl-2 is an apoptosis antagonist, has been well-demonstrated to be an important factor for prolonging cell survival, and may be related to the occurrence of cancer [1-7]. The effects of Bcl-2 expression on immune cells has also been shown in some aspects, such as the effects on $CD8^+$ T cells [8], T and B lymphopoiesis [9], T cell development and regulation [10,11] and Germinal center responses [12]. Previous studies including ours [13-15] have shown that apoptosis contributes to the reduction of $CD8^+$ and CD 4^+ T lymphocyte frequencies in splenocyte culture of aged mice. It was further shown that increased apoptosis in aging was mediated by Fas and Fas-L, TNF-α and TNF receptors [13-15]. Different from T lymphocytes, the total IgG, IgM and IgA –presenting B cells are all increased in old mice compared to the young mice, which results in an increasing immunoglobulin level in aging [16]. However, whether *in vivo* expression of Bcl-2 is involved in these changes in T ($CD3^+$, $CD4^+$ and $CD8^+$) and B ($CD19^+$) cell frequencies in aged mice is not yet known. In this study, freshly isolated peripheral lymphocytes were used to investigate the relationships between the Bcl-2 expression and apoptosis as well as survival in T and B lymphocytes in old and young mice. Freshly isolated cells are likely to produce results that reflect *in vivo* events.

Respiratory viral infectious diseases, such as influenza and RSV infections, cause significantly higher morbidity and mortality in aged people [17-21]. More than 90% of deaths caused by influenza occur in old people [22,23]. It has been demonstrated in both clinical and animal experiments that both cellular and humoral immunity to influenza virus and RSV infection declined significantly in aged subjects [14,24-31]. We previously observed that, following stimulation with influenza virus *in vitro*, the frequencies of $CD4^+$ and $CD8^+$ T cells from spleens of old mice were significantly lower than those from the young mice [14,24-31]. Such a virus stimulation-related changes in T cells from aged mice was also associated with increased apoptosis. To further clarify whether this also occurs *in vivo*, influenza virus-primed old and young mice were challenged with the same dose of virus and the peripheral lymphocytes from these mice were kinetically assessed for the T cell subsets and the correlations among the frequencies of T cell subsets, apoptosis markers, expression of Bcl-2 and age. The "real time" evaluations allowed us to avoid tissue culture-mediated effects and to more accurately assess the dynamics of the immune response *in vivo*, as well as to assess the role of age-associated abnormalities on the immune response to influenza virus infection. Similar analysis was conducted for B cells since hypergammaglobinemia is prevalent in aging [16].

Materials and Methods

Old (22-24 months) and young (2-4 months) BALB/c (H-2^d) mice were purchased from Charles River Laboratories under a contractual arrangement with the National Institute on Aging. These animals were housed in specific pathogen-free certified rooms in cages covered with barrier filters with sentinel cages for monitoring infections. The Baylor Animal Protocol and Research Committee approved use of animals according to principles expressed in the National Institutes of Health, USPHS, *Guide for the Care and Use of Laboratory Animals*. 8 old mice and 8 young mice (total 16 mice) were respectively equally divided into two groups, and the mice in one of each two groups were primed with 10 MID_{50} of influenza A/Taiwan/1/86 (H1N1) virus or with control medium by intranasal inoculation [16,32,33]. Two months later, the virus-primed 4 mice in each group were challenged with the same dose of influenza A/Taiwan/1/86 (H1N1) virus by the same administration methods as priming.

Antibodies

Rat monoclonal antibodies specific for mouse CD3, CD8 and CD4 conjugated to PE (or PerCP) and FITC, respectively were purchased from BD Pharmingen, San Diego, CA. For detection of apoptosis, Annexin V-PE contained in an apoptosis detecting kit was used according to the manufacturer's instructions (BD Pharmingen, San Diego, CA). Anti-TNFR-p55-PE, anti-TNFR-p75-PE (Caltag Laboratories, Buriinggame,

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CA) and anti-FasL-FITC (BD Pharmingen, San Diego, CA) monoclonal antibodies were combined with anti-CD8-PE reagent for detection of TNFR-p55 (TNFRI) and FasL expression on CD8$^+$ T cells.

Isolation of peripheral blood lymphocytes

For the mice with virus challenge, 0.25 ml of whole blood drawn retro-orbitally was drawn each time once every other day before and days 2, 4 and 6 after challenge and each whole blood sample was mixed with 1 ml 1.4% of sodium citrate PBS. For the mice without virus challenge, 0.75 ml of whole blood drawn retro-orbitally was drawn and each blood sample was mixed with 3 ml 1.4% of sodium citrate PBS. Cells were then spun down and the plasma was removed. After the cells were re-suspended with 5% FBS RPMI 1640, the peripheral blood lymphocytes were isolated by gradient centrifugation using Lympholyte (Cedarlanen, Ontario, Canada) and following manufacturer's instruction. Before staining, cells were washed twice, re-suspended with PBS in the concentration of 10×10^6/ml.

Determination of T and B cell frequencies using flow cytometry Intracellular cytokine flow cytometry (ICF) for detecting Bcl-2

Prior to and 2, 4 and 6 days following priming and challenging with influenza virus, the peripheral blood lymphocytes were isolated as described above. Peripheral lymphocytes were stained for the surface markers CD3, CD4, CD8, CD19, Fas-L, TNF receptor (TNFR) I, TNFR II and Annexin V as described previously [14,16]. Following CD3, CD4, CD8 and CD19 staining, cells were washed with PBS and permeabilized in Permeabilizing Solution 2 (BD Pharmingen) for 10 min by following manufacturer's instruction. They were then stained with anti-mouse Bcl-2 with reagents contained in the BD FastImmune ICF kits for 30 min at room temperature in the dark according to manufacturer's instructions. The cells were stored at 4°C in the dark and analyzed within 24 h. Three-color flow cytometry was used in the examination of the splenocytes.

Analysis of data

Comparisons of differences among mean cell frequencies of different groups were made using the ANOVA procedure (STATVIEW Software, SAS Institute, Inc, Cary, NC, USA). The correlations among age (old and young), Bcl-2 expression and T or B cell frequencies were tested using multiple correlation analysis [14]. $P<0.05$ level was considered significant for the difference between comparison groups and the correlations among factors.

Results

Association of decreased CD3$^+$ cell frequency and the reduced expression of Bcl-2 on T lymphocytes in aged mice

Lymphocytes were isolated from peripheral blood of old and young mice and stained immediately with appropriate reagents. Two-color flow cytometry was used to determine CD3$^+$ cell frequency and Bcl-2$^+$ cell frequency. CD3$^+$ cell frequency was significantly lower in old mice than in the young mice (Figure 1a). The frequency of Bcl-2$^+$ T cells

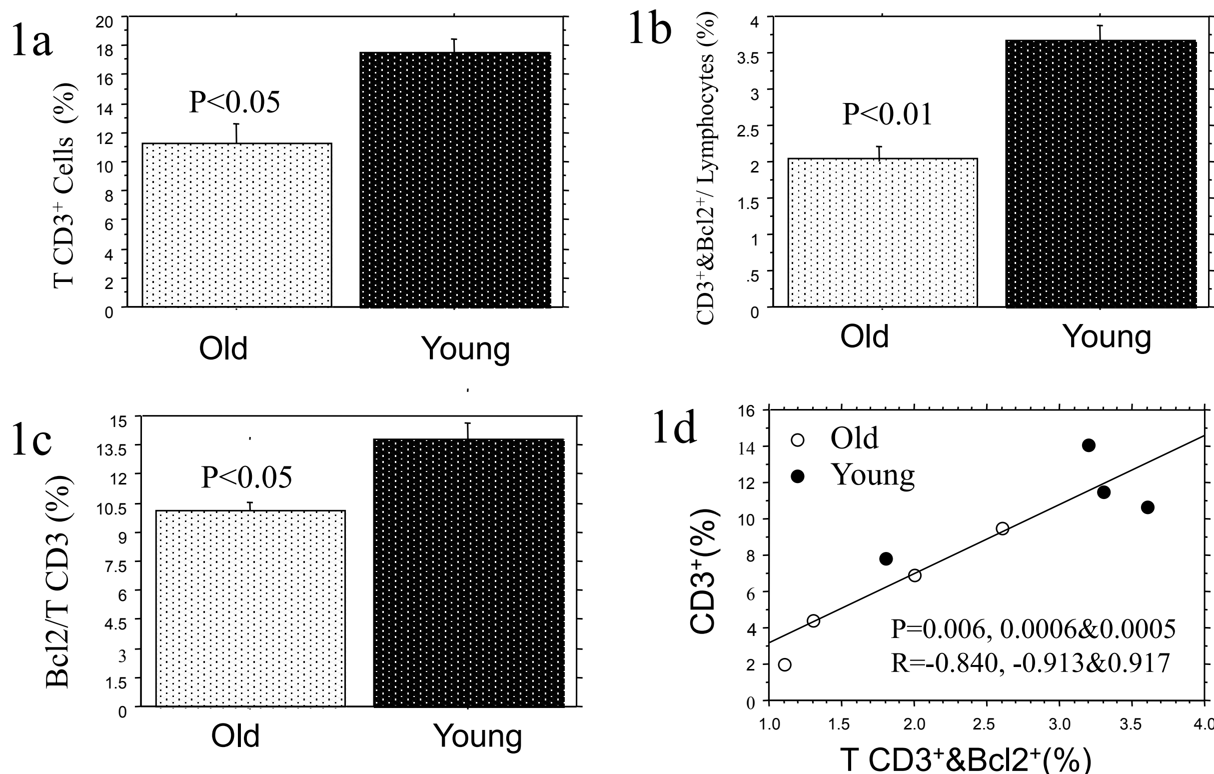

Figure 1: Expression of Bcl-2 on CD3$^+$ cells in old and young mice. Four old and four young (24 and 4 months of age, respectively) mice were bled and peripheral lymphocytes were assessed for the frequencies (mean ± SE of percentage) of CD3$^+$ cells **(a)** and the cells positive for both CD3 and Bcl-2 (CD3$^+$Bcl-2$^+$ cells) **(b)** using 2-color flow cytometry and the Bcl-2 positive rate of CD3$^+$ cells (CD3$^+$Bcl-2$^+$/total CD3$^+$ cells) (mean ± SE of percentage) was calculated **(c)**. Old mice exhibited significantly decreased frequencies of CD3$^+$ cells ($P<0.01$) and CD3$^+$ Bcl-2$^+$ cells ($P<0.01$), but Bcl-2 positive rates of CD3$^+$ cells had no significant difference between old and young mice. The relationships among the frequencies of CD3$^+$ cells, CD3$^+$ Bcl-2$^+$ double positive cells and age (old and young) of mice were analyzed with multiple correlations **(d)**. The frequencies of CD3$^+$Bcl-2$^+$ cells positively correlated with those of CD3$^+$ cells (R=0.948, P<0.0001), while CD3$^+$ and CD3$^+$Bcl-2$^+$ cells and age (R=-0.792, P=0.016) as well as frequencies of CD3$^+$ cells and age (R=-0.857, P=0.004) are all in negative correlation.

($CD3^+$ $Bcl\text{-}2^+$ cells) in total lymphocytes, were also significantly reduced in the old mice in comparison to young mice (Figure 1b). Similarly, the Bcl-2 positive rate of $CD3^+$ cells ($CD3^+$ $Bcl\text{-}2^+$/total $CD3^+$ cells), which reflects the proportion of $CD3^+$ $Bcl\text{-}2^+$ cells in T cells, was also significantly reduced in the old mice (Figure 1c). Multiple correlation analysis (Figure 1d) showed that frequencies of $CD3^+$ cells and $CD3^+$ $Bcl\text{-}2^+$ cells were in positive correlation ($P<0.0001$, $R=0.948$), but the age (young and old) of mice and the frequency of $CD3^+$ cells ($P=0.016$, $R=-0.792$) as well as the age (young and old) of mice and the frequency of $CD3^+$ $Bcl\text{-}2^+$ cells ($P<0.005$, $R=-0.857$) were all in negative correlation.

Unlike what has been seen in cultured splenocytes, expression of apoptosis markers including Annexin V, FasL, TNFRp75 and TNFRp55 on peripheral T cells were not significantly different between old and young animals, in which Annexin V and FasL were all at very low levels (<1%, data not shown). These data suggest that the reduced $Bcl\text{-}2^+$ cells may lead to the declined frequency of T cells in aging since $Bcl\text{-}2^+$ has been previously demonstrated to play an important role in prolonging the T cell survival [8-10].

Association of decreased $CD4^+$ cell frequency and the reduced expression of Bcl-2 on T lymphocytes in aged mice

$CD4^+$ cell and $CD4^+$ $Bcl\text{-}2^+$ cell frequency were also determined by flow cytometry. As shown for $CD3^+$ T cells, the frequency of $CD4^+$ cells and the cells positive for both CD4 and Bcl-2 ($CD4^+$ $Bcl\text{-}2^+$ cells) in total lymphocytes were significantly lower in old than young mice (Figures 2a and 2b). Also consistent with $CD3^+$ cells , the Bcl-2 positive rate of $CD4^+$ cells ($CD4^+$ $Bcl\text{-}2^+$/total $CD4^+$ cells) was significantly lower in old mice ($p<0.05$) compared to the young (Figure 2c). Multiple correlation analysis (Figure 2d) showed that the frequencies of $CD4^+$ cells and $CD4^+$ $Bcl\text{-}2^+$ cells were in positive correlation ($p<0.0005$, $R=0.917$), while the age (young and old) of mice and the frequency of $CD4^+$ cells ($p<0.001$, $R=-0.913$) as well as the age (young and old) of mice and the frequency of $CD4^+$ $Bcl\text{-}2^+$ cells ($p<0.01$, $R=-0.840$) were all in negative correlation. There also were no significant differences in apoptosis markers Annexin V, FasL, TNFRp75 and TNFRp55 on T cells between old and young mice, in which Annexin V and FasL were all at very low levels (<1%, data not shown). This suggests that Bcl-2 expression also exerts effects on $CD4^+$ cells, a major portion of $CD3^+$ cells.

Association of decreased $CD8^+$ cell frequency and the reduced expression of Bcl-2 on T lymphocytes in aged mice

Similar to $CD4^+$ cells and $CD3^+$ cells, the frequencies of $CD8^+$ cells and the cells positive for both CD8 and Bcl-2 ($CD8^+$ $Bcl\text{-}2^+$ cells) in total lymphocytes were all lower in old mice than in young mice (Figures 3a and 3b). Also as shown for $CD4^+$ T cells, the Bcl-2 positive rate of $CD8^+$ cells ($CD8^+$ $Bcl\text{-}2^+$/total $CD8^+$ cells) was also significantly lower in old mice compared to the young mice (Figure 3c). This suggests that reduced $Bcl\text{-}2^+$ expression in $CD8^+$ T cells from old mice may have

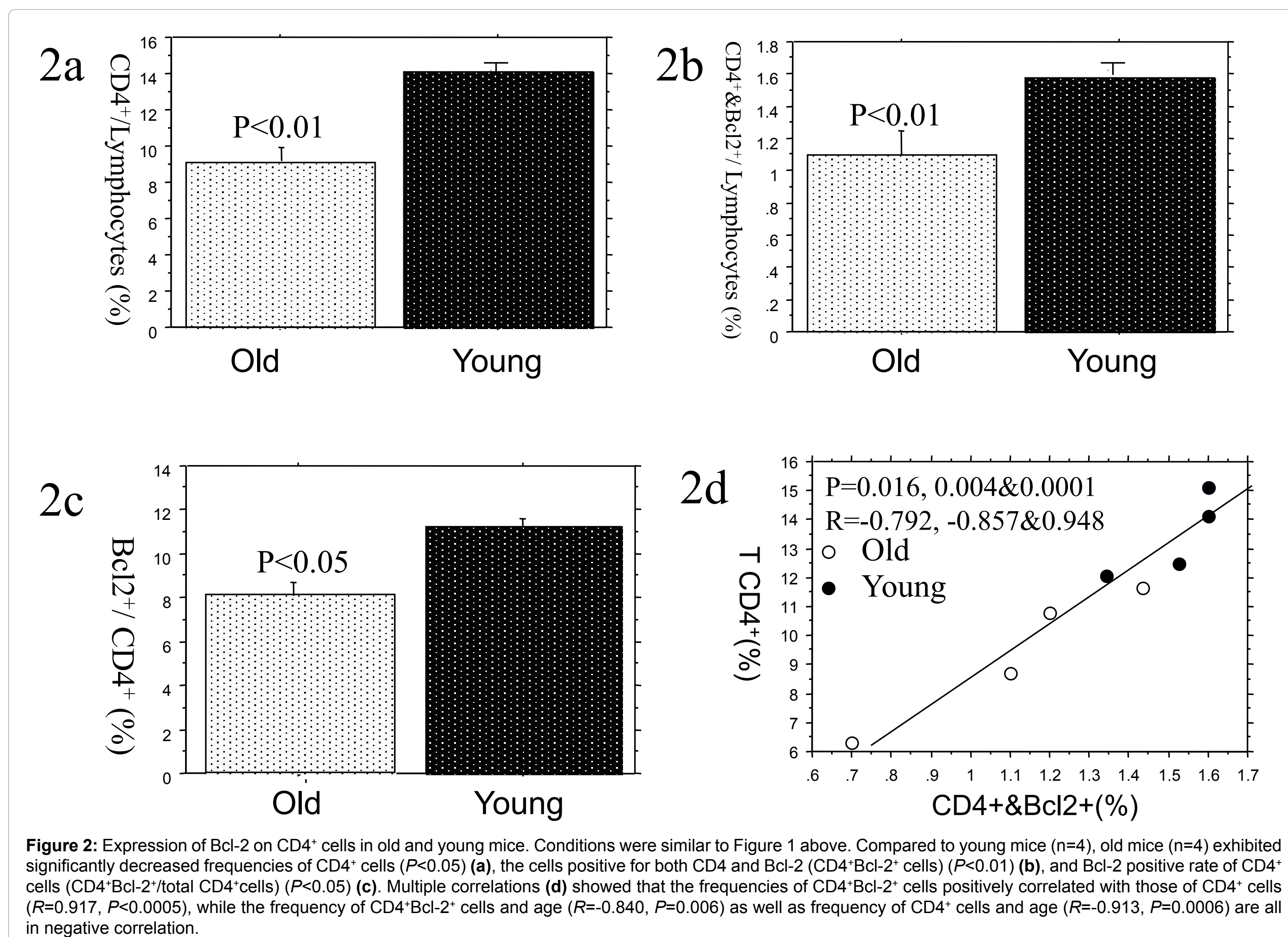

Figure 2: Expression of Bcl-2 on $CD4^+$ cells in old and young mice. Conditions were similar to Figure 1 above. Compared to young mice (n=4), old mice (n=4) exhibited significantly decreased frequencies of $CD4^+$ cells ($P<0.05$) **(a)**, the cells positive for both CD4 and Bcl-2 ($CD4^+Bcl\text{-}2^+$ cells) ($P<0.01$) **(b)**, and Bcl-2 positive rate of $CD4^+$ cells ($CD4^+Bcl\text{-}2^+$/total $CD4^+$cells) ($P<0.05$) **(c)**. Multiple correlations **(d)** showed that the frequencies of $CD4^+Bcl\text{-}2^+$ cells positively correlated with those of $CD4^+$ cells ($R=0.917$, $P<0.0005$), while the frequency of $CD4^+Bcl\text{-}2^+$ cells and age ($R=-0.840$, $P=0.006$) as well as frequency of $CD4^+$ cells and age ($R=-0.913$, $P=0.0006$) are all in negative correlation.

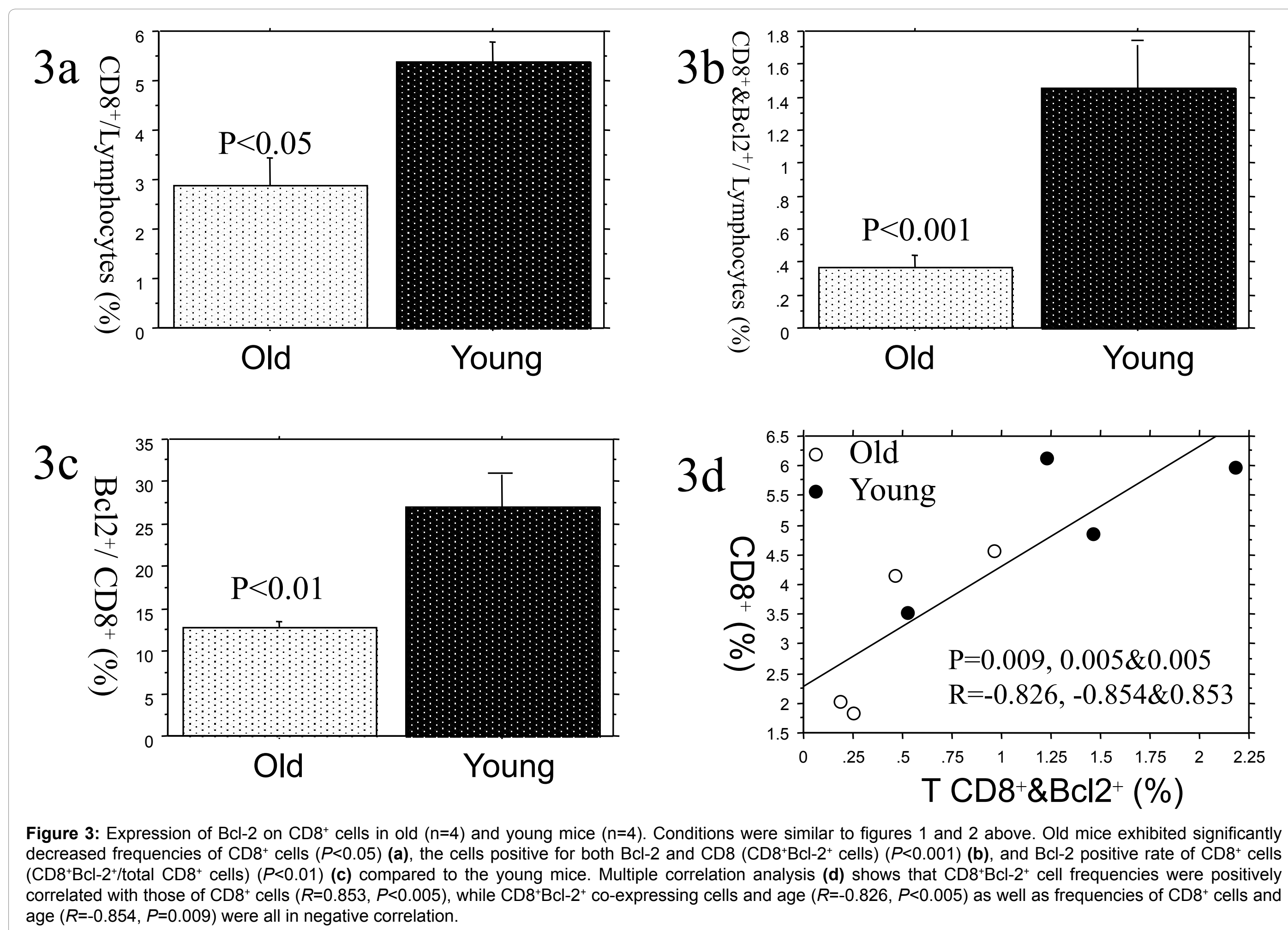

Figure 3: Expression of Bcl-2 on CD8⁺ cells in old (n=4) and young mice (n=4). Conditions were similar to figures 1 and 2 above. Old mice exhibited significantly decreased frequencies of CD8⁺ cells (*P*<0.05) **(a)**, the cells positive for both Bcl-2 and CD8 (CD8⁺Bcl-2⁺ cells) (*P*<0.001) **(b)**, and Bcl-2 positive rate of CD8⁺ cells (CD8⁺Bcl-2⁺/total CD8⁺ cells) (*P*<0.01) **(c)** compared to the young mice. Multiple correlation analysis **(d)** shows that CD8⁺Bcl-2⁺ cell frequencies were positively correlated with those of CD8⁺ cells (*R*=0.853, *P*<0.005), while CD8⁺Bcl-2⁺ co-expressing cells and age (*R*=-0.826, *P*<0.005) as well as frequencies of CD8⁺ cells and age (*R*=-0.854, *P*=0.009) were all in negative correlation.

important consequences on their survival and functions. Multiple correlation analysis (Figure 3d) also showed that the frequencies of $CD8^+$ cells and $CD8^+$ $Bcl-2^+$ cells were in positive correlation (P<0.005, R=0.853), while the age (young and old) of mice and the frequency of $CD8^+$ cells (P<0.005, R=-0.854) as well as the age (young and old) of mice and the frequency of $CD8^+$ $Bcl-2^+$ cells (P<0.01, R=-0.826) were all in negative correlation. As shown for $CD3^+$ and $CD4^+$ cells, no significant differences were observed between old and young mice with regards to the expression of FasL, TNFRp75 and TNFRp55 on $CD8^+$ T cells, in which Annexin V and FasL were all at very low levels (<1%, data not shown).

Reduced Bcl-2 expression and $CD4^+$ and $CD8^+$ T cell response to influenza virus challenge in virus-primed old mice

Mice were primed with 10 MID_{50} of influenza A/Taiwan/1/86 (H1N1) virus and challenged with the same dose of virus two months later [16]. Prior to and 2, 4 and 6 days after virus challenge, all mice were bled and peripheral lymphocytes were harvested for Bcl-2 and T cell subsets determination. The frequencies of $CD4^+$ and $CD8^+$ T cells were all significantly increased after virus challenge in both old and young mice, but the old mice reached the response peaks on day 4 post-challenge, two days later than did young mice (Figures 4a and 4d). It is noteworthy that the peak levels of $CD4^+$ and $CD8^+$ T cells were still lower in old mice than those in young mice. These differences, between the old and the young, shown for peaks of $CD4^+$ and $CD8^+$ cell responses were similar to those shown for the pre-challenge levels of $CD4^+$ and $CD8^+$. The frequencies of $CD4^+$ and $CD8^+$ T cells co-expressing Bcl-2 ($CD4^+$ $Bcl-2^+$ and $CD8^+$ $Bcl-2^+$) were displayed with the same pattern as shown for $CD4^+$ cells and $CD8^+$ cells in response to virus challenge (Figures 4c and 4f), that is, old mice had significantly lower cell frequencies for these subsets, lower peak levels and later peak times. As shown above, the Bcl-2 positive rates of $CD4^+$ and $CD8^+$ were significantly lower at all time points in old mice in comparison to young mice (Figures 4b and 4e). Even though there were increases in their Bcl-2 proportions over time in old mice, they never achieved the same levels as young mice. However, such increases resulted in closer levels of $CD4^+$ and $CD8^+$ T cells on day 6 post-challenge. The reduction in Bcl-2 proportions in $CD4^+$ and $CD8^+$ cells of young mice between days 2 and 4 post virus challenge suggested that the increase of Bcl-2 negative T cells of the circulating pool in the early stage of T cell responses to virus challenge is also not ignorable. The reductions of $CD4^+$ and $CD8^+$ cells following the response peaks were likely due to activation-induced cell death (AICD) mediated by virus stimulation and/or because these T cells rapidly left circulating pool, which also appeared earlier in young mice than in the old as, did the peaks.

The increased $CD19^+$ B cell frequency associated with increased Bcl-2 expression in elderly mice

Lymphocytes were isolated from peripheral blood of old and young mice. Two-color flow cytometry was used to determine $CD19^+$ cell

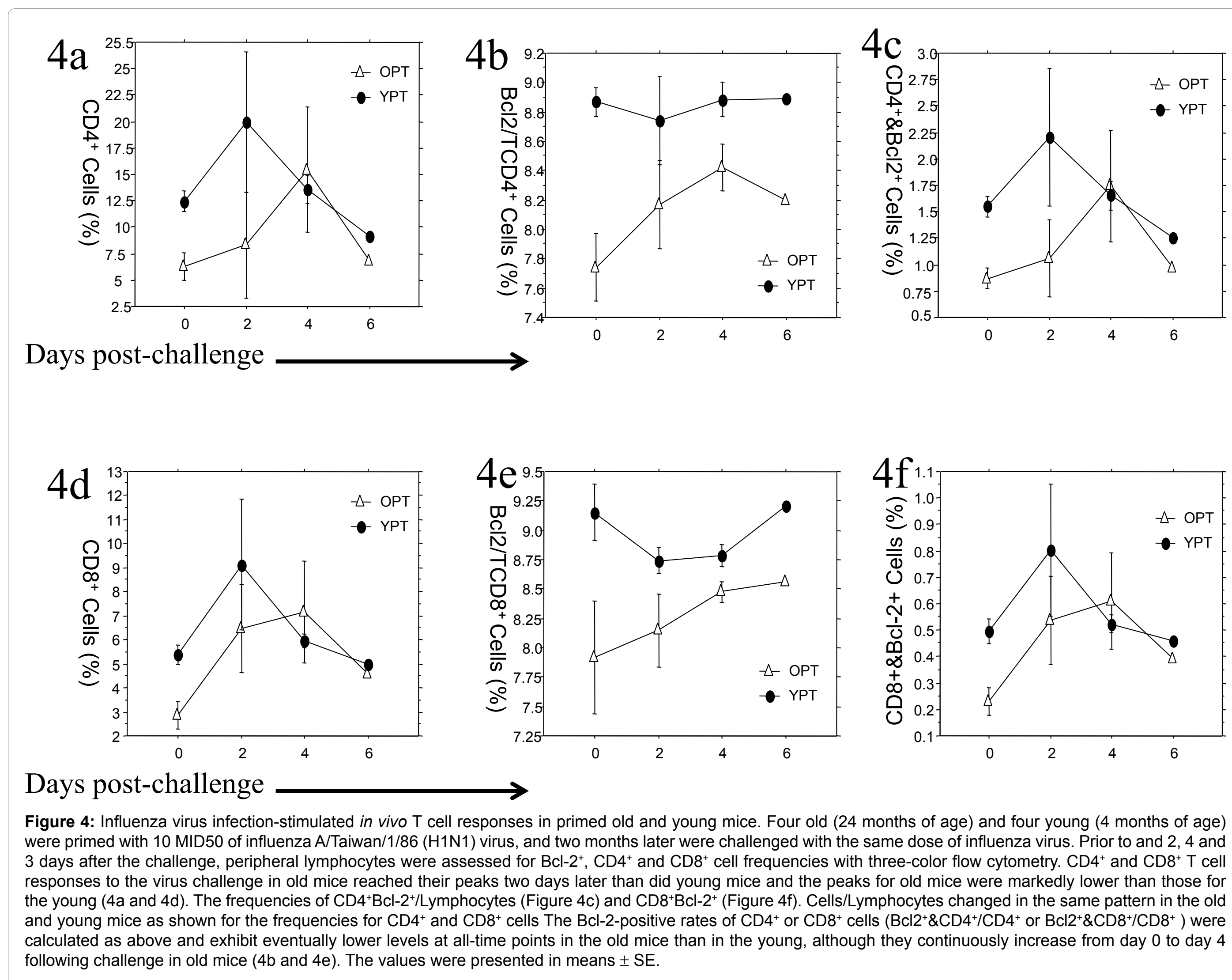

Figure 4: Influenza virus infection-stimulated *in vivo* T cell responses in primed old and young mice. Four old (24 months of age) and four young (4 months of age) were primed with 10 MID50 of influenza A/Taiwan/1/86 (H1N1) virus, and two months later were challenged with the same dose of influenza virus. Prior to and 2, 4 and 3 days after the challenge, peripheral lymphocytes were assessed for Bcl-2+, CD4+ and CD8+ cell frequencies with three-color flow cytometry. CD4+ and CD8+ T cell responses to the virus challenge in old mice reached their peaks two days later than did young mice and the peaks for old mice were markedly lower than those for the young (4a and 4d). The frequencies of CD4+Bcl-2+/Lymphocytes (Figure 4c) and CD8+Bcl-2+ (Figure 4f). Cells/Lymphocytes changed in the same pattern in the old and young mice as shown for the frequencies for CD4+ and CD8+ cells The Bcl-2-positive rates of CD4+ or CD8+ cells (Bcl2+&CD4+/CD4+ or Bcl2+&CD8+/CD8+) were calculated as above and exhibit eventually lower levels at all-time points in the old mice than in the young, although they continuously increase from day 0 to day 4 following challenge in old mice (4b and 4e). The values were presented in means ± SE.

frequency and Bcl-2+ cell frequency. Different from CD3+, CD4+ and CD8+ T cells, CD19+ cell frequency was significantly increased ($p<0.05$) in old mice compared to the young mice (Figure 5a). The frequencies of the cells positive for both CD19 and Bcl-2 (CD19+ Bcl-2+ cells) ($p<0.001$) and the Bcl-2 positive rate of CD19+ cells (CD19+ Bcl-2+/total CD19+ cells) ($p<0.005$) were also significantly higher in the old mice than those in the young mice (Figures 5b and 5c). Multiple correlation analysis (Figure 5d) showed that frequencies of CD19+ cells and CD19+ Bcl-2+ cells ($p<0.05$, $R=0.706$). The age (young and old) of mice and the frequency of CD19+ cells ($p<0.05$, $R=0.755$) as well as the age (young and old) of mice and the frequency of CD19+ Bcl-2+ cells ($p<0.005$, $R=0.930$) were all in positive correlation (Figure 5d). These data suggest that increased Bcl-2 expression may play a role in the increased frequency of B cells observed in aging. The kinetics of CD19+ B cells responses to *in vivo* influenza virus challenged were not evaluated due to the limited cell number. However, immunoglobulin-expressing B cells from spleen cells from old mice exhibited a significantly higher expression of Bcl-2 than young mice following *in vitro* stimulation with influenza virus (data not shown).

Discussion

Bcl-2, as an apoptosis antagonist, plays a important role in prolonging cell life span and over expression of Bcl-2 is a causative factor in cell malignant proliferation [34-36]. In our previous studies, it was demonstrated that the decreased frequencies of CD8+ and CD4+ cells in cultured splenocytes of old mice were associated with increased apoptosis in T cells [14]. Such changes in aged mice occurred on both influenza virus-stimulated and non-stimulated cells of influenza virus primed mice [14]. Similar results were obtained in a human study in which the cultured CD4+ and CD8+ cells from peripheral blood underwent more severe apoptosis in elderly persons than in the young, and the Bcl-2 expression in CD4+ and CD8+ cells of aged individuals were decreased in comparison with the young [14,32]. In addition, the T cells exhibited increased expression of apoptosis mediators, Fas, Fas-L, TNF-α and TNF receptors as shown in our previous studies [14,32]. Those findings led present study to examine if direct correlations exist between *in vivo* T cell frequencies and Bcl-2 expressions and whether Bcl-2 expression contributes to the CD4+ or CD8+ cell frequency changes in T cell responses to virus infection *in vivo*. In addition, the analysis included expression of Bcl-2 in peripheral B lymphocytes as these cells have hitherto not been evaluated in aged mice.

Consistent with the data from cultured lymphocytes [14,32], the frequencies of CD3+ cells as well as the frequencies of T cell subsets,

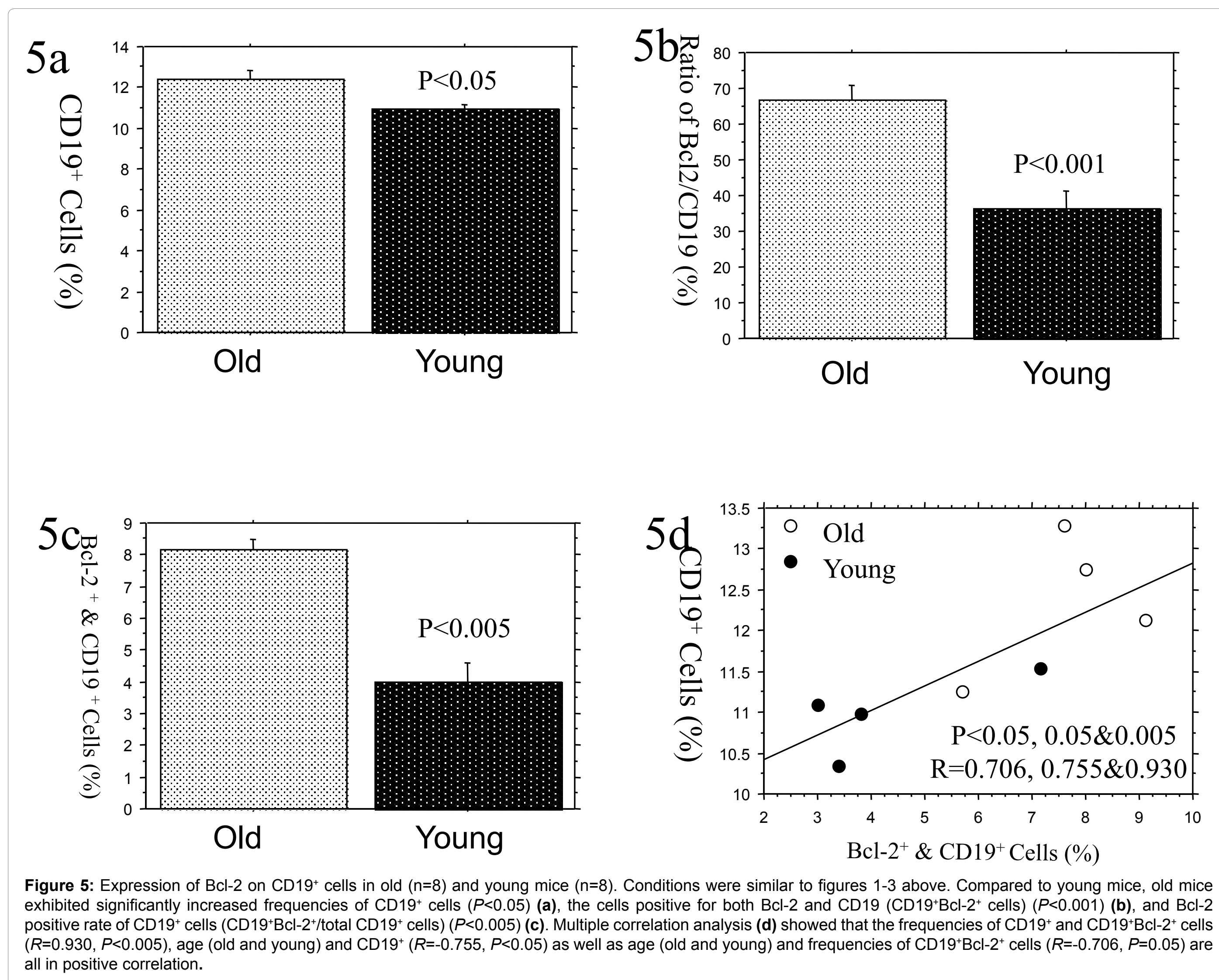

Figure 5: Expression of Bcl-2 on CD19$^+$ cells in old (n=8) and young mice (n=8). Conditions were similar to figures 1-3 above. Compared to young mice, old mice exhibited significantly increased frequencies of CD19$^+$ cells (P<0.05) **(a)**, the cells positive for both Bcl-2 and CD19 (CD19$^+$Bcl-2$^+$ cells) (P<0.001) **(b)**, and Bcl-2 positive rate of CD19$^+$ cells (CD19$^+$Bcl-2$^+$/total CD19$^+$ cells) (P<0.005) **(c)**. Multiple correlation analysis **(d)** showed that the frequencies of CD19$^+$ and CD19$^+$Bcl-2$^+$ cells (R=0.930, P<0.005), age (old and young) and CD19$^+$ (R=-0.755, P<0.05) as well as age (old and young) and frequencies of CD19$^+$Bcl-2$^+$ cells (R=-0.706, P=0.05) are all in positive correlation.

CD4$^+$ and CD8$^+$ cells were all reduced in aged mice compared to the young mice. However, we could not find any significant differences in these fresh T cells between old and young mice in Annexin V binding assay used for measuring early stage apoptosis [14,32]. This is likely due to lack of appreciable accumulation of these cells *in vivo* as the body is very efficient in eliminating apoptotic cells [37,38]. By the same analogy, we also did not find any significant differences for the expression of Fas-L, TNF receptor I and TNF receptor II on these T cells, because they were all in very low frequencies (<1%). In contrast, Bcl-2 expression on CD3$^+$, CD4$^+$ and CD8$^+$ T cells significantly reduced in old mice in comparison to the young, and frequencies of CD3$^+$ cells and CD3$^+$ Bcl-2$^+$ cells were in positive correlation. These data suggested that the decreased expression of Bcl-2 might play a role in the declining *in vivo* T cell frequency in aging. The significant decreases of Bcl-2 positive rates of CD4$^+$ and CD8$^+$ T cells in aged mice were further suggestive of the effects of Bcl-2 expression on T cell population during aging.

In cultured splenocytes, CD8$^+$ and CD4$^+$ cells from old mice declined in response to influenza virus stimulation as did to non-stimulation [14]. In our kinetic observations on CD4$^+$ and CD8$^+$ cells responses to influenza virus challenge in primed mice, these T cell subsets exhibited the same response pattern but with a different response kinetics in old and young mice. The responses in old mice peaked 2 days later than in young mice and the peak response was markedly lower in old mice than young mice. These data indicate that T cell response to virus infection in immunized old animals were not only delayed but also weakened. Figures 4a and 4d revealed that such delay and decline of T cell responses were coincident with the levels of CD4$^+$ and CD8$^+$ cells before challenge. Although the frequencies of Bcl-2-expressing CD4$^+$ and CD8$^+$ cells in the old mice increased continuously in the response to virus challenge, they did not reach the levels of young mice. This is not surprising because these frequencies were significantly lower in old mice prior to virus challenge (day 0). Considering the function of the Bcl-2 as a antagonist of apoptosis, the combined results of reduced frequencies of CD4$^+$ and CD8$^+$ cells as well as CD4 and CD8 co-expressing Bcl-2 suggest that the reduced Bcl-2 expression might play a role in the lower (compared to the young) and postponed T cell response peaks due to its associated lower initial T-cell level in aged mice. The present results are consistent with other studies on the effects of Bcl-2 expression on occurrence of cancer [1-7], on CD8$^+$ T cells [8], T and B lymphopoiesis [9], T cell development and

regulation [10,11] and germinal center responses [12]. The studies are novel in establishing a link between deficient *in vivo* T cell responses to a viral infection and reduced Bcl-2 expression on the peripheral T cells in old mice.

It should be noted that the T cells increased in circulating pool were mainly released from lymphoid organ, such as spleen and lymph nodes. Only a small portion (normally <1%) of these lymphocytes were from those activated directly by antigen stimulation. Because of the limited cell numbers in our current studies, the effects of Bcl-2 on antigen-specific T cells were not evaluated, in which the assay, such as tetramer assay, should be conducted with much more cells for a reliable result. In addition, Bcl-2 positive rates in $CD4^+$ and $CD8^+$ all slightly dropped on day 2 following the virus challenge in young mice, but these was not seen in the old. The possible reason for this phenomenon could be that some T lymphocytes released to circulating pool might contain more Bcl-2 negative cells in the young mice. Even so, Bcl-2 expression in $CD4^+$ and $CD8^+$ T cells of young mice were still significantly higher than in old mice at all-time points (Figures 4b and 4e), suggesting a potential role of Bcl-2 expression in maintaining T cell levels in the circulating pool following virus infection.

The mechanism of the effects of Bcl-2 on T cell development and regulation [10,11] has been well documented. In contrast to results for T cells in aging, our current data demonstrate a significantly increased frequency of $CD19^+$ B cells in peripheral blood and enhanced expression of Bcl-2 in B cells of aged mice compared to the young mice. This is supported by our previous study, in which the increased immunoglobulin-expressive B cell frequency in the old mice was associated with hyperimmunoglobuliemia [16]. However, in the studies of Mireille Verdier et al. [39], it was not found significant difference in Bcl-2 expression between old and young mice. The possible reasons for the difference between their results and ours could be the age of the old mice used in the experiments. The age of the old mice in their study was in a big range (15-21 months), but ours were in very narrow range, all between 24 and 25 months of age. In addition, they may have different definition for peripheral B lymphocyte because the testing cells in their study were from spleen or peyer's patches as described in the "Materials and Methods" section of their publication while the cells used in our current study were from mouse peripheral blood.

To further confirm the up-regulation role of Bcl-2 in of B cells, the peripheral mononuclear cells from 4 aged mice were cultured in ZYX bioreactor [16,40] with RPMI1640 media containing Bcl-2 inhibitor, 10 μM Venetoclax (ABT199) [41] and other components as we previously described [16]. The results showed that the total B cells and Bcl-2 positive B cells were respectively 42.1% and 36.8% reduced ($P<0.01$and 0.05) and Annexin V positive cells respectively 26.4% and 19.2% increased ($P<0.01$ in both) when compared to the culture without Venetoclax. This further supports that Bcl-2 played a role in regulation of B cells by the inhibition of apoptosis and could contribute to the imbalance of B cells in aging.

In summary, the frequencies of T cell ($CD3^+$) and T cell subsets ($CD4^+$ and $CD8^+$ cells) from peripheral blood were all decreased in old mice in contrast to young mice. The decreased $CD3^+$, $CD4^+$ and $CD8^+$ T cells in aged mice were highly associated with the reduced expression of Bcl-2. The lowered Bcl-2 expression level did not only affect T cell response by lowering the initial response points in aged mice but is also associated with the T cell levels in the late stage of responses with a shortened life span of the stimulated $CD4^+$ and $CD8^+$ cells in the circulating pool. B cells from aged mice, in the contrary,

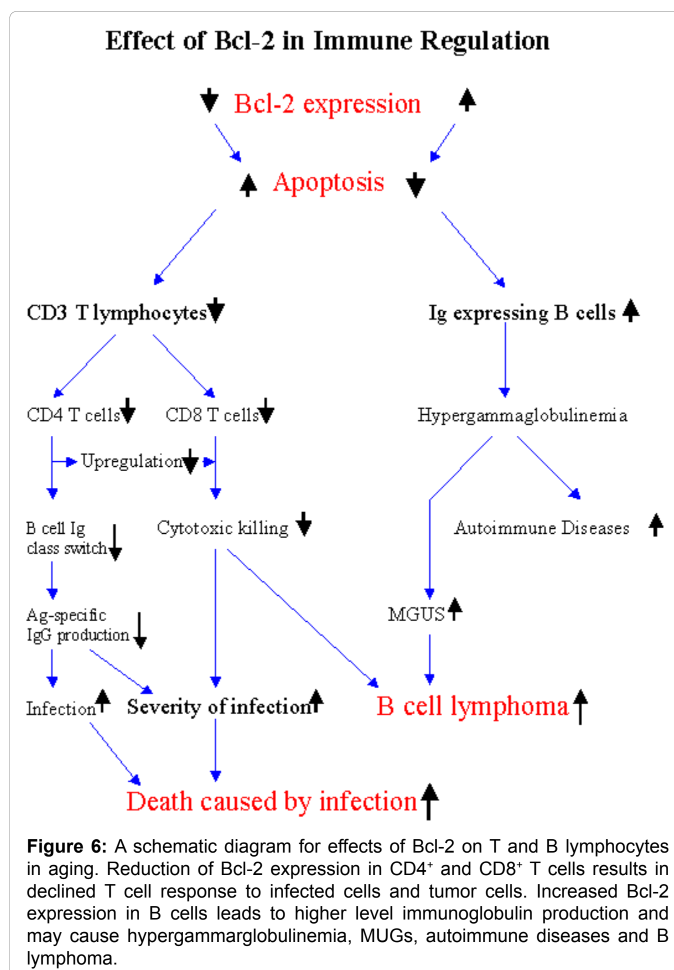

Figure 6: A schematic diagram for effects of Bcl-2 on T and B lymphocytes in aging. Reduction of Bcl-2 expression in $CD4^+$ and $CD8^+$ T cells results in declined T cell response to infected cells and tumor cells. Increased Bcl-2 expression in B cells leads to higher level immunoglobulin production and may cause hypergammarglobulinemia, MUGs, autoimmune diseases and B lymphoma.

were up-regulated by Bcl-2. Thus, Bcl-2 may play an important role in the imbalance of T and B cells by the inhibition of apoptosis and prolonging the cell survival in both physiological procedure and pathological procedure (Figure 6).

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References

1. Reed JC, Haldar S, Cuddy MP, Croce C, Makover D (1990) Deregulated BCL2 expression enhances growth of a human B cell line. Proc Nat Acad Sci USA 87: 3660-3664.
2. Reed JC, Haldar S, Cuddy MP, Croce C, Makover D, et al. (1990) BCL2-mediated tumorigenicity of a human T-lymphoid cell line: synergy with MYC and inhibition by BCL2 antisense. Proc Nat Acad Sci USA 88: 8661-8665.
3. Strasser A, Whittingham S, Vaux DL, Bath ML, Adams JM, et al. (1991) Enforced BCL2 expression in B-lymphoid cells prolongs antibody responses and elicits autoimmune disease. Proc Nat Acad Sci USA 89: 7295-7299.
4. Alnemri ES, Robertson NM, Fernandes TF, Croce CM, Litwack G, et al. (1992) Overexpressed full-length human BCL2 extends the survival of baculovirus-infected Sf9 insect cells. J Nat Cancer Inst 84: 1789-1793.
5. Keating MJ (1999) Chronic lymphocytic leukemia. Semin Oncol 26: 107-114.
6. Green MH, Petit-Frčre C, Clingen PH, Bentham G, Cole J, et al. (1999) Possible effects of sunlight on human lymphocytes. J Epidemiol 9: S48-57.
7. Fernandez-Vazquez A (2001) Primary cutaneous large B-cell lymphoma: the relation between morphology, clinical presentation, immunohistochemical markers, and survival. Am J Surg Pathol 25: 307-315.

8. Ohteki T, Maki C, Koyasu S (2001) Overexpression of Bcl-2 differentially restores development of thymus-derived CD4-8+ T cells and intestinal intraepithelial T cells in IFN-regulatory factor-1-deficient mice. J Immunol 166: 6509-6513.

9. Matsuzaki Y, Nakayama K, Nakayama K, Tomita T, Isoda M, et al. (1997) Role of bcl-2 in the development of lymphoid cells from the hematopoietic stem cell. Blood 89: 853-862.

10. Nakajima H, Leonard WJ (1999) Role of Bcl-2 in alpha beta T cell development in mice deficient in the common cytokine receptor gamma-chain: the requirement for Bcl-2 differs depending on the TCR/MHC affinity. J Immunol 162: 782-790.

11. Williams O, Norton T, Halligey M, Kioussis D, Brady HJ (1998) The action of Bax and bcl-2 on T cell selection. J Exp Med 188:1125-1133.

12. Alabyev B, Manser T (2002) Bcl-2 rescues the germinal center response but does not alter the V gene somatic hypermutation spectrum in MSH2-deficient mice. J Immunol 169: 3819-3824.

13. Telford WG, Miller RA (1999) Aging increases CD8 T cell apoptosis induced by hyperstimulation but decreases apoptosis induced by agonist withdrawal in mice. Cell Immunol 191: 131-138.

14. Zhang Y, Wang Y, Gilmore X, Xu K, Chen M, et al. (2002) Apoptosis and reduced influenza A virus specific CD8+ T cells in aging mice. Cell Death Differ 9: 651-660.

15. Herndon FJ, Hsu HC, Mountz JD (1997) Increased apoptosis of CD45RO- T cells with aging. Mech Ageing Dev 94: 123-134.

16. Zhang Y, Wang Y, Zhang M, Liu L, Mbawuike IN (2016) Restoration of Retarded Influenza Virus-specific Immunoglobulin Class Switch in Aged Mice. J Clin Cell Immunol 7:403.

17. Falsey AR, Treanor JJ, Betts RF, Walsh EE (1992) Viral respiratory infections in the institutionalized elderly clinical and epidemiologic findings. J Am Geriatr Soc 40: 115-119.

18. Agius G, Dindinaud G, Biggar RJ, Peyre R, Vaillant V, et al. (1990) An epidemic of respiratory syncytial virus in elderly people: clinical and serological findings. J Med Virol 30: 117-127.

19. Mlinaric-Galinovic G, Falsey AR, Walsh EE (1996) Respiratory syncytial virus infection in the elderly. Eur J Clin Microbiol Infect Dis 15: 777-781.

20. Falsey AR (1995) Respiratory syncytial virus and influenza A infections in the hospitalized elderly. Journal of Infectious Diseases 172: 389-394.

21. Han LL, Alexander JP, Anderson LJ (1999) Respiratory syncytial virus pneumonia among the elderly: an assessment of disease burden. J Infect Dis 179: 25-30.

22. Sprenger MJ, Van Naelten MA, Mulder PG, Masurel N (1989) Influenza mortality and excess deaths in the elderly, 1967-82. Epidemiol Infect 103: 633-641.

23. Carrat F, Valleron AJ (1995) Influenza mortality among the elderly in France, 1980-90: how many deaths may have been avoided through vaccination? J Epidemiol Community Health 49: 419-425.

24. Biro J, Beregi E (1980) The influence of influenza vaccinations on human peripheral blood lymphocytes relating to aging. Aktuelle Gerontologie 10: 319-322.

25. Ershler WB, Moore AL, Socinski MA (1984) Influenza and aging: age-related changes and the effects of thymosin on the antibody response to influenza vaccine. J Clin Immunol 4: 445-454.

26. Felser JM, Raff MJ (1983) Infectious diseases and aging: immunologic perspectives. J Am Geriatr Soc 31: 802-807.

27. Powers DC, Belshe RB (1994) Vaccine-induced antibodies to heterologous influenza A H1N1 viruses: effects of aging and "original antigenic sin". J Infect Dis 169: 1125-1129.

28. Boon AC, Fringuelli E, Graus YM, Fouchier RA, Sintnicolaas K, et al. (2002) Influenza A virus specific T cell immunity in humans during aging. Virology 299: 100-108.

29. Zhang Y, Wang Y, Gilmore X, Xu K, Mbawuike IN (2001) Independent and synergistic effects of interleukin-18 and interleukin-12 in augmenting cytotoxic T lymphocyte responses and IFN-gamma production in aging. J Interfer Cytokine Res 21: 843-850.

30. Looney RJ, Falsey AR, Walsh E, Campbell D (2002) Effect of aging on cytokine production in response to respiratory syncytial virus infection. J Infect Dis 185: 682-685.

31. Meyer KC (2001) The role of immunity in susceptibility to respiratory infection in the aging lung. Respir Physiol 128: 23-31.

32. Zhang Y, Pacheco S, Acuna CL, Switzer KC, Wang Y, et al. (2002) Immunoglobulin A-deficient mice exhibit altered T helper 1-type immune responses but retain mucosal immunity to influenza virus. Immunology 105: 286-294.

33. Zhang Y, Wang Y, Gilmore X, Mbawuike IN (2016) Analysis of replaceable amino acids in influenza A virus M2 protein. Br Microbiol Res J 15: 1-16.

34. Aggarwal S, Gupta S (1998) Increased apoptosis of T cell subsets in aging humans: altered expression of Fas (CD95), Fas ligand, Bcl-, and Bax. J Immunol 160: 1627-1637.

35. Tsujimoto Y, Cossman J, Jaffe E, Croce CM (1985) Involvement of the bcl-2 gene in human follicular lymphoma. Science 228: 1440-1443.

36. Yip KW, Reed JC (2008) Bcl-2 family proteins and cancer. Oncogene 27: 6398-6406.

37. Oyaizu N, McCloskey TW, Coronesi M, Chirmule N, Kalyanaraman VS, et al. (1993) Accelerated apoptosis in peripheral blood mononuclear cells (PBMCs) from human immunodeficiency virus type-1 infected patients and in CD4 cross-linked PBMCs from normal individuals. Blood 82: 3392-3400.

38. McCloskey TW, Oyaizu N, Coronesi M, Pahwa S (1994) Use of a flow cytometric assay to quantitate apoptosis in human lymphocytes. Clin Immunol Immunopathol 71: 14-18.

39. Verdier M, Malissein E, Munteanu E, Jayat-Vignoles C, Ratinaud MH, et al. (2006) Aged Mice Exhibit Distinct Peripheral B-Cell Phenotypes Differing in Apoptotic Susceptibility: An Ex Vivo Analysis. Cytometry A 69: 999-1009.

40. Zhang Y, Wang Y, Wang Z, Zhang M, Wang Z (2016) Cancer Specific CTL Expansion with ZYX Bioreactor. J Clin Cell Immunol 7:398.

41. Weiss J, Gajek T, Köhler BC, Haefeli WE (2016) Venetoclax (ABT-199) Might Act as a Perpetrator in Pharmacokinetic Drug–Drug Interactions. Pharmaceutics 8: 5.