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Apoptosis and reduced influenza A virus specific CD8+ T cells in aging mice

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Abstract

Some studies have reported increased apoptosis in CD8$^+$ T cells from aged mice. We previously demonstrated diminished virus-specific CD8$^+$ cytotoxic T lymphocyte (CTL) activity in aged mice in comparison to young mice. The present study investigated the role of apoptosis in age-related influenza virus-specific CD8$^+$ CTL deficiency. Splenocytes from influenza-primed aged and young mice were stimulated *in vitro* with virus. The CD8$^+$ T cell/total lymphocyte ratios correlated with CTL activity and were significantly decreased and increased in aged and young mice, respectively. Fas, FasL, TNF-α and TNFR-p55 expression, measured by flow cytometry, ELISA and/or RT–PCR, were significantly elevated in aged mice. Apoptotic CD8$^+$ T cells (Annexin V binding) were also elevated in aged mice. IL-12 treatment increased CD8$^+$ CTL activity and IFN-γ production but did not affect apoptosis. Thus, apoptosis may contribute to reduced influenza virus-specific CD8$^+$ T cell frequency, CTL deficiency and increased influenza disease in aging.

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Keywords: aging; apoptosis; influenza virus; CD8$^+$ T cells

Abbreviations: AICD, activation-induced cell death; CTL, cytotoxic T lymphocytes; DD, death domain

Introduction

Apoptosis or programmed cell death is now recognized as a major mechanism for elimination of activated T cells during an immune response to viral infections.[1–3] Highly activated and proliferating CD8$^+$ T cells generated during a viral infection are highly susceptible to apoptosis by a process of activation-induced cell death (AICD).[4–7] This serves to maintain homeostasis of the immune system, so that during the resolution phase of infection, a proportion of effector CD8$^+$ T cells are deleted by apoptosis while some virus-specific CD8$^+$ T cells escape apoptosis and are retained as memory cells.[8–11] These memory CD8$^+$ T cells respond more vigorously after subsequent viral infections and have been shown in recent studies to persist throughout the life of an animal.[9,12] Influenza virus-specific CD8$^+$ CTL activity is significantly lower among aged mice when compared to young mice.[13–17] The occurrence of high levels of excess morbidity and mortality among individuals 65 years of age or older during annual influenza epidemics,[18–23] has been attributed to the diminished influenza virus-specific CD8$^+$ CTL activity in this population.[24–30] Animal studies from several laboratories, including ours have demonstrated that influenza virus-specific CD8$^+$ CTL functional deficiency contributes to lowered ability to clear influenza virus infection and to increased mortality from influenza virus infection in aging.[15–17,31] Although implied from above results, it is not known if the frequency of influenza virus-specific memory CD8$^+$ CTL is altered in aging, and if so, whether it is caused by excess apoptosis.

Previous studies have shown increased age-related apoptosis in T cells in mice and humans[32–35] while others have observed a decrease in apoptosis or no effect.[36] Of note, recent results from Effros's laboratories indicate that even though CD28$^-$ T cells are significantly increased in elderly person, these senescent T cells are quite resistant to apoptosis.[37–39] These conflicting data may be due to the variation in the activating agents used in the various studies. Thus, by evaluating CD8$^+$ T cells induced by influenza virus stimulation, we should obtain data that is relevant to deficient virus-specific CD8$^+$ CTL activity in aging. Apoptosis may also differentially affect different subpopulations of T cells since aged mice and elderly human populations contain different subsets of T cells when compared to the young.[40–44]

TNF-α is secreted by activated macrophages and lymphocytes. TNF-α normally stimulates the proliferation and differentiation of cells. However, TNF-α/TNF-αR (CD120a, CD120b) interaction can mediate apoptosis in a variety of cells types.[45–47] Fas (CD95/APO-1), a transmembrane glycoprotein, is expressed on almost all cell types. Fas ligand (FasL) is highly expressed on activated T cells and cross-links with Fas to induce apoptosis.[47–51] Both TNF-α/TNF-αR- and Fas-FasL-mediated apoptosis are regulated by Bcl-2 and Bcl-2 homologues.[52] T cells from aged mice and elderly humans have been shown to exhibit increased expression of the above cell death mediators.[32,46,47,53,54] However, this has not been evaluated in the context of virus-specific CD8$^+$ CTL. The objective of the present study, was to determine if the frequency of influenza virus-specific memory CD8$^+$ T cells was decreased in old mice and if so, whether the decrease was mediated by apoptosis. It was predicted that the accumulated activated

influenza virus-specific memory CD8+ T cells among aged mice will be highly susceptible to apoptosis and this may account for the depletion of influenza antigen-specific CD8+ T cells. It was also postulated that treatment with IL-12, which enhances CTL responses and IFN-γ,[24,55–60] might reduce apoptotic cell death in CD8+ T cells from aged mice.

Results

Correlation of CD8+ T cell frequency with CTL activity

Our previous data showed that influenza A virus-specific memory CD8+ CTL exhibited significantly reduced cytotoxicity against virus-infected targets cells and produced profoundly lower IFN-γ in comparison to young mice.[17,57] It was not clear whether this was due to reduced functional activity of individual cells or to reduced frequency of memory CD8+ T cells. To investigate this, splenic lymphocytes were isolated from aged and young Balb/c mice previously primed 2 to 3 months earlier by influenza A/Taiwan/1/86 (H1N1) virus infection and were then stimulated *in vitro* with influenza A/Beijing/353/89 (H3N2) virus. The H1N1 priming and H3N2 stimulation scheme permitted us to measure CD8+ CTL responses directed primarily against the internal nucleoprotein (NP) and matrix (M1) proteins which are the major CTL antigens without interference from antibodies to surface hemagglutinin (HA) and neuraminidase (NA) molecules. Six days after stimulation, dead cells were depleted and CD8+ T cells were purified using AutoMacs mini cell sorter. To ascertain purity, unseparated and separated cells were stained with anti-CD8-PE and anti-CD4-FITC reagent (BD Bioscience, San Diego, CA, USA) and then analyzed using two-color flow cytometry. Results of a typical flow cytometric analysis showed that prior to purification, CD8+ and CD4+ T cells were 13.4 and 70.3%, respectively (data not shown). After purification, CD8+ T cells increased to 98.2% while CD4+ T cells were not detectable. In contrast, the CD8− fraction contained 0.2% CD8+ and 84.2% CD4+ cells, respectively. A majority of the CD8+ T cells were CD44hi (data not shown), indicative of memory phenotype. The ratio of CD8+ T cells to total lymphocytes was calculated.

Lysis of A/Beijing/353/89-infected P815 (H-2d) target cells by unseparated and purified CD8+ T cells was assessed in a 4-h 51-Cr release assay. Both aged and young mice displayed an E:T ratio-dependent per cent specific lysis of virus-infected target cells (Figure 1A). As previously shown, young mice exhibited significantly higher CTL activity than aged mice ($P<0.0001$). The ratio of CD8+ T cells to total lymphocytes was plotted against per cent CTL lysis by purified CD8+ T cells at the 40:1 E:T ratio (Figure 1B). Note that purified CD8+ T cells exhibited a markedly higher level of lysis than unpurified effector CTL as expected. As shown above, purified CD8+ T lymphocytes from young mice exhibited significantly higher levels of CTL activity than old mice ($P<0.01$). A positive correlation between CD8+ CTL activity and the ratio of CD8+ T cells to total lymphocytes was observed ($r=0.738$, $P<0.01$). Since the same numbers of CD8+ T cells were used in the CTL assay, the data suggest that reduced CTL activity by CD8+ T cells from old mice was due to reduced number of influenza virus specific-CD8+ CTLs and/or diminished activity of individual cells.

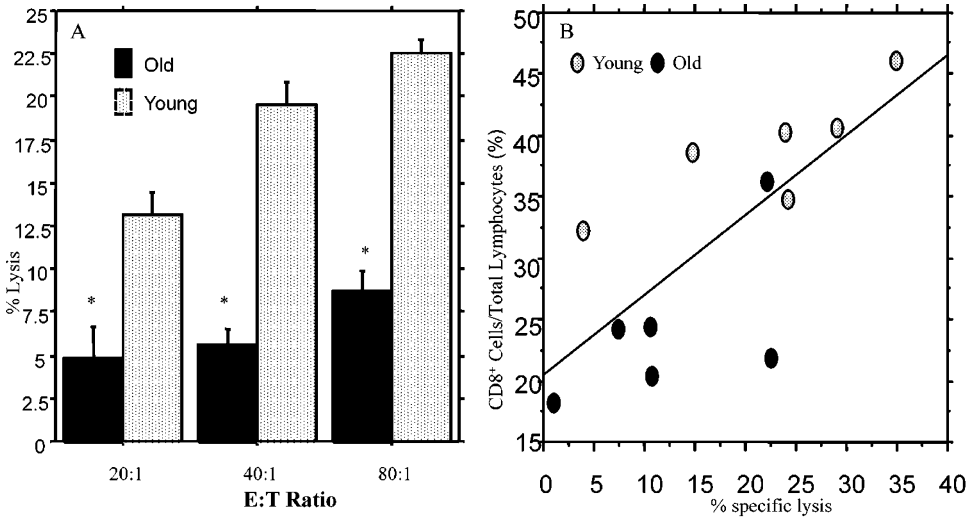

Figure 1 Age-determined relationship between CD8+ T cell/total lymphocyte ratios and CTL activity. Splenic lymphocytes from mice primed with influenza A/Taiwan/1/86 (H1N1) virus were stimulated with influenza A/Beijing/353/89 (H3N2) virus for 6 days. Dead cells were depleted and purified CD8+ T cells were obtained using Automacs. Lysis of influenza A/Beijing/353/89 virus-infected P815 (H-2d) target cells was then assessed in a 4-h chromium release assay. Data represent mean ± S.E.M. of per cent specific lysis using unseparated CTL at indicted E:T ratio for six mice per group (**A**). * Indicates that aged mice had significantly lower CTL than young mice ($P<0.0001$). Calculated CD8+/total lymphocytes ratios (per cent) determined by flow cytometry were plotted against levels of per cent specific lysis by purified CD8+ CTL from old and young mice (**B**). Data are for per cent specific lysis at 40:1 E:T ratio in two separate experiments utilizing six individual mice per group. A significant positive correlation between levels of per cent specific lysis and CD8+/total lymphocytes ratios was shown ($r=0.738$, $P<0.01$)

Memory CD8+ T cells from aged mice are refractory to influenza virus activation

Following secondary influenza A virus challenge of mice previously primed with virus, memory CD8+ T cells respond, proliferate and mediate virus clearance.[1,9,61,62] Thus, *in vitro* stimulation of splenocytes from influenza-primed mice with virus should mimic the *in vivo* situation and result in increased number of CD8+ T cells. To determine whether the CD8+ T cell frequency increases in response to influenza stimulation, CTL cultures were harvested on days 3 and 6, stained with anti-CD8-PE and anti-CD4-FITC reagent and analyzed using two-color flow cytometry. The total number of splenic lymphocytes obtained from primed mice was typically higher in aged than in young mice ($152 \pm 30 \times 10^6$ *versus* $80 \pm 3 \times 10^6$; $P < 0.05$). In contrast, the number of viable lymphocytes and CD8+ T cells obtained 6 days following *in vitro* stimulation with virus was higher in young mice in comparison to aged mice (data not shown). Nonetheless, the CD8+ T cell/total lymphocyte ratios among old and young mice were similar in freshly harvested unstimulated spleen cells (data not shown) and 3 days following culture (Figure 2). However, by day 6, the CD8+ T cells ratio was significantly lower in aged mice than young mice. In fact, while the ratio increased for young mice, it was unchanged in aged mice. These results suggest that CD8+ T cells from aged mice were refractory to stimulation by influenza virus. CD4+ T cells exhibited a similar pattern of reduction as CD8+ T cells from aged mice (data not shown).

To determine whether the stimulation with influenza virus was causing a depletion of CD8+ T cells in aged mice, we compared the ratios in virus-stimulated and unstimulated cultures on day 6. The data showed that the reduction in CD8+ T cell ratio was similar in unstimulated and influenza-stimulated cells from aged mice (data not shown). Since the frequency of CD8+ T cells in uncultured fresh spleen cells were similar in aged and young mice, the present results suggest that the reduction in CD8+ T cells from aged mice was not due to increased activation-induced programmed cell death. Rather, since aged cells were not proliferating in response to antigen stimulation, they probably lacked the capacity to survive well in culture and therefore exhibit increased spontaneous apoptosis.

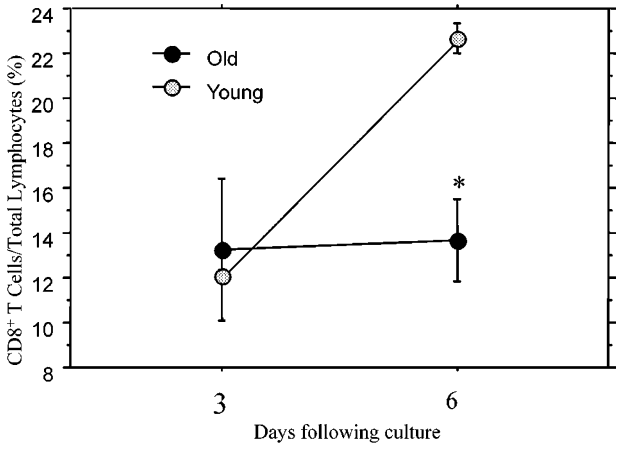

Figure 2 Changes in CD8+ T cell/total lymphocyte ratios in response to influenza virus stimulation over time. Cells were stimulated with influenza virus and harvested on the 3rd and 6th day of culture. The frequency of CD8+ T cells was determined by flow cytometry. Values for mean ± S.E.M. for CD8+ T cell/lymphocyte ratios for old and young mice are shown (six mice per group). *Value for old mice was significantly lower than young mice on day 6 ($P < 0.01$)

Increased apoptosis in CD8+ T cells from old mice

Annexin V binding to membrane phospholipid phosphatidylserine (PS) on the outer cell membrane was used to measure cells undergoing early stages of apoptosis. CD8+ T cells from old and young mice were stained with anti-CD8-PE and Annexin V-FITC reagents and analyzed by flow cytometry. The level of Annexin V+/CD8+ T cells in freshly isolated spleen cells were negligible in both old and young mice (data not shown). However, as shown in Figure 3, Annexin V+/CD8+ T cell frequency increased significantly in the aged mice from day 3 to day 6 when compared to the young mice. This suggests that apoptosis in CD8+ T cells from aged mice contributed to the reduction in the frequency of CD8+ T cells in total lymphocytes. Since the increase in Annexin V positive CD8+ T cells in aged mice was similar in both influenza-stimulated and un-stimulated cells (Figure 4), the present results suggest that excess apoptosis must be a common phenomenon during the aging process.

Mediators of apoptosis

Cross linking of Fas and FasL induces cell death.[63–65] TNF-α and TNF receptors (TNFR) also play very important roles in apoptosis. The cytoplasmic domain of TNFR1 (p55) contains a death domain (DD), and mediates signals that induce T cell activation by inducing NF-κB as well as signals for apoptosis.[66] The TNFRII (p75) does not contain a DD, but

Figure 3 Increased apoptosis in CD8+ T cells from aged mice following influenza virus stimulation *in vitro*. Spleen cells from mice primed with influenza A/Taiwan/1/86 (H1N1) virus were stimulated with influenza A/Beijing/353/89 (H3N2) virus. On days 3 and 6, virus-induced CTL were depleted of dead cells and stained for CD8 antigen and Annexin V. The frequency of Annexin V+/CD8+ T cells was assessed by flow cytometry. Values are for mean ± S.E.M. for per cent positive cells (six individual mice per group). *Values were significantly higher in aged mice in comparison to young mice ($P < 0.05$)

has been suggested to act by concentrating the ligand and then transferring it to TNFRI which then mediates the apoptosis.[67] In this study, intracellular Fas and TNF-α expression and cell surface FasL and TNFRI (p55) expression were determined by flow cytometry 6 days following influenza virus stimulation. In addition, TNF-α and TNFRII (p75) mRNA expression in purified influenza virus-specific CD8+ T cells was measured using RT–PCR. ELISA was used to measure TNF-α protein secreted in CTL culture supernatants on days 3 and 6. The results show that both Fas and FasL were significantly elevated in old mice when compared to the young mice (Figure 5A). This suggests that Fas and FasL partook in the acceleration of apoptosis of virus-specific CD8+ T cells in aging. CD8+ T cells from old mice exhibited markedly higher level of intracellular TNF-α as well as TNFRI when compared to the young mice (Figure 5B). Figure 6 shows that the frequency of influenza virus-specific CD8+ T cells expressing TNFRI was significantly increased from day 3 to day 6 in old mice but progressively decreased in young mice from day 3 to day 6. The levels of CD8+ T cells expressing Fas, FasL, TNF-α or TNFRI in freshly isolated spleen cells were very low, similar in old and young mice and significantly lower than cultured cells (data not shown). The ELISA results (Figure 7) show that the levels of TNF-α protein were identical in cultures from aged and young on day 3 but decreased to a much lower level in young mice than in aged mice on day 6. In addition, the RT–PCR results show that CD8+ T cells from aged mice had a significantly higher expression of TNF-α-specific mRNA than young mice (Figure

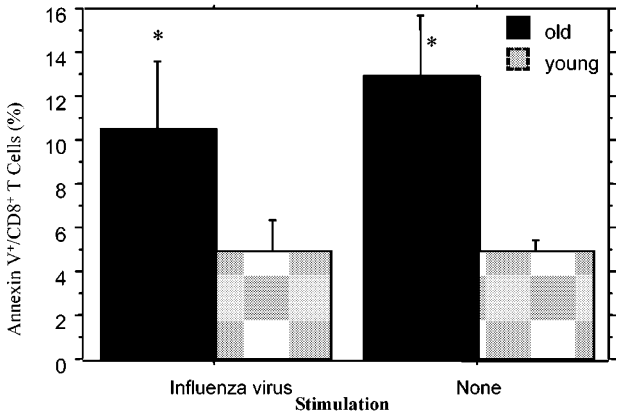

Figure 4 Effect of influenza virus stimulation *in vitro* on Annexin V+/CD8+ T cell ratios. Spleen cells from primed mice were stimulated with influenza A/Beijing/353/89 virus or not for 6 days. Values representing mean ± S.E.M. of per cent positive cells for six mice per group are shown. *Significantly higher in old *versus* young mice ($P<0.01$)

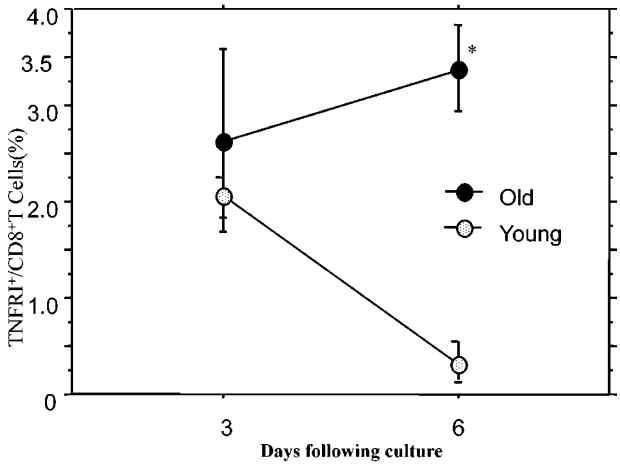

Figure 6 Increasing TNFRI+/CD8+ T cells in influenza-stimulated cultures in aged mice. Spleen cells were obtained from six aged and young mice each and stimulated with influenza virus individually. Cells were harvested on the 3rd and 6th day of culture and the expression of TNFRI on CD8+ T cells measured by dual color flow cytometry. Data are mean ± S.E.M. of per cent positive cells for six mice per group. *Significantly higher in aged mice when compared to young mice ($P<0.001$)

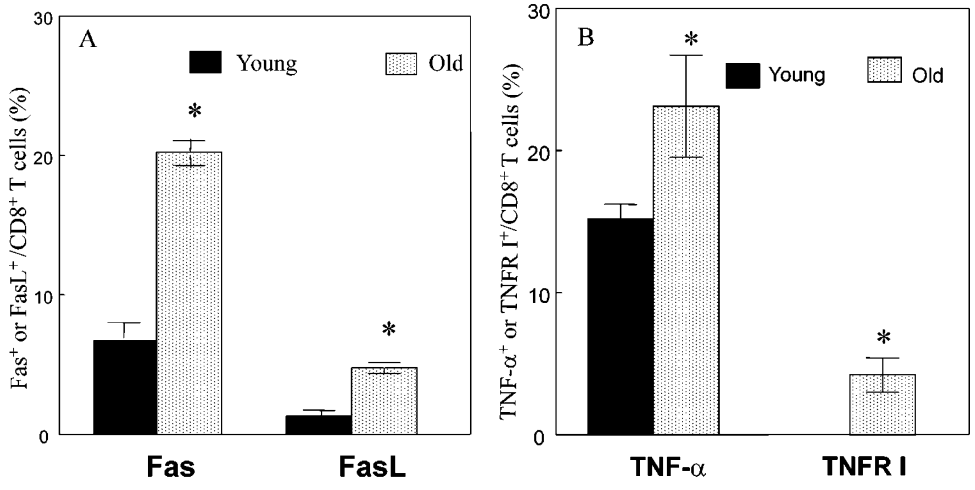

Figure 5 Enhanced expression of cell death mediator molecules in aged mice. Influenza virus-stimulated CTL cultures were harvested on day 6 and the expression of Fas and FasL (**A**) and TNF-a and TNFRI (p55) molecules (**B**) on CD8+ T cells were measured using flow cytometry. *Significantly higher in old than in young mice ($P<0.05$–0.01)

8). TNFRII mRNA expression varied from mouse to mouse and was generally similar among the few aged and young mice tested.

Effects of rIL-12 treatment on CTL responses, apoptosis and apoptosis mediators

We showed previously that IL-12 enhanced influenza virus-specific memory CD8+ CTL activity in humans[24,55] and recently that IL-12 with or without IL-18 augmented CTL responses in aged mice.[57,68] Other studies have demonstrated the ability of IL-12 to inhibit apoptosis and increase T cell functions.[69–72] Therefore, the ability of IL-12 to inhibit apoptosis and reverse CTL functions was evaluated as a means to understand the mechanism and effects of apoptosis on CTL functions in aged mice. Before proceeding, the optimal dose of IL-12 to be utilized was determined in young mice. Splenic lymphocytes from two young mice were stimulated with influenza A virus and treated with 0.2, 1, 5 or 25 μg/ml of murine rIL-12 at initiation of culture. Figure 9 shows that the frequency of CD8+ T cells (A) was slightly increased by IL-12 treatment in a dose-dependent manner (A) ($P<0.0001$). Based on above results, influenza virus-specific CTL was generated using splenocytes from influenza-primed old and young mice in the presence or absence of 25 μg/ml of rIL-12 (optimal dose). Six days later, the CTL were harvested and analyzed. IL-12 treatment slightly increased the frequency of Fas+/CD8+ and FasL+/CD8+ T cells in young mice while decreasing them in aged mice (data not shown). However, IL-12 treatment had no effect on apoptosis (Annexin V binding) (B) and TNF-α protein production (data not shown). In contrast, both IFN-γ (C) production and CTL responses (D) were significantly augmented by IL-12 treatment in both old and young mice.

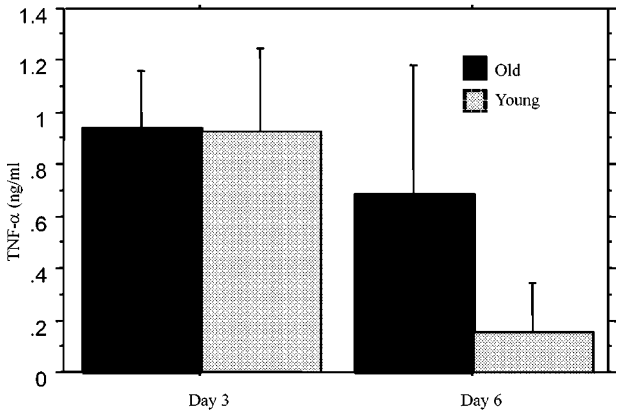

Figure 7 Effect of age on the TNF-α protein production in CTL culture. Influenza virus-induced CTL culture supernatants were harvested on days 3 and 6 and the level of TNF-α measured by ELISA. Data are mean$\pm$S.E.M. ng/ml) for four mice per group

Discussion

Our previous data had shown that influenza virus-specific CD8+ CTL functional activity was profoundly compromized in aged mice when compared to young mice as indicated by diminished target cell lysis and IFN-γ production.[16,17,57] In the present study, the frequency of memory CD8+ T cells following *in vitro* influenza virus stimulation was demonstrated to be significantly lower in aged mice in comparison to young mice. In addition, influenza virus-specific CD8+ T cells from old mice exhibited significantly increased apoptosis as indicated by elevated Annexin V binding and increased expression of the cell death signaling molecules, Fas, FasL, TFN-α and TNFRI as determined by flow cytometric analysis. Up-regulated production of TNF-α protein by CD8+ T cells from old mice was further confirmed by ELSA and at the mRNA level by RT–PCR. These results are in agreement with a majority of studies showing increased apoptosis in aging.[11,32,34,46,47,50,73–75] Several of the above studies have evaluated apoptosis induction after polyclonal mitogen stimulation with anti-CD3 monoclonal antibody, Fas-FasL or TNF-α-TNFαR cross-linking with antibody.[32,34,35,46,75,76] It has

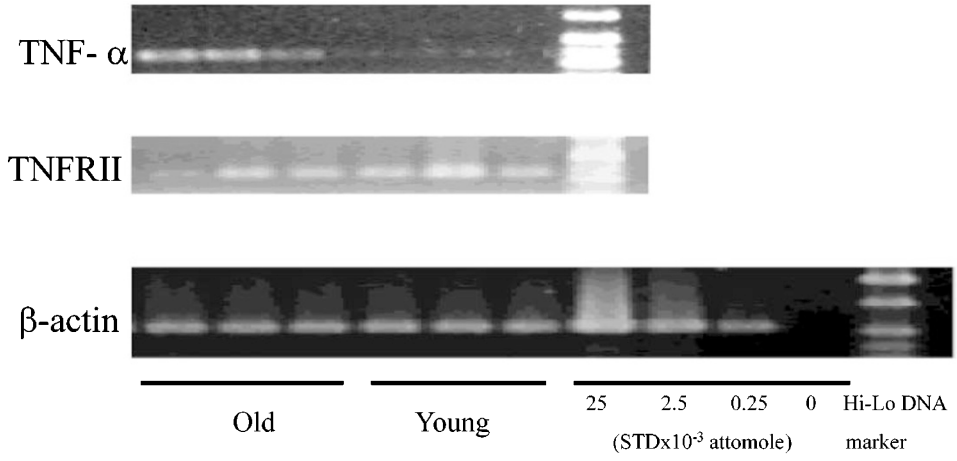

Figure 8 TNF-α and TNFRII (p75) mRNA expression in CD8+ T cells from aged and young mice. Total RNA was isolated from purified CD8+ T cells induced by influenza A virus stimulation (day 6). Following reverse transcription, the cDNA was normalized for β-actin content and TNF-α- and TNFR-p75-specific primers were amplified using PCR. Products were separated using agarose gel electrophoresis. Data for three mice per group are shown

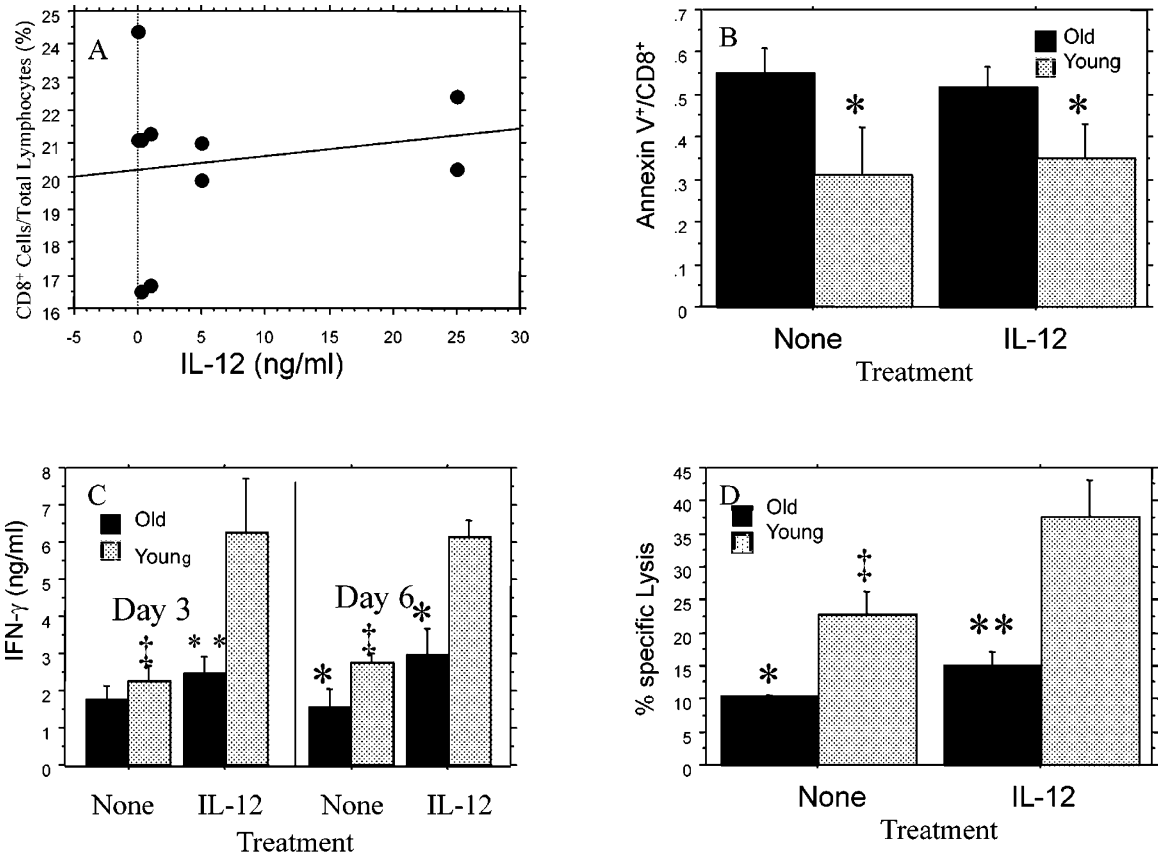

Figure 9 Effects of IL-12 on apoptosis, IFN-γ production and CD8$^+$ CTL responses. Splenocytes from two young mice previously primed with influenza virus were stimulated with virus with varying concentrations of mouse rIL-12 for 6 days. The frequency of CD8$^+$ T cells was determined by flow cytometry (**A**). CTL was then generated using splenocytes from influenza-primed aged and young mice in the presence or absence of 25 ng/ml of rIL-12. The frequency of Annexin V$^+$/CD8$^+$ T cells was determined by flow cytometry (**B**). Supernatants harvested on days 3 and 6 were measured for levels of IFN-γ using ELISA (**C**). CD8$^+$ T cells were purified using Automacs and tested for lysis of influenza A/Beijing-infected P815 target cells in a 4-h ^{51}Cr release assay (**D**).[57,98] Values are mean $\pm$ S.E.M. for per cent Annexin V/CD8$^+$ T cells, ng/ml (IFN-γ) and per cent specific lysis for six mice per group from two separate experiments (**B**–**D**). Asterisk denotes level of significant differences between old and young mice (*$P<0.05$; **$P<0.001$); ‡significantly higher in IL-12-treated *versus* untreated groups ($P<0.05$–0.01)

also been shown that virus infections induce apoptosis in T cells by AICD.[5–8,11,77–79] Virus-induced apoptosis is necessary for elimination of excess activated T cells and for generation of memory cells.[5,6,8,11,77–80] The present study, however, represents an initial evaluation of virus-induced apoptosis in the context of aging and demonstration of elevated apoptosis in influenza virus-specific CD8$^+$ T cells in aged mice. The apoptosis seen in this study is not caused by direct infection of CD8$^+$ T cells by virus since the mice were primed with influenza 2–3 months earlier. It is most likely due to activation induced programmed cell death (AICD) because the CTL were induced with influenza-infected autologous lymphocytes in the context of MHC class I molecules. Since CD8$^+$ T cells from aged mice and humans are more susceptible to normal apoptosis and influenza virus-induced CD8$^+$ T cells also exhibit elevated apoptosis, it is not surprising that CD8$^+$ T cells from aged mice will display functional abnormalities when compared to young mice. This includes reduced MHC class I-restricted CTL responses and diminished Th1 cytokine (IFN-γ) production. Recent studies have shown that subsets of human T cells undergo extensive apoptosis after exposure to influenza virus.[81] Also, influenza virus infection causes profound apoptosis in both CD8$^+$ and CD4$^+$ T cells in mice.[1,2,82] These results suggest that apoptosis caused by influenza virus infection and AICD induced by virus antigen activation in CD8$^+$ T cells constitutes a major burden and likely to result in the CD8$^+$ CTL deficiency observed in aged mice.[14–17,31,57] In elderly persons, the deleterious effects of apoptosis may be even more profound as influenza epidemics occur annually. This may explain the severely reduced CD8$^+$ CTL responses in this population.[24,27,29,30,83] Increased susceptibility of the elderly to excess influenza morbidity and mortality has been attributed to diminished virus-specific CD8$^+$ CTL responses.[24,26–28,37,84–87] Therefore strategies that reduce apoptosis may also reduce depletion of CD8$^+$ T cells in aging and enhance the overall CTL response.

Increased expression of death signaling molecules, such as TFN-α and TNFR as well as Fas and FasL, have been reported as described here.[32,39,43,47,50,74,76] Cross-linking of TFN-α with TNFRI or Fas with FasL will result in increased apoptosis and may cause exaggerated depletion of

activated virus-specific CD8+ CTL and consequently lead to reduced functional activity. Reduced frequency of virus-specific CD8+ T cells in aged mice as observed here is consistent with such a tenet. Alternatively, apoptotic CD8+ T cells may fail to respond to virus antigen stimulation. In fact, the data shown in Figure 2 shows that while the frequency of CD8+ T cells from young mice was increasing with time, those from aged mice remained unchanged and unresponsive to influenza stimulation. In addition, CD8+ T cells from both old and young mice showed increases in Annexin V binding; the rate of increase in aged mice was significantly higher. Since those cells exhibiting the characteristics of early apoptosis (Annexin V binding) will go on to die, the net effect is a disproportionately increased depletion of activated CD8+ T cells from aged mice in comparison to young mice. It is interesting to note that the cell surface expression of TNFRI increased with time in CD8+ T cells from aged mice while decreasing in young mice, consistent with increased apoptosis. TNFRII mRNA was not affected by aging and may therefore not play a major role in age-related apoptosis observed in the present study. Future studies will evaluate the level of apoptosis in different subsets of CD4+ and CD8+ T cells, including CD44, CD62L and CD44RB and correlate it with functions. This is important because apoptosis may also differentially affect different subpopulations of T cells since aged mice and elderly human populations contain different subsets of T cells when compared to the young.[40–44] In fact, in preliminary studies in humans, we found the CD45RO+/CD8+ T cells from elderly persons exhibited significantly higher levels of apoptosis than those from young persons (unpublished results). The effects of apoptosis on the generation of antigen-specific CD8+ T cells will be conducted using the tetramer assay which accurately quantifies MHC class I-peptide specific T cells. It is also essential to conduct these studies *in vivo* to determine the relevance of the present *in vitro* findings.

One of the strategies to reverse CTL deficiency in aging could be to inhibit apoptosis using exogenous IL-12 as shown in HIV AIDS patients.[73,88,89] In a similar study, IL-12 antagonism was shown to enhance T cell apoptosis, inhibit Th1 immune responses and promote allograft survival in hepatic transplant studies in mice.[90] The results presented here, however, show that while IL-12 significantly increased CTL responses and IFN-γ production in CD8+ T cells, it failed to reduce apoptosis and expression of cell death signaling molecules. It is possible that Annexin V binding assay, which measures the early stages of apoptosis, was not sensitive enough to detect the effects of IL-12 treatment in the present study. Studies to evaluate the effects of IL-7 and IL-15, alone or in combination with IL-12, on age-related apoptosis and CTL function, are in progress. This is based on recent findings that IL-7 inhibited and reversed age-related increase in apoptosis in mice[91] while IL-15 was shown to enhance the survival of CD56+ (NK cells), CD4+ and CD8+ T cells of HIV+ individuals.[92,93] Although not evaluated in the present study, Bcl-2 and Bcl-X_L expression is expected to be decreased in CD8+ T cells from aged mice relative to young mice.[11,32,46,76,78] Thus, assessment of the effects of these various cytokines an the expression of apoptosis mediators and inhibitors will provide insight into the mechanisms of apoptosis in aging and its effect on virus-specific CD8+ CTL functions.

In conclusion, the present results showed that in addition to functional CTL deficiency, the frequency of influenza A virus-specific CD8+ T cells was profoundly decreased in old mice than in young mice and correlated with increased apoptosis. The concurrent increases in the expression of cell death mediators, including Fas, FasL, TNF-α and TNFR-p55, in influenza virus-specific CD8+ T cells from old mice, suggest an important role for apoptosis in regulating age-related T cell immuno-senescence. Increased apoptosis is likely to contribute to decreased frequency of influenza virus-specific CD8+ T cells in old mice and to their declining CTL activity. Thus, immunotherapeutic strategies that reduce apoptosis, such as IL-12, IL-7, IL-15 treatment alone or in combination, may prove useful in enhancing virus-specific CD8+ responses and reducing the impact if viral infections in the elderly population.

Materials and Methods

Mice and influenza virus infection

Old (20–22 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*. The old and young mice were primed with a sublethal dose (0.05 LD_{50}) of influenza A/Taiwan/1/86 (H1N1) virus using small particle aerosolization as previously described.[17,94]

Generation and assay of influenza virus-specific CTL responses

Two to three months after priming, influenza A virus-specific CTL activity was generated by stimulating splenic lymphocytes with influenza A/Beijing/89 virus (H3N2)-infected autologous cells *in vitro* for 6 days as previously described.[17,57] When indicated, recombinant (r) murine IL-12 (rIL-12) (R & D Systems, Minneapolis, MN, USA) was added at the initiation of CTL culture at 0.2–25.0 ng/ml. The effector cells induced were then assayed for MHC class I-restricted CTL lysis of influenza A/Beijing/353/89 virus-infected P815 (H-2^d) target cells in a 4-h chromium release assay as previously described.[17,94–96]

Purification of T cell subpopulations

CD8+ T cells were purified using the magnetic affinity cell sorting method.[55] Briefly, effector cells (10^7) were incubated with 20 μl of magnetic CD8a (Ly-2) MicroBeads™ (Miltenyi Biotec, Auburn, CA, USA) for 30 min at 4°C and washed. After passing through a column placed in the magnetic field of an AutoMACS mini cell sorter (Miltenyi Biotec), purified CD8+ T cells were obtained by positive selection. CD8− cells (CD4+ T cells, B cells and macrophages) were also eluted. The frequency of CD8+ and CD4+ cells in each fraction was

determined by dual color flow cytometry (Beckman Coulter, Miami, FL, USA). The $CD8^+$ T cells isolated were $\geqslant$95% pure.[57,97]

T cell frequency and apoptosis determination using flow cytometry

Three and 6 days following stimulation with influenza virus, bulk effector CTL were depleted of dead cells using Lympholyte M (Cedarlane Laboratories, Hornby, Ontario Canada) gradient centrifugation. The cells were stained with a rat monoclonal antibody specific for mouse CD8 and CD4 conjugated to PE (or PerCP) and FITC, respectively. For detection of apoptosis, the cells were next stained with Annexin V-PE contained in an apoptosis detecting kit, according to the manufacture's instructions (BD Pharmingen, San Diego, CA, USA). Anti-TNFR-p55-FITC and ant-FasL-FITC monoclonal antibodies were combined with anti-CD8-PE reagent for detection of TNFR-p55 (TNFRI) and FasL expression on $CD8^+$ T cells. Rat anti mouse CD44 (Pgp-1)-PE was used to measure memory $CD8^+$ T cells. Stained cells were stored at 4°C in the dark and analyzed within 24 h using two- or three-color flow cytometry (Beckman Coulter, Miami, FL, USA). Similar analyses were conducted with freshly harvested, unstimulated spleen cells (day 0).

Intracellular cytokine flow cytometry (ICF) for detecting TNF-α and Fas

Cytotoxic T lymphocytes induced as above were washed in Permeabilizing Solution 2 (BD Pharmingen) for 10 min. They were then stained with anti-mouse CD8-FITC reagent followed by anti-mouse TNF-α-PE and anti-Fas-PE 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 using two-color flow cytometry.

ELISA for IFN-γ and TNF-α

Supernatants from influenza CTL cultures were harvested on days 3 and 6 and tested for secreted cytokines according to the sandwich ELISA method[57] using the Duoset® ELISA development kits (R&D Systems, Minneapolis, MN, USA). Each kit contained a capture antibody matched with a biotinylated detection antibody, a recombinant cytokine standard and streptavidin-HRP reagent. The assays were performed according to manufacturer recommendations.

Reverse transcription and polymerase chain reaction (RT–PCR) for TNF-α and TNFR-p75

Total cellular RNA was isolated from purified $CD8^+$ T cells using the Trizol Protocol according to manufacturer's specifications. mRNA was then purified using Oligotex® mRNA Purification kit (Qiagen, Valencia, CA, USA). First strand cDNA reverse transcription and cDNA amplification using gene specific primers were performed as previously described.[55,57] β-Actin was used as a positive control. The sense and anti-sense primer sequences used, respectively, were as follows: mβ-actin, 5′-GTG GGC CGC TCT AGG CAC CAA-3′ and 5′-CTC TTT GAT GTC ACG CAC GAT TTC-3′; mTNF-α: 5′-TTCTGTCTACTGAACTTCGGGGTGATCGGTCC-3′ and 5′ -GTAT-GAGATAGCAAATCGGCTGACGGTGTGGG-3′; mTNFRII (TNFR-p75): 5′-GCAAGCACAGATGCAGTCTG-3′ and 5′-GGTCAGAGCTGC-TACAGACG-3′.

PCR products were quantitated as previously described.[57] Briefly, serial 10-fold dilutions of standard DNA for mouse β-actin (Clontech Laboratories, Palo Alto, CA, USA) and the cDNA of target genes were amplified with appropriate primers for 30 cycles. The PCR products were then separated using gel electrophoresis, visualized using ethidium bromide and photographed using a digital camera (DC120, Eastman Kodak Company, Rochester, NY, USA). The pixel intensity of the band images and the quantity of each gene product were extrapolated from its standard curve.[57] β-Actin served as a positive control for each sample. Because standard DNA for TNF-α and TNFR-p75 were not available, the relative pixel intensity for each sample was standardized based on the β-actin content.

Analysis of data

Comparisons of differences between mean CTL lysis (for each E : T ratio), cell frequencies (per cent) and cytokine titers were made using the ANOVA procedure (STATVIEW Software, SAS Institute, Inc, Cary, NC, USA). Correlations between CTL and cell frequencies and apoptosis were tested using Spearman correlation analysis. A difference between comparison groups of $P<0.05$ level was considered significant.

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