An Aged Mouse Model for RSV Infection and Diminished CD8$^+$ CTL Responses[1]

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Recent studies indicate that respiratory syncytial virus (RSV), like influenza, causes significant morbidity and mortality among elderly persons. There are currently no animal models to study the effects of aging on RSV disease and immunity. This manuscript provides an initial description of such a model. Aged and young BALB/c mice (22–24 and 2–4 months, respectively) were infected with 10^4 $TCID_{50}$ of RSV A2. RSV was detected by culture in lung and nose wash specimens obtained 4–6 days following infection at a slightly higher titer in old mice in comparison with young mice. RT-PCR assay detected RSV in the lungs and nose washes of all mice on 4, 8, and 21 days postinoculation, with only a slightly less frequency in young mice. Splenic lymphocytes from old mice exhibited significantly lower RSV-specific MHC class I-restricted CD8$^+$ CTL responses ($P < 0.01$–0001), and reduced IFN-production ($P < 0.03$) than young mice. Conversely, IL-4 production was somewhat elevated in old mice. These results demonstrate diminished RSV virus-specific CD8$^+$ CTL responses and IFN-γ production in old mice in comparison with young. It is speculated that the deficient RSV-specific CTL responses may account for the increased morbidity and mortality from RSV infections in elderly persons. Although detailed histopathological, virological, and immunological analyses are incomplete at present, the old BALB/c RSV infection model described provides an opportunity to evaluate the role of CD8$^+$ CTL and cytokines in RSV disease in aging. [Exp Biol Med Vol. 227(2):133–140, 2002]

Key words: respiratory syncytial virus; CD8$^+$ CTL; IFN-γ; aging

Respiratory syncytial virus (RSV) is the major cause of lower respiratory tract infections in infants and young children. It is also the leading viral etiologic agent of bronchiolitis in infants and pneumonia in children less than 3 years old. Fifty percent of children become infected with RSV before the age of 1 and almost all are infected before the age of 5. However, not only the young are infected with this virus. Recent studies indicate that RSV is a major cause of morbidity and mortality among elderly persons (1–4). Indeed, it is second only to influenza as a cause of excess morbidity and mortality in persons greater than 65 years of age (5, 6). Animal studies have shown that antibodies to the surface attachment (G) and fusion (F) proteins are protective. Moreover, virus-specific serum neutralizing antibodies correlate with reduction in primary infection and reinfection in children (7). The elderly mount antibody responses comparable with that of young adults in both quantity and quality (8). These results and other studies (3) indicate that excess RSV disease in the elderly is not due to a significant defect in antibody immunity.

Cellular immune responses (CMI) are thought to play an important role in clearing RSV infection. CMI-deficient children shed RSV for prolonged periods (several months), whereas normal children shed for only 7–21 days (9). Immunosuppressed and transplant patients are also unable to clear virus, and this often leads to pneumonia and death (10–12). Elderly persons exhibit varying levels of cellular immunodeficiencies, and these may contribute to RSV disease in that population (13). A role for T cells in RSV immunity is supported by animal studies. Athymic nude mice and mice immunosuppressed by cyclophosphamide or γ-irradiation shed virus persistently and can be cured by adoptive transfer of immune T cells (14–16). Virus-specific cytotoxic T lymphocytes (CTL) promote clearance of RSV from the lungs of infected normal mice. However, they may also enhance acute and sometimes fatal pulmonary disease (15).

Cytokines are important in regulating immune responses to viruses. Studies in adult humans and children have suggested that naturally acquired RSV induces a Th1

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memory T cell response (17, 18). They also suggest that a predominant Th2-like cytokine response to RSV infection occurs in infants and children (17). This is in contrast to animal studies where primary RSV infection induces Th1 cytokines predominantly. It is thought that Th2 cytokines, particularly IL-4 and IL-5, mediate allergic airways sensitization by activation of virus-specific IgE, which in turn causes RSV-induced lung eosinophilia and airways hyperresponsiveness (AHR) (18, 19). Lung macrophages release inflammatory mediators, including IL-1, IL-6, IL- 10, and TNF-α that may be responsible for RSV-induced fever and malaise. In addition, T cells can produce pro-inflammatory mediators such as IFN-γ, Regulation upon Activation Normal T cell Expressed and Secreted (RANTES), and macrophage inflammatory protein-α (MIP-α) that can attract and activate mast cells, basophils, and eosinophils during RSV infection. A recent study found that IL-6 and IL-8 responses were more frequent and were found at higher levels in the plasma samples of more severely ill RSV-infected infants (20). These findings suggest that cytokines have important regulatory roles in RSV immunity and immunopathology.

Other studies, as well as our own, have shown that elderly persons exhibit significant deficiency in influenza virus-specific $CD8^+$ T cell response when compared with young persons (21–24). Aged mice are more susceptible to influenza virus infection and are also deficient in CTL response to influenza. In addition, they express less IFN-γ and more IL-4 (25–27). This had led to studies aimed at understanding the role of T cells and cytokines in influenza disease in aging.

There are currently no animal models to study the effects of aging on RSV disease. However, BALB/c mice have been extensively used to study RSV-induced pathogenesis and immune correlates to RSV infection (28, 29). Thus, adult (>10 weeks old) and aged (22–24 months old) BALB/c ($H\text{-}2^d$) mice were selected for use in these studies, whose major goal was to define the effect of aging on RSV infection in mice. The studies also aimed to evaluate the role of $CD8^+$ CTL response and the characteristics of Th1 and Th2 cytokine responses to RSV infection in old mice because it has been hypothesized that old mice may be more susceptible to RSV infection and disease than young mice because they are deficient in RSV-specific $CD8^+$ CTL response. The key Th1 and Th2 cytokine responses selected for study were FN-γ (Th1) and IL-4 (Th2).

Materials and Methods

Experimental Design. Aged and young mice were infected intranasally with 10^4 $TCID_{50}$ of RSV A2 (50 μl per animal). Lung and nose wash specimens were obtained for virus titration 4, 6, 8, and 21 days postinoculation. Two lobes of each lung were transpleurally lavaged using 3 ml of modified Eagle's medium (MEM). The resulting lavage fluids were assayed for RSV levels as described above. The remaining three lobes were homogenized using a minibead beater (Biospec Products, Bartlesville, OK). These were used for RSV detection using the RT-PCR assay. Spleens were removed on Day 21 for CTL and cytokine assays. RSV infection was defined by the occurrence of isolation of RSV by tissue culture and/or detection of a 243-bp segment of the F1 subunit of the F gene of RSV AB by RT-PCR. The experiments were repeated three times.

Mice. Old (20–22 months) and young (2–4 months) BALB/c mice were purchased from Charles River Laboratories (Wilmington, MA) 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. Use of animals was approved by the Baylor Animal Protocol and Research Committee according to principles expressed in the National Institutes of Health, USPHS, *Guide for the Care and Use of Laboratory Animals.*

Tissue Culture. HEp-2 cells (CCL 23; American Type Culture Collection [ATCC], Rockville, MD) were used to grow and test for the presence of RSV. This cell line was originally obtained from the ATCC and was serially passaged in MEM supplemented with 10% fetal calf serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin sulfate, 2 mM L-glutamine, and 0.2% sodium bicarbonate whenever they became confluent. MEM supplemented with 2% FCS was used to maintain HEp-2 cell cultures and in all virus and antibody assays. P815 ($H\text{-}2^d$), a mouse mastocytoma line, and EL-4 ($H\text{-}2^b$), a mouse lymphoma line, were maintained using standard cell culture procedures.

Viruses. RSV A2, an A subtype RSV, was obtained from the ATCC (catalog no. VR1302). Challenge pools of this virus were prepared by infecting monolayers of HEp-2 cells. When the infected monolayers exhibited approximately 90% syncytia formation, the cells and medium from the monolayers were collected, pooled, and clarified by centrifugation (450*g*). The resulting supernatant fluids were passed through a 0.45 μm filter, portioned, and stored at −70°C.

RSV pools for CTL assays were prepared as previously described (30). Briefly, HEp-2 cells were grown to confluency and were infected with RSV Bernett (RSV A2) using a multiplicity of infection (MOI) of 1.0 in the presence of 100 g/ml Lipofectamine (Gibco/BRL, Bethesda MD). After 90 min, the inoculum was removed. (Lipofectamine is a polycationic liposome and has been shown to significantly enhance the ability to induce RSV-specific CTLs [30] by enhancing the expression of viral antigens on cells [I. Mbawuike, unpublished data]). RPMI-1640 medium supplemented with 1% FBS was added to the cells. The infected cells were cultured in a 37°C (5% CO_2) incubator for 3 days. At that time, the virus was harvested, stabilized with 0.5% BSA, and stored at −196°C in liquid nitrogen. Virus titers were determined by plaque assay using Hep-2 cells as previously described (31). Titers ranged from 1×10^7 to 2×10^8 plaque forming units (pfu)/ml. All virus stocks were determined to be free of bacteria and mycoplasma.

Collection of Lungs and Nose Washes. Mice were sacrificed using air saturated with CO_2. The lungs from each animal were removed intact with thoracic trachea, trimmed of detectable lymph nodes, and rinsed in sterile saline. Ten milliliters of 5% FCS-MEM was then injected into each lobe transpleurally with a syringe. After removing the lungs, each mouse was decapitated and the lower jaw was removed from each head. Nose washes were collected by pushing 1 ml of 5% FCS-MEM through each nostril and capturing effluent from the posterior opening. Lung fluids and nose washes were assessed for virus levels as described below.

Virus Quantification. Serial 0.5 $\log_{10}$ dilutions of each test sample in 2% FCS-MEM were added to microtiter plates containing 3×10^3 HEp-2 cells/well and were incubated at 36°C (5% CO_2). On Day 7, the wells were scored by visual inspection for the formation of syncytia. Endpoints were calculated using the method of Karber (32). The amount of virus present in each suspension was expressed as median tissue culture infectious dose ($TCID_{50}$/ml) for nose wash, or geometric mean virus titers (GMT; $\log_{10}$ $TCID_{50}$/g lung) for lung tissue. The minimum detectable virus concentration was 1.3 $\log_{10}$/g lung and 1.6 $\log_{10}$ per nose wash.

RT-PCR for RSV Detection and Identification. Four sets of oligonucleotide primers were designed to detect RSV A, RSV B, or both subtypes. Total RNA was extracted from Hep-2 grown RSV Bernettt (RSV A) using the Trizol technique. Recovered RNA was then extracted by the phenol/chloroform procedure. RT-PCR was performed in two steps using an RNA PCR kit from Perkin Elmer (Norwalk, CT) (27). Following cDNAs synthesis, the following primers were used to amplify target gene sequences by PCR: a) F: 5′-TTAACCAGCAAAGTGTTAGA3′ and R: 5′-TTTGTTATATAGGCATATCATTG-3′; b) F: 5′-GATGTTACGGTGGGGAGTCT-3′ and R: 5′-GTACACTGTAGTTAATCACA3′; c) F: 5′-GTCTTACAGCCGTGATTAGG-3′ and R: 5′-GGGCTTTCTTTGGTTACTTC-3′; and d) F: 5′-AATGCTAAGATGGGGAGTTC-3′, R: 5′-GAAATTGAGTTAATGACAGC-3. These primers, respectively, corresponded to a 243-bp fragment amplified from a segment of the F1 subunit of the F gene of RSV A and B (33, 34), a 334-bp fragment from subunit N of RSV A, a conserved 838-bp fragment from sequences of N and P genes of RSV A and B, and a 183-bp fragment from N sequence of RSV B (negative control) (35). The PCR products (including RSV A2 standards) were electrophoresed on a 1.5% agarose gel, stained with ethidium bromide, and photographed using a digital camera (DC120, Eastman Kodak, Rochester, NY). The PCR products of cDNA synthesized from RSV A2 RNA and amplified with the above primers detected the predicted 243-bp segment of the F1 subunit of the F gene of RSV A, conserved 838-bp sequence of N and P genes of RSV A and B, and 334-bp subunit N of RSV A (data not shown). The 183-bp sequence of N of RSV B did not produce a product from RSV A2 sample indicating specificity. Negative control and blank samples did not produce any bands for the two primers tested.

Detection of RSV in Experimental Specimen by RT-PCR. Total RNA was extracted from nose wash specimen and lung homogenate after pelleting at 10,000*g* using the Trizol technique. Recovered RNA was then precipitated and washed as previously described (27). Following reverse transcription, a 243-bp of the F1 subunit of the F gene was amplified from the cDNA using specific primers as described above. Subtyping and identification of RSV were performed for some specimens as described above.

RSV CTL Induction and Assay. Splenocytes were infected with RSV A2 in the presence of 100 g of Lipofectamine for 3 hr to prepare stimulator cells as previously described (30). After washing, stimulator cells were cocultured with autologous responder cells at a ratio of 1:10 in a 37°C (5% CO_2) incubator for 6 days. Target cells were prepared by infecting P815 ($H\text{-}2^d$) or EL-4 ($H\text{-}2^b$) cell line with RSV Bernett as above and cultured for 3 hr. At that time, the cells were washed, incubated overnight, and then labeled with 100 Ci of ^{51}Cr (Na_2CrO_4, specific activity, 400–1200 Ci/mM, Dupont/NEN Research Products, Boston, MA). The labeled cells were incubated at 37°C with rocking for an additional 6 hr. After washing, they were mixed with syngeneic and allogeneic target cells infected with RSV A2 or influenza A/Hong Kong/68 (H3) virus. ^{51}Cr release was determined in a 4-hr ^{51}chromium release assay (30, 36, 37). The percentage of specific lysis was calculated from the following formula:

$$[(\text{experimental release} - \text{spontaneous release})/(\text{maximal release} - \text{spontaneous release})] \times 100$$

Spontaneous release was obtained by incubating target cells with medium alone, and maximal release was obtained by lysis of target cells with 1% Triton X-100 (30). Virus specificity and MHC class I restriction of CTL response were determined by including target cells infected with influenza A/Hong Kong/68 virus and EL-4 target cells ($H\text{-}2^d$) infected with RSV A2, respectively.

Depletion and Purification of T Cell Subpopulation. $CD8^+$ T cells were purified using the magnetic affinity cell sorting method (38). Briefly, effector cells (10^7) were incubated with 20 μl of magnetic CD8a (Ly-2) MicroBeads™ (Miltenyi Biotec, Auburn, CA) 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). The $CD8^+$ T cells isolated were ≥95% pure (21, 30).

ELISA for IFN-γ and IL-4. Supernatants from RSV CTL cultures harvested on Days 4 and 6 were tested for secreted cytokines using the sandwich ELISA method and

Duoset ELISA development kits (R&D Systems, Minneapolis, MN). 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's recommendations. After incubation with peroxidase-conjugated streptavidin, the assay was developed with ABTS substrate kit for horseradish peroxidase (Zymed Laboratories, South San Francisco, CA). The absorbance of the color in each well was read at a wavelength of 415 nm using an automatic microplate reader (Molecular Devices, Sunnyvale, CA). The data was collected in a SOFTmax data reduction software (Molecular Devices). Murine recombinant IFN-γ and IL-4 were used to generate standard curves. The amount of cytokine in the test samples was extrapolated from the standard curves and was expressed as nanograms per milliliter of cytokine (22, 38).

T Cell Frequency and Apoptosis Determination Using Flow Cytometry. Six days following stimulation with RSV, 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 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 manufacturer's instructions (BD PharMingen, San Diego, CA). Stained cells were stored at 4°C in the dark and analyzed within 24 hr using three-color flow cytometry (Beckman Coulter).

Analysis of Data. The Mann Whitney nonparametric analysis was used to compare geometric mean titers and $TCID_{50}$ of virus in different groups. Lung fluid and nose wash samples with undetectable virus titers were assigned a value of 0.8 or 1.1, respectively, for statistical evaluation. Differences in the frequency of infections were evaluated by the chi-square test. Comparisons of differences between mean CTL lysis (for each E:T ratio) and cytokine titers were made by a two-tailed unpaired *t* test procedure. These tests were performed using STATVIEW Software (SAS Institute, Cary, NC). A difference between comparison groups of $P < 0.05$ level was considered significant.

Results

RSV-infectivity. Virus was detected in every lung and nose wash collected on Day 4 and day 6 after virus inoculation, regardless of the age of the mouse. Virus titers were similar on both days in the respective age groups and were, therefore, combined for analysis. Mean lung virus titers were only slightly higher among old mice than young mice ($P = 0.07$; Fig. 1). In contrast, the levels of virus in the nose washes of old and young mice were equivalent. Viable virus was detected in only one young mouse on Day 8 and in none of the aged mice. The RT-PCR assay detected RSV in lung tissue specimen from all mice, regardless of their age, on Days 4 (or 6), 8, and 21 postinoculation (data not shown). RSV was present in most nose washes with a slightly lower frequency in young mice where only one of four mice expressed RSV on Day 8. RSV in selected Day 4 specimens was identified as RSV A by RT-PCR using RSV A N-specific primers (data not shown).

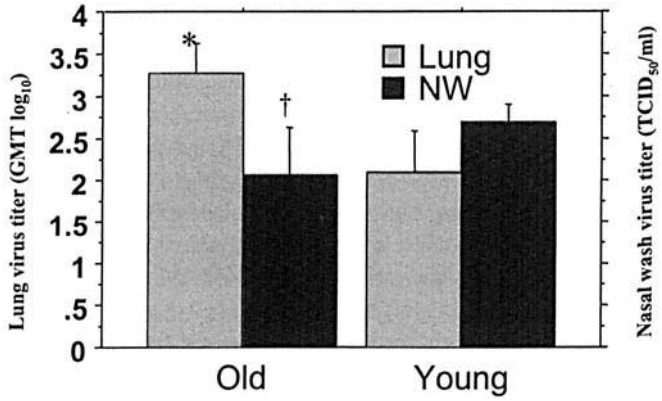

Figure 1. Comparison of RSV titers in lungs and nose washes of experimentally infected aged and young BALB/c mice. Lungs and nose washes were obtained from RSV A2-infected mice. Virus was titrated on Hep-2 cells. Values are GMT log_{10}/g of lung and $TCID_{50}$/ml of nose wash performed in three separate experiments (10–12 mice per group). An asterisk indicates that lung virus titers were significantly higher than nose wash virus in old mice ($P < 0.01$); † signifies that lung virus titers were higher in old than young mice ($P < 0.07$).

Diminished RSV-Specific CTL Response In Old Mice. Twenty-one days following RSV infection, splenic lymphocytes were stimulated with RSV A2 for 6 days. The effector cells induced were separated into $CD8^+$ T cells and $CD8^-$ ($CD4^+$, B cells, and macrophages) using AutoMacs. The $CD8^+$ purified fraction was comprised of >95% $CD8^+$ T cells and <2% of $CD4^+$ cells. The $CD8^-$ fraction was comprised of <2% $CD8^+$ cells and >80% of $CD4^+$ cells (FACS data not shown). Figure 2 shows the CTL activity against P815 (H-2^d) target cells infected with RSV A2. As the bars in this figure indicate, significant $CD8^+$-mediated lytic activity (35–72%) was measured in purified $CD8^+$ and unseparated cells. However, significantly greater activity was seen in the $CD8^+$ fraction. In contrast, the $CD8^-$ fraction had minimal lytic activity against RSV-infected target cells. The levels of specific lysis by both the $CD8^+$ and the unseparated fractions were significantly lower in old mice when compared with mid and young mice ($P < 0.02$-001). Control cells not stimulated with RSV or stimulated with influenza A/HK/68 exhibited minimal lysis in both old and young mice (data not shown).

$CD8^+$ CTL Activity Is RSV-Specific And MHC Class I-Restricted. To assess virus-specificity, purified RSV-induced $CD8^+$ were tested for lysis of P815 (H-2^d) target cells infected with RSV or influenza A/HK/68 (H3N2). Figure 3 shows that the $CD8^+$ CTLs lysed P815 target cells infected with RSV, but exhibited minimal activity (i.e. <5%) against influenza virus-infected cells. The RSV-induced $CD8^+$ effector cells also did not lyse allogeneic EL-4 (H-2^b) target cells infected with RSV or influenza, indicative of MHC-class I restriction.

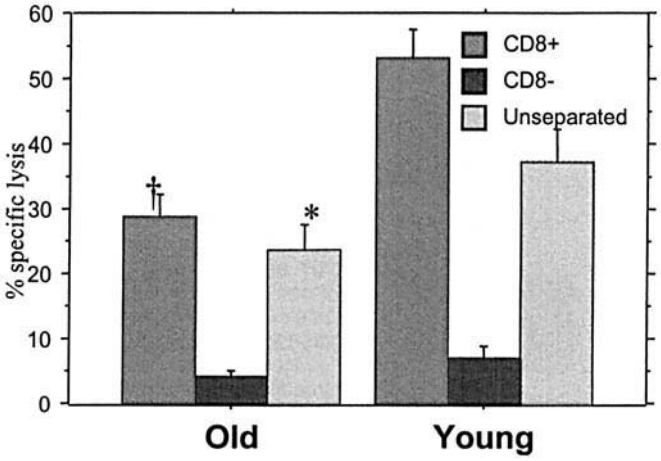

Figure 2. Comparison of RSV-specific CTL responses in experimentally infected aged and young mice. Splenic lymphocytes from RSV-infected mice were obtained on Day 21 and were stimulated with RSV A2 for 6 days. Unseparated $CD8^+$ and $CD8^-$ T cells were obtained from these suspensions and were tested for lysis of RSV-infected P815 ($H\text{-}2^d$) target cells in a 4-hr ^{51}chromium release assay using effector-to-target (E:T) ratios of 50:1, 25:1, and 12.5:1. Mean of the percentage of specific lysis ± SEM for 50:1 E:T ratio for 10–12 mice per group in three separate experiments are presented. An asterisk and † signify lower mean ^{51}Cr release in old mice when compared with young mice in $CD8^+$ ($P < 0.01$) and unseparated ($P < 0.0001$) fractions, respectively.

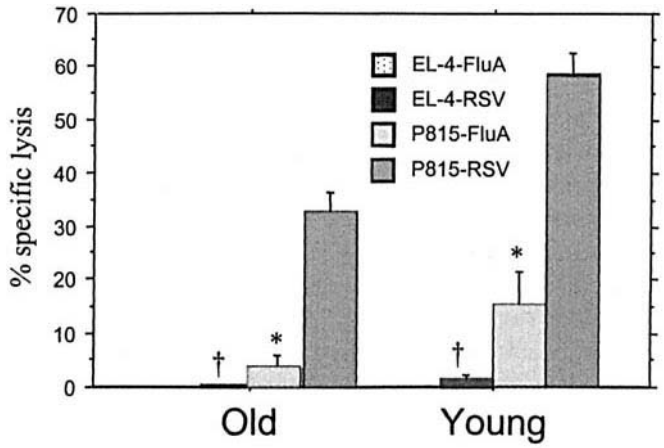

Figure 3. Specificity and MHC class I restriction of CTL response. RSV-induced $CD8^+$ CTL were assessed for lysis of P815 ($H\text{-}2^d$) and EL-4 ($H\text{-}2^b$) target cells infected with RSV A2 or influenza A/Beijing/89 (H3N2; FluA) virus. Data are for four mice per group. An asterisk signifies significant differences in the percentage of specific lysis for P815-RSV vs P815-FluA target cells ($P < 0.001$–0001). † signifies a significant difference in the percentage of specific lysis of P815-RSV vs EL-4-RSA target cells ($P < 0.0005$–0.0001). Lysis of EL-4-FluA target cells was not detectable.

Reduced T Cell Number In Aged Mice. $CD8^+$ and $CD4^+$ T cells were analyzed by flow cytometry to determine if there were age-associated alterations in these T cell subsets. The percentage of $CD8^+$ and $CD4^+$ T cells was similar among old and young mice prior to stimulation (Fig. 4, A and B). However, following stimulation with RSV, the percentage of $CD8^+$ and $CD4^+$ T cells decreased in aged mice, whereas they increased in young mice. The frequency of RSV-induced $CD8^+$ and $CD4^+$ T cells was markedly (though not significantly) higher in young when compared with old mice. Old mice exhibited much less increase in $CD8^+$ T cells (Fig. 4C) ($P = 0.019$) and $CD4^+$ T cells (Fig. 4D) than young mice. Further analysis showed that a higher frequency of $CD8^+$ and $CD4^+$ T cells from old mice exhibited Annexin V binding following RSV stimulation, an indication of increased apoptosis (Table I). These results suggest that $CD8^+$ and $CD4^+$ T cells from old mice were refractory to RSV stimulation or were depleted by activation-induced programmed death (apoptosis).

Th1 And Th2 Cytokine Responses. Day 6 RSV CTL culture supernatants from above were analyzed for levels of IFN-γ and IL-4 using ELISAs (Fig. 5). Similar but low levels of IFN-γ were produced in unstimulated lymphocyte culture of old and young mice (A). Following stimulation with RSV, significant amounts of IFN-γ were produced in lymphocyte cultures from both age groups, compared with unstimulated cultures ($P = 0.023$–0.003). However, IFN-γ levels in old mice were significantly lower when compared with young mice ($P = 0.027$). Similar levels of IL-4 were induced in unstimulated and RSV-stimulated culture (B). However, the levels of IL-4 were somewhat higher in old when compared with young mice ($P = 0.083$). These results are consistent with previous observation in the influenza virus system demonstrating reduced IFN-γ and increased IL-4 production in old mice when compared with young mice (25, 27).

Discussion

An aged BALB/c mouse model has been established for RSV infection. Intranasal challenge with 10^4 $TCID_{50}$ resulted in infection in 100% of aged (22–24 months) and young (2–4 months) mice. Aged mice were apparently more susceptible than young mice to RSV infection, as indicated by higher pulmonary virus titers early in infection (4–6 days). No differences were observed when the more sensitive RT-PCR assay was used. With RT-PCR assay, RSV was detected in the lungs and nose washes of most mice for up to 21 days. MHC class I-restricted $CD8^+$ CTL responses to RSV were significantly lower in old mice when compared with young mice. Furthermore, IFN-γ production was significantly reduced in old mice, whereas IL-4 was somewhat increased.

BALB/c is a good mouse model for evaluating the aging effects of RSV infection because the model appears to reproduce some features of human disease. Previous studies have shown younger BALB/c mice (<8 weeks old) are less susceptible to RSV infection than older (>8 weeks old) mice (28, 29). Involvement of host genetic factors has been indicated because BALB/c and other $H\text{-}2^d$ mice tend to have higher lung RSV titers than other strains of mice (39). The current data suggest that aged mice have a slightly higher lung virus load than younger mice. However, additional studies need to be conducted with higher doses of virus to determine if old mice are indeed more susceptible to RSV infection than young mice. Histopathological evaluation

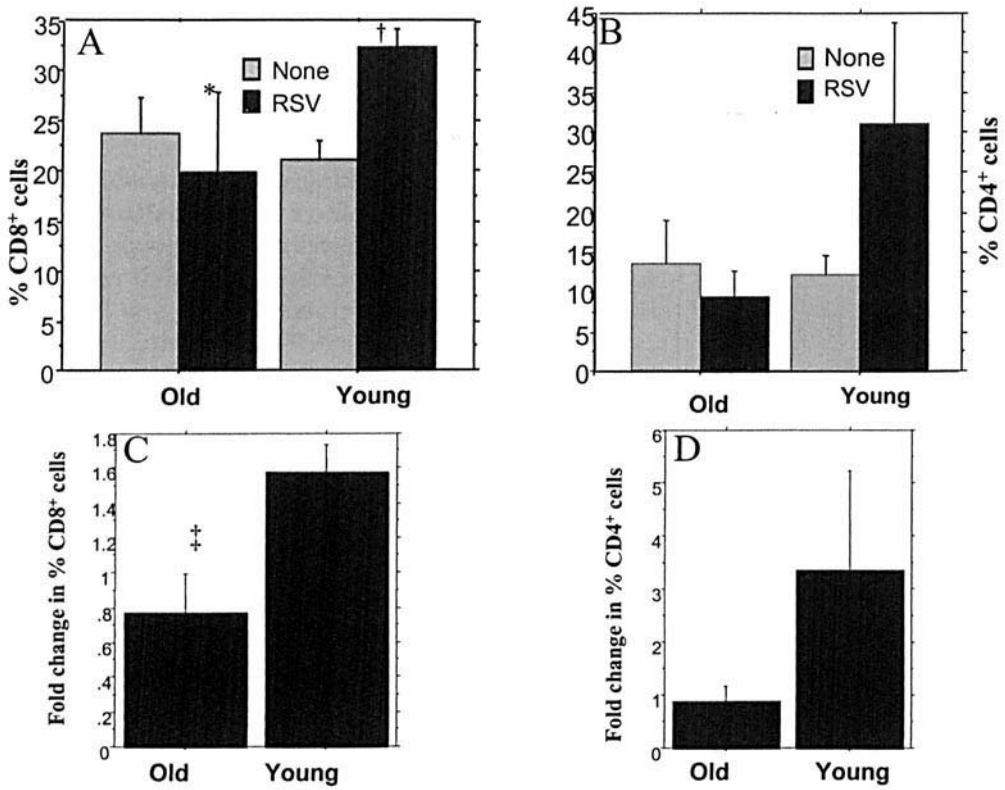

Figure 4. Comparison of $CD8^+$ and $CD4^+$ T cell frequencies in old and young mice. Unstimulated or RSV-stimulated lymphocytes were stained and analyzed by flow cytometry as described in "Materials and Methods." Values are mean ± SEM of the percentage of positive $CD8^+$ (A) and $CD4^+$ (B) T cells for four mice per group. Calculated fold changes in the T cell frequencies are shown in C and D. † signifies significant differences in the frequency of $CD8^+$ T cells in young mice in RSV-stimulated vs unstimulated ($P = 0.005$). An asterisk indicates that $CD8^+$ T cells from RSV-stimulated cultures were lower in old than young mice ($P = 0.133$). ‡, signifies a significant difference in the change in $CD8^+$ T cells in old vs young mice following RSV stimulation ($P = 0.0187$).

Table I. Annexin V Binding in $CD4^+$ and $CD8^+$ T Cells[a]

Age	Cell Fraction	Stimulation	
		None	RSV
Old	$CD4^+$	22.6 ± 1.9[b]	18.8 ± 3.4
	$CD8^+$	16.4 ± 1.4	16.7 ± 0.9
Young	$CD4^+$	11.2 ± 1.4	12.4 ± 3.8
	$CD8^+$	17.0 ± 1.8	12.1 ± 3.9

[a]Unstimulated (None) or RSV-stimulated lymphocytes (RSV) were stained and analyzed by flow cytometry as described in "Materials and Methods." Values for means ± SEM of the frequency (%) of $CD4^+$/Annexin V^+ and $CD8^+$/Annexin V^+ T cells for three to four mice per group are presented.
[b]$CD4^+$/Annexin V^+ T cells in unstimulated cultures were lower in old than young mice ($P < 0.005$).

may also help to determine if old mice display an increased RSV disease. Studies on CTL responses to RSV in humans are limited and no information is available on these responses in elderly persons. However, in a recent study, it was observed that the young adults (mean age of 34 ± 5 years) exhibited significantly higher levels of RSV CTL responses than the elderly persons (mean age years 71 ± 1 years; our unpublished data). These results and the present data suggest that RSV-specific $CD8^+$ CTL and IFN-γ response deficits may contribute to increased RSV morbidity and mortality seen in the elderly. It should be acknowledged, though, that the results obtained with the present primary RSV infection study in mice might differ from results in elderly persons who have experienced multiple episodes of RSV infection. The significance of the long persistence of RSV RNA seen the present studies is unclear. However, the persistence was the same for old and young mice. Future studies could look at just how long RSV RNA persists and if there are differences in young and aged mice. In addition, quantitative RT-PCR assay could be used to determine if there are differences in virus load.

Deficient MHC class I-restricted $CD8^+$ CTL response coupled with reduced IFN-γ production in RSV-infected aged mice suggest a Th1 or cytotoxic T lymphocyte type one (Tc1) deficiency. Clearly, a major consequence of such a deficiency would be a reduced ability to clear virus infection, possibly resulting in higher morbidity and mortality. Age-related $CD8^+$ CTL deficiency may result from lack of clonal expansion or depletion of activated cells by apoptosis. The results show that RSV stimulation caused an increase in the frequency of $CD8^+$ T cells and decreased apoptosis (Annexin V binding) in young mice. Even though RSV stimulation did not affect Annexin V binding in aged mice, it nonetheless caused a substantial decrease in $CD8^+$ CTL frequency. This indicates an overall net increase in apoptosis in old mice relative to young mice. It is possible that evaluating apoptosis prior to dead cell depletion might produce a clearer result. $CD8^+$ CTL responses have been shown in several studies to mediate virus clearance in RSV-infected mice (39–42). Ongoing studies will evaluate a direct role for $CD8^+$CTL in virus clearance and prevention of morbidity and mortality in old mice. It should be stated, though, that $CD8^+$ T cells have also been implicated in RSV immunopathology (43). Pulmonary histopathology was not assessed in the present studies. In contrast to IFN-γ, which

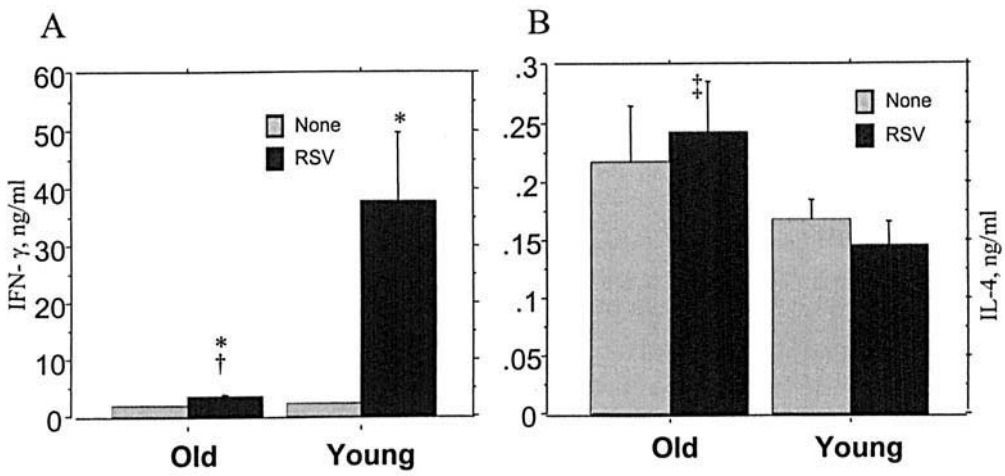

Figure 5. Comparison of IFN-γ and IL-4 responses to RSV infection in old and young mice. Lymphocytes were stimulated with RSV for 6 days as described above. The CTL cell culture supernatants were harvested on Day 6 and were tested for IFN-γ (A) or IL-4 (B) by ELISA. Data for mean levels of cytokine (ng/ml) ± SEM are presented for four mice per group. IFN-γ: An asterisk indicates significant differences between RSV-stimulated and unstimulated cultures in young mice (P = 0.023) or old mice (P = 0.003). A † indicates that IFN-γ levels were significantly lower in old than young mice in RSV-stimulated cultures (P = 0.027). ‡ signifies higher IL-4 levels in RSV-stimulated cultures from old compared with young mice (P = 0.083).

stimulates virus-specific $CD8^+$ CTL responses, IL-4 suppresses them. Recent studies have shown that immunization of mice with RSV M2-vaccinia construct expressing IL-4 resulted in significant down-regulation of RSV-specific $CD8^+$ CTL response as well as IFN-γ production by $CD8^+$ T cells (44, 45). Also, transgenic mice overexpressing IL-4 had diminished RSV-specific $CD8^+$ CTL response and exhibited delayed virus clearance (46). In the present study, it was observed that old mice produced an elevated IL-4 compared with young mice and this might have contributed to their reduced $CD8^+$ CTL response. RSV-induced $CD4^+$ T cells may play a role in virus clearance, but they are not thought to be the primary effector cells in RSV infections (47, 48). Reduced percentage of $CD4^+$ T cells in RSV-infected old mice could result in reduced antibody responses. However, available data suggest that the severe clinical manifestations of RSV disease in the elderly are not due to a significant defect in antibody responses (3, 8).

The present results are consistent with those for influenza virus and suggest that deficient RSV-specific CTL response and a Th1 (IFN-γ) to Th2 (IL-4) cytokine switch appear to occur in aging. The old BALB/c mouse model will permit us to perform systematic evaluation of the role of the different arms in immune response in control of RSV disease in aging. Results from such studies will provide insight into the mechanisms of increased RSV disease in elderly persons.

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