Th1 Cytokines Facilitate CD8-T-Cell-Mediated Early Resistance to Infection with *Mycobacterium tuberculosis* in Old Mice

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Numerous immunological defects begin to emerge as an individual ages, the consequence of which is heightened susceptibility to infectious diseases. Despite this decline in immune function, old mice display an early transient resistance to *Mycobacterium tuberculosis* infection in the lung, which is dependent on CD8 T cells and gamma interferon (IFN- γ) production. In this study, we investigated the mechanism of resistance by examining the CD8-T-cell phenotype and function in old naïve and *M. tuberculosis*-infected mice. Pulmonary CD8 T cells from naïve old mice expressed cell surface markers of memory in addition to receptors for several Th1 cytokines. Stimulation of lung cells from naïve old mice with a combination of Th1 cytokines (interleukin-2 [IL-2], IL-12, and IL-18) resulted in nonspecific production of IFN- γ by memory CD8 T cells. Following aerosol infection with *M. tuberculosis*, the lungs of old mice contained significantly more IL-12, IL-18, and IFN- γ than the lungs of young mice contained. Together, these data demonstrate that the increased and early production of Th1 cytokines in the lungs of *M. tuberculosis*-infected old mice, in combination with CD8 T cells that can nonspecifically produce IFN- γ , leads to transient control of *M. tuberculosis* growth in the lungs of old mice. Further characterization of this mechanism should provide essential information regarding the aging immune system and should contribute to the development of novel strategies to decrease the morbidity and mortality of the aging population associated with infectious diseases.

The most significant consequence of immunosenescence is enhanced susceptibility to, and increased mortality from, infectious diseases. Elderly individuals are particularly susceptible to respiratory diseases, including influenza, pneumonia, and tuberculosis (3, 10, 25, 32, 33, 41). In the United States, nearly 90% of influenza and pneumonia deaths (1) and 20% of all reported cases of tuberculosis (42) are in the elderly. Because of this increased susceptibility to infection, in combination with decreased vaccine efficacy (24, 28), poor antimicrobial drug tolerance (40), and a projected doubling of the elderly population by 2025 (18), a better understanding of immune responses in the elderly is essential. More importantly, identification of immune mechanisms that remain intact in old age could provide important information for the design of more efficacious vaccines and immunotherapies for the growing elderly population.

It is well established that generation of both CD4-T-cell-mediated immunity (6, 26, 29, 30) and Th1-cytokine-mediated immunity (8, 9, 14, 34) is critical for the control of *Mycobacterium tuberculosis* infections, and indeed, the increased susceptibility of old mice to infection with *M. tuberculosis* is associated with impaired CD4-T-cell-mediated immunity (27, 38). Interestingly, previous studies have clearly shown that old mice express a transient early resistance to low-dose aerosol infection with *M. tuberculosis* (7, 38), and in contrast to what occurs in young mice, in which the importance of CD8 T cells emerges during the chronic stage of disease (37), early control of *M. tuberculosis* in old mice has been associated with CD8 T cells. The proportion of CD8 T cells isolated from the lungs of old

mice (both before and soon after infection) that were capable of producing gamma interferon (IFN- γ) in response to T-cell receptor cross-linking was significantly higher than the proportion of CD8 T cells isolated from the lungs of young mice that were able to do this (38), and subsequent experiments showed that the novel control identified in old mice was dependent on both CD8 T cells (38) and IFN- γ (39). Additional characterization of this mechanism of early resistance in old mice should provide information regarding both the aging immune response to infection and the control of tuberculosis.

It has been reported that antigen-specific CD8-T-cell responses are impaired in the lungs of old mice (11). This finding makes it difficult to reconcile the role of CD8 T cells in the lungs of old mice during an infection with *M. tuberculosis*; however, Berg et al. have reported that a minor population of memory CD8 T cells from young mice can be induced to secrete IFN- γ in an antigen-independent manner when the cells are cultured with Th1 cytokines (4). In addition, memory CD8 T cells were shown to provide protection during an infection with *Listeria monocytogenes* in the absence of cognate antigen (5), demonstrating that a nonspecific stimulus was sufficient for generating a memory CD8-T-cell-mediated protective immune response. Based on the distinct ability of memory CD8 T cells to nonspecifically produce IFN- γ , coupled with the association between CD8 T cells and the expression of early resistance to *M. tuberculosis* (38, 39) (a potent inducer of Th1 immunity (13)), we hypothesized that the transient early resistance observed in old mice during *M. tuberculosis* infection could be induced by nonspecific stimulation of CD8 memory T cells in the aging lungs.

In this study we demonstrated that memory CD8 T cells from naïve old mice could be nonspecifically stimulated to produce IFN- γ in response to Th1 cytokines and that this

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response was significantly amplified compared to the response in young mice. In addition, we found that within 5 to 8 days of an aerosol challenge with *M. tuberculosis* the levels of mRNA expression and proteins of key Th1 cytokines were elevated in the lungs of old mice. Together, these data suggest that infection with *M. tuberculosis* drives a heightened Th1-type cytokine response in the lungs of old mice that results in IFN- γ production from bystander activated CD8 T cells. These early immune events in the lungs result in transient control of the bacterial burden. Targeting CD8 T cells with postexposure Th1 cytokine therapeutics may therefore be an effective strategy for boosting immunity in the elderly.

MATERIALS AND METHODS

Mice. Specific-pathogen-free female C57BL/6 or BALB/c mice were purchased from Harlan when they were 6 to 8 weeks old (Indianapolis, IN) or when they were 18 months old through a contract with the National Institute on Aging (supplied by Harlan). Mice were maintained with sterilized water and chow ad libitum and were acclimatized for at least 1 week prior to manipulation in microisolator cages located in either a standard vivarium for all noninfectious studies or in a biosafety level 3 facility for all studies using *M. tuberculosis*. Old mice were carefully examined at necropsy, and mice with visible tumors were excluded from the study. Procedures were approved by The Ohio State University Institutional Laboratory Animal Care and Use Committee.

Cell isolation and purification. Lungs were perfused through the pulmonary artery with phosphate-buffered saline containing 50 U/ml of heparin (Sigma; St. Louis, MO) and placed into Dulbecco's modified Eagle's medium (500 ml; Mediatech, Herndon, VA) supplemented with 10% heat-inactivated fetal bovine serum (Atlas Biologicals, Ft. Collins, CO), 1% HEPES buffer (1 M; Sigma), 1% L-glutamine (200 nM; Sigma), 10 ml of a 100 $\times$ MEM nonessential amino acid solution (Sigma), 5 ml of a penicillin/streptomycin solution (50,000 U penicillin and 50 mg streptomycin; Sigma), and 0.1% β -mercaptoethanol 2-hydroxyethyl-mercaptan (50 mM; Sigma). A single-cell suspension was obtained as described previously (38).

For CD8⁺ cell purification, pools of lung cells from five old or five young mice were overlaid onto HISTOPAQUE 1083 (Sigma) and centrifuged at 400 $\times$ g for 30 min without braking to obtain live mononuclear cells. Adherent cells were removed by incubation at 37°C with 5% CO₂ for 1 h. CD8⁺ cells were isolated using CD8a MicroBeads (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. Cells were passed over two columns to increase the purity of CD8⁺ cells, which was more than 94% for all samples.

Flow cytometry. Unless indicated otherwise, all analyses were performed with lung cells obtained from individual mice. Cell suspensions were adjusted to obtain a concentration of 1 $\times$ 10⁷ cells/ml in fluorescence-activated cell sorting buffer (deficient RPMI [Irvine Scientific, Santa Ana, CA] supplemented with 0.1% sodium azide [Sigma]) and incubated on ice for 30 min. All antibodies were obtained from BD Biosciences (San Jose, CA) unless indicated otherwise. A total of 1 $\times$ 10⁶ cells were incubated with 0.5 μ g of Fc Block for 5 min at 4°C. Cells were subsequently labeled with 0.5 μ g of specific fluorescently labeled or purified antibody for 30 min at 4°C in the dark, followed by two washes with fluorescence-activated cell sorting buffer. For secondary labeling of purified antibodies, cells were incubated with 0.5 μ g of phycoerythrin (PE)-conjugated anti-Armenian and Syrian hamster antibody or Alexa Fluor 488-conjugated anti-goat antibody (Molecular Probes, Eugene, OR) for 30 min at 4°C in the dark and washed twice. Samples were examined with a LSRII flow cytometer, and data were analyzed using the FACSDiva software (BD Biosciences). Lymphocytes were gated according to their forward and side scatter profiles, and CD8 T cells were identified by the presence of specific fluorescently labeled antibody to CD8 in combination with T-cell receptor β or CD3 ϵ . Isotype controls were included in each experiment (for both young and old lung cells) and were used to set gates for analysis. The cell surface markers and cytokines analyzed were purified CD119, interleukin-12R β 2 (IL-12R β 2), or IL-18R α (R&D Systems, Minneapolis, MN); IFN- γ -fluorescein isothiocyanate; CD45RB-PE; CD3 ϵ -peridinin chlorophyll protein-Cy5.5; CD44-allophycocyanin (APC), CD8a-APC, or TCR β -APC; CD8a-PE-Cy7 or IFN- γ -PE-Cy7; and CD8a-APC-Cy7. Intracellular staining for IFN- γ was performed using a Cytofix/Cytoperm kit according to the manufacturer's instructions (BD Biosciences).

In vitro cell culture. Single-cell suspensions from individual lungs were cultured at a concentration of 2.5 $\times$ 10⁶ cells/ml in 96-well tissue culture plates for

16 h in a humidified incubator at 37°C with 5% CO₂. Cells were cultured with either medium alone, anti-CD3 and anti-CD28 (1 μ g/ml and 0.1 μ g/ml, respectively; BD Biosciences), specific cytokines (100 ng/ml IL-2 [R&D Systems], 5 ng/ml IL-12 [PeproTech, Rocky Hill, NJ], 100 ng/ml IL-15 [PeproTech], or 10 ng/ml IL-18 [R&D Systems]), or combinations of cytokines. Monensin (3 μ M; BD Biosciences) was added for the final 4 h of incubation. Cultured cells were processed for flow cytometric analysis as described previously.

In vivo cell proliferation. Mice were inoculated intraperitoneally with 100 μ l bromodeoxyuridine (BrdU) (10 mg/ml) 14 h prior to tissue collection. Lung tissue was processed as described previously, and cells from individual mice were labeled with fluorescein isothiocyanate-conjugated anti-BrdU used according to the manufacturer's instructions (BD Biosciences).

ELISA. Whole-lung homogenates were collected 12 days postinfection and frozen at -80°C. Samples were thawed and clarified by centrifugation to remove tissue debris. The levels of IL-12p40 and IL-18 in supernatants were determined using OptEIA enzyme-linked immunosorbent assay (ELISA) sets according to the manufacturer's instructions (BD Biosciences).

Bacterial infections. *M. tuberculosis* Erdman (= ATCC 35801) was obtained from American Type Culture Collection (Manassas, VA) and grown in Proskauer-Beck liquid medium containing 0.05% Tween 80 to the mid-log phase. Bacterial suspensions were frozen in aliquots at -80°C. Mice were infected aerogenically with a low dose of *M. tuberculosis* Erdman (50 to 100 CFU), and CFU were enumerated as previously described (38). Data were expressed as log₁₀ mean number of CFU recovered per organ ($n = 4$ or 5).

Real-time PCR. Right middle lung lobes were homogenized in 1 ml of Ultra-Spec (Biotecx, Houston, TX) and frozen rapidly at -80°C. Total RNA was isolated by following the manufacturer's instructions, and 1 mg was reverse transcribed using an Omniscript RT kit (QIAGEN, Valencia, CA). Real-time PCR was performed with an ABI PRISM 7700 (Applied Biosystems, Foster City, CA), using TaqMan predeveloped assay reagents for 18S rRNA, IFN- γ , IL-12p40, and IL-2 (Applied Biosystems) and functionally validated QuantiTect reagents for 18S, IL-18, and IL-15 (QIAGEN). The Delta Delta cycle threshold method was used for relative quantification of mRNA expression; in this analysis 18S was used as an endogenous normalizer and naïve young mice were used as the calibrator (2).

Statistical analysis. Statistical significance was determined using the Prism 4 software (GraphPad Software, San Diego, CA). The unpaired two-tailed Student *t* test was used for comparisons of young and old mice.

RESULTS

Increased number of memory CD8 T cells in the lungs of naïve old mice. It has been reported previously that the number of memory T cells increases with age (21). To confirm that there were increased numbers of CD8 memory T cells in the lungs of naïve old mice, lung cells were isolated from young and old mice using gentle enzymatic digestion and were subjected to flow cytometric analysis. As anticipated, the proportion of resident CD8 T cells isolated from the lungs of naïve old mice that were CD44^{hi} CD45RB^{lo} was higher than the proportion of CD8 T cells isolated from the lungs of naïve young mice that were CD44^{hi} CD45RB^{lo} (Fig. 1A). The absolute numbers revealed that naïve old mice had significantly more resident CD44^{hi} CD45RB^{lo} CD8 T cells (Fig. 1B) in their lungs than their young counterparts had.

Pulmonary CD8 T cells from naïve old mice nonspecifically produce IFN- γ in response to Th1 cytokines. Antigen-independent secretion of IFN- γ , mediated by IL-12 and IL-18, has been found in naïve young mice by using a minor subset of splenic CD8 T cells that brightly express CD44 (4). Because of the substantial number of CD8 memory T cells present in the lungs of naïve old mice, we examined whether Th1 cytokines could similarly stimulate lung CD8 T cells from naïve old mice to nonspecifically produce IFN- γ . Cells were isolated from the lungs of individual naïve young or old mice and cultured overnight with medium alone, with anti-CD3/CD28, with IL-12, with IL-18, or with a combination of IL-12 and IL-18. IFN- γ -

FIG. 1. Old mice have more memory CD8 T cells in their lungs than young mice have. Single-cell suspensions from lungs of naïve young and old mice were stained with fluorescently conjugated antibodies against CD3, CD8, CD44, and CD45RB. Percentages were determined and expressed in representative dot plots (A), and they were used to calculate absolute numbers for each individual mouse (B). The data are the means $\pm$ standard errors for three or four mice and are representative of three independent experiments. The asterisks indicate that the *P* value is <0.0005 , as determined by Student's *t* test.

producing CD8 T cells were detected by intracellular flow cytometry. Anti-CD3/CD28 was included as a positive control in an independent well for each sample and, as anticipated, induced CD8 T cells from old mice to produce IFN- γ (Fig. 2A). Overnight culture with a combination of IL-12 and IL-18 resulted in a substantial increase in the proportion of CD8 T cells from the lungs of old mice that could produce IFN- γ (Fig. 2A). In contrast, only a minor proportion of CD8 T cells produced IFN- γ in response to culture with either IL-12 or IL-18 alone (Fig. 2A), demonstrating that synergy between these two cytokines was required.

It has also been reported that in young mice addition of IL-2 can enhance the IL-12- and IL-18-driven IFN- γ production by CD8⁺ CD44^{hi} T cells (4). Indeed, addition of IL-2 to lung cell cultures from young and old mice resulted in significant increases in the proportion of CD8 T cells that could produce IFN- γ (Fig. 2B and E). When the data were converted to

absolute numbers, it was evident that naïve old mice had significantly more CD8 T cells in their lungs that could secrete IFN- γ in response to IL-12, IL-18, and IL-2 than young mice had (Fig. 2D). Lung cells were also incubated with a combination of IL-2 and IL-12 or a combination of IL-2 and IL-18 (Fig. 2C). There was a moderate but significant increase in the proportion of CD8 T cells from the lungs of old mice that produced IFN- γ when they were incubated with either IL-2 and IL-12 or IL-2 and IL-18 (Fig. 2C).

It has been reported previously that CD4 T cells from old mice are deficient in the capacity to secrete IL-2 in response to a stimulus (16, 17, 31), which led to the conjecture that our observations may be irrelevant in the context of a biological stimulus. To examine this deficiency in more detail in our *in vitro* assays, we replaced IL-2 with IL-15, which has been shown to stimulate proliferation of memory CD8 T cells in young mice (43) and, like IL-2, augments IFN- γ production by

FIG. 2. IFN- γ secretion by CD8 T cells isolated from the lungs of naïve old mice. Single-cell suspensions from the lungs of naïve young and old mice were cultured for 16 h with medium, individual cytokines, or combinations of cytokines. Monensin was added to the cultures for the final 4 h. Cells were stained with anti-CD8 and anti- β TCR, fixed, permeabilized, and stained with anti-IFN- γ . The percentages of CD8⁺ IFN- γ ⁺ T cells were determined (A to C) and used to calculate absolute numbers (D). (E) Representative dot plots for young and old lung samples stimulated with IL-2/IL-12/IL-18. The data are means $\pm$ standard errors for five mice and are representative of two independent experiments. One asterisk indicates that the *P* value is <0.05 and two asterisks indicate that the *P* value is <0.005 , as determined by Student's *t* test. ND, not detected.

FIG. 3. IFN- γ production by purified CD8 T cells. Purified CD8⁺ cells from the lungs of naïve young and old mice were cultured for 16 h with a combination of IL-2, IL-12, and IL-18. Monensin was added to the cultures for the final 4 h. Cells were stained with fluorescently conjugated antibodies against CD8, CD3 ϵ , CD44, and CD45RB, fixed, permeabilized, and stained with anti-IFN- γ . The percentages of CD8⁺ IFN- γ ⁺ T cells were determined for young and old mice (A). CD8⁺ IFN- γ ⁺ T cells were gated and analyzed further for CD44 and CD45RB expression, and the percentages of CD44^{hi} CD45RB^{lo} IFN- γ ⁺ CD8⁺ T cells were determined (B). The data are from a pool of five mice and are representative of two independent experiments.

IL-12- and IL-18-stimulated CD8 T cells (4). In this analysis IL-15 amplified the stimulating capacities of IL-12 and IL-18 equally, and the levels obtained were comparable to the levels observed with IL-2 (Fig. 2B and C). Together, these data show that IL-12 is essential for nonspecific production of IFN- γ by CD8 T cells from the lungs of old mice and that its action can be synergistically enhanced by addition of IL-2, IL-15, or IL-18.

Purified pulmonary CD8 T cells from naïve old mice produce IFN- γ in response to Th1 cytokines in the absence of antigen-presenting cells. To conclusively demonstrate that Th1 cytokines could stimulate CD8 T cells directly, CD8⁺ cells were purified (purity, >94%) from the lungs of naïve young and old mice and stimulated overnight with IL-2, IL-12, and IL-18 in the absence of antigen-presenting cells. As observed with whole-lung cultures, the proportion of CD8 T cells from naïve old mice that produced IFN- γ in response to the Th1 cytokine cocktail was significantly higher than the proportion

of CD8 T cells from naïve young mice that produced IFN- γ in response to the Th1 cytokine cocktail (Fig. 3A). CD8⁺ IFN- γ ⁺ T cells were gated and then analyzed for CD44 and CD45RB expression. In contrast to the cells in young mice, the overwhelming majority of IFN- γ -producing CD8 T cells in old mice expressed a memory phenotype (Fig. 3B). Together, these data conclusively demonstrated that naïve old mice have a significant population of memory CD8 T cells in their lungs that, in the presence of specific Th1 cytokines, are capable of antigen-independent IFN- γ production.

CD8 T cells from the lungs of old mice express receptors for Th1 cytokines. Because of the heightened reactivity of pulmonary CD8 T cells from naïve old mice to IL-2/IL-15, IL-12, and IL-18, we hypothesized that these cells also express increased levels of Th1 cytokine receptors. CD8 T cells isolated from the lungs of naïve old mice exhibited increases in both the proportion and the absolute number of cells that brightly expressed

FIG. 4. CD8 T cells express Th1 cytokine receptors. Single-cell suspensions from the lungs of individual naïve young and old mice were stained with anti-CD8, CD3ε, and IL-2Rβ (A and D), IL-18Rα (B and E), or IFN-γR (C and F). (A to C) Representative histograms for young and old lung samples. (D to F) Percentages were determined and used to calculate absolute numbers. The data are means $\pm$ standard errors for five mice and are representative of two independent experiments. One asterisk indicates that the *P* value is <0.05 , two asterisks indicate that the *P* value is <0.005 , and three asterisks indicate that the *P* value is <0.0005 , as determined by Student's *t* test. FITC, fluorescein isothiocyanate.

IL-2Rβ (CD122) (Fig. 4A and D) and IL-18Rα (Fig. 4B and E). No significant difference between young and old mice was observed for IL-12Rβ2 or the IL-2Rα chain (data not shown). Despite our inability to detect differences between young and

old mice for surface expression of IL-12Rβ2 on CD8 T cells, it is clear from the *in vitro* data presented previously that purified CD8 T cells from naïve old mice are highly responsive to IL-12. An analysis of the IFN-γ receptor was also performed, as it has

FIG. 5. Th1 cytokine production in the lungs of old mice during infection with *M. tuberculosis*. Young and old mice were aerogenically infected with a low dose of *M. tuberculosis* Erdman. At specific times after infection the right middle lung lobe was removed, homogenized in Ultraspec, and frozen at -80°C . RNA was isolated and reverse transcribed, and cDNA for IL-12p40 (A) and IFN- γ (B) was amplified by real-time PCR. The data are means $\pm$ standard errors for the relative quantity of message from five mice at each time and are representative of three independent experiments. 18S was used as an endogenous normalizer, and naïve young mice were used as the calibrator. At the early times after infection (0 to 12 days) the total numbers of lung cells isolated from young and old mice were statistically indistinguishable (data not shown). (C and D) On day 12 postinfection aliquots of lung homogenate were analyzed to determine the levels of IL-12p40 (C) and IL-18 (D) protein by ELISA. The data are means $\pm$ standard errors for five mice per group and are representative of two independent experiments. An asterisk indicates that the *P* value is <0.05 , as determined by Student's *t* test.

been shown previously that addition of IFN- γ in T-cell cultures can enhance Th1-cytokine-driven CD8 $^{+}$ CD44 $^{\text{hi}}$ T-cell proliferation (35). As observed with IL-2R β and IL-18R α , naïve old mice showed increases in both the proportion and the absolute number of pulmonary CD8 T cells that expressed the IFN- γ receptor (Fig. 4C and F). The combined data suggest that the increased expression of Th1 cytokine receptors on pulmonary CD8 T cells from naïve old mice can facilitate the responsiveness of this T-cell population to Th1 cytokines and likely contributes to the increased capacity of the cells to produce IFN- γ in response to IL-2/IL-15, IL-12, and IL-18.

Levels of Th1 cytokines are elevated in the lungs of old mice following infection with *M. tuberculosis*. We found that CD8 T cells from the lungs of naïve old mice are capable of producing IFN- γ in vitro in response to a Th1 cytokine cocktail. We therefore hypothesized that the capacity of CD8 T cells in the lungs of old mice to secrete IFN- γ and subsequently limit the growth of *M. tuberculosis* should be facilitated by the presence of Th1 cytokines in the lungs of *M. tuberculosis*-infected old mice. Old and young mice were infected aerogenically with *M.*

tuberculosis, and the relative quantities of IL-12p40, IFN- γ , IL-2, IL-15, and IL-18 mRNA in the lungs were determined using real-time PCR. During the first 12 days of infection, increased expression of IL-12p40 mRNA was detected in the lungs of old mice as early as day 5, and the expression consistently peaked by day 8 in three independent experiments (Fig. 5A). On day 8 postinfection the level of IL-12p40 mRNA expression in the lungs of old mice was more than 40-fold higher than the level in the lungs of young mice (Fig. 5A). A similar trend was observed for IFN- γ mRNA levels, and the peak expression in the lungs of old mice 8 days postinfection was 70-fold higher than the expression detected in the lungs of young mice (Fig. 5B). IL-12p40 and IFN- γ mRNA expression transiently declined in young and old mice at 10 to 12 days postinfection (Fig. 5A and B); however, analysis of lung tissue at later times postinfection revealed that not only did the IL-12p40 and IFN- γ mRNA levels increase, but, as expected, young mice were capable of producing substantial levels of IL-12p40 and IFN- γ mRNA at these later times postinfection (data not shown). We were unable to detect any consistent

differences in IL-2, IL-15, or IL-18 mRNA expression between young and old mice throughout the first 12 days of infection with *M. tuberculosis* (data not shown).

Lung homogenates were also analyzed for the production of cytokine protein by ELISA. Homogenates from old mice (day 12 postinfection) contained at least twofold more IL-12p40 than homogenates from young mice contained (Fig. 5C). Although we were unable to detect differences between the level of IL-18 mRNA expression in young mice and the level of IL-18 mRNA expression in old mice after infection, old mice produced significantly more IL-18 protein in the lungs 12 days postinfection than young mice produced (Fig. 5D), demonstrating that bioactive IL-18 was available in the lung. Together, these data conclusively establish that soon after infection the cytokines required for nonspecific IFN- γ production by CD8 memory T cells in vitro not only are present in the local lung environment but also are more abundant in the lungs of *M. tuberculosis*-infected old mice.

Similar to the results obtained with naïve mice, throughout the first 12 days of infection with *M. tuberculosis* (days 2, 5, 8, 10, and 12), significantly higher proportions of CD8 T cells from the lungs of old mice produced IFN- γ upon culture with IL-2, IL-12, and IL-18 (Fig. 2B and data not shown), demonstrating that early during an *M. tuberculosis* infection CD8 T cells from old mice are more responsive to Th1 cytokines than CD8 T cells from young mice are. As demonstrated with naïve mice, the levels of Th1 cytokine receptor expression also remained elevated in CD8 T cells isolated from the lungs of old mice at the same early times after infection (Fig. 4 and data not shown).

Old mice show early resistance to *M. tuberculosis* infection in the lung. Previous experiments have shown that old mice express transient resistance to *M. tuberculosis* 2 to 3 weeks postinfection, which is dependent on CD8 T cells and IFN- γ (38, 39). Since pulmonary CD8 T cells from naïve old mice were capable of producing significant amounts of IFN- γ in response to Th1 cytokines and since the same cytokines were found in the aging lungs soon after infection with *M. tuberculosis*, we investigated whether the transient resistance seen in old mice was apparent earlier than previously demonstrated. The bacterial loads in the lungs of *M. tuberculosis*-infected mice were determined at various times postinfection. No differences in bacterial load were observed for the first 5 days postinfection (data not shown), arguing against the hypothesis that there is differential bacterial uptake in young and old mice during the initial infection. By day 8 postinfection, the lungs of old mice contained fewer bacteria than the lungs of young mice contained, and the bacterial burden remained consistently lower for 21 days postinfection (Fig. 6A). In some experiments a statistically significant difference in the bacterial loads in the lungs of young and old mice was observed as early as 8 days postinfection (data not shown). Such differences in the number of CFU, while not consistently statistically significant, suggest that immune events can occur extremely early in the lungs of old mice, which can be detected by a reduction in the bacterial load.

To determine whether the lung environment in old mice was conducive to local proliferation of CD8 T cells following aerosol infection with *M. tuberculosis*, BrdU was administered intraperitoneally to young and old mice. By day 15 postinfection the lungs of old mice had a higher proportion of dividing CD8

T cells than the lungs young mice had (Fig. 6C). Although there was considerable variation between individual mice, which resulted in a lack of statistical significance, we consistently found more proliferating CD8 T cells in the lungs of old mice at 10 and 15 days postinfection (Fig. 6B). These data suggest that during the first 2 weeks of an infection with *M. tuberculosis* the CD8 T cells in the lungs of old mice can proliferate in response to local stimuli and are more responsive than the CD8 T cells in the lungs of young mice.

DISCUSSION

In the current study we investigated the immunological mechanisms that contribute to the transient early resistance to infection with *M. tuberculosis* expressed by old mice. Our data show that in naïve old mice there is a significant number of CD8 T cells in the lungs that have a memory T-cell phenotype and express receptors for Th1 cytokines. We also found that CD8 T cells isolated from the lungs of naïve old mice nonspecifically produced IFN- γ in response to in vitro stimulation with a combination of IL-12 and IL-18. The synergistic effect of IL-12 and IL-18 could be further enhanced by addition of either IL-2 or IL-15. Since *M. tuberculosis* is a strong inducer of Th1 cytokines in vivo, we hypothesized that following aerosol infection with *M. tuberculosis* the aging lung environment is conducive to stimulation of memory CD8 T cells. This hypothesis was supported by the finding that IL-12p40 and IL-18 levels were elevated in the lungs of old mice soon after an aerosol challenge with *M. tuberculosis*. The levels of the IFN- γ message were also elevated in the lungs of old mice, demonstrating that our in vitro observations were directly relevant to in vivo infection of old mice with *M. tuberculosis*.

The ability of CD8 T cells to contribute to innate immune responses through the nonspecific production of IFN- γ has been described for young mice (4, 5, 19, 20), and memory CD8 T cells from young mice rapidly produce IFN- γ in response to infectious agents such as *Burkholderia pseudomallei* (20) and *L. monocytogenes* (4, 5, 20) or in response to direct stimulation with IL-12 and IL-18 (4, 5, 19, 20). These observations were made with a minor subset of memory CD8 T cells in young mice, yet these cells are clearly capable of influencing the outcome of disease. Such findings led us to speculate that the increasing number of memory CD8 T cells in the lungs of old mice could also influence the outcome of aerosol infection with *M. tuberculosis*. Nonspecific immune mechanisms could transiently bypass the antigen-specific immunity that is normally required to control an infection with *M. tuberculosis* and could account for the reduced bacterial load found in the lungs of old mice. The same mechanism may also account for the enhanced control that old mice exhibit upon infection with other intracellular pathogens, such as *Leishmania major* (12) and *L. monocytogenes* (23), in which Th1 cytokine production is also essential for protection.

For the nonspecific stimulation of memory CD8 T cells to be relevant in vivo, the Th1 cytokines capable of driving IFN- γ production in vitro need to be present in the lungs of old mice following *M. tuberculosis* infection. Indeed, our data demonstrate that both IL-12p40 and IL-18 protein could be detected. More significantly, old mice were actually capable of producing more Th1 cytokines in response to infection with *M. tubercu-*

FIG. 6. Early resistance of old mice to *M. tuberculosis* infection. Young and old mice were infected with a low dose of *M. tuberculosis* Erdman by aerosol, and bacterial burdens in the lungs were determined (A). The data are means $\pm$ standard errors for five mice per group and are representative of four independent experiments. BrdU was administered intraperitoneally 14 h before lung cells were harvested. (B and C) Single-cell suspensions from lungs were stained with fluorescently conjugated antibodies against CD3e, CD8, and BrdU. (C) Representative dot plots for lung cells from young and old mice on day 15 postinfection. (B) Percentages were determined and used to calculate absolute numbers. The data are representative of two independent experiments. One asterisk indicates that the *P* value is <0.05 and three asterisks indicate that the *P* value is <0.0005 , as determined by Student's *t* test.

losis than young mice produced. Both IL-12p40 and IFN- γ mRNA were detected in the lungs of old mice 5 days postinfection, and the levels peaked 8 days postinfection, at which point the level of expression far exceeded that found in young mice. These findings are contrary to those of Cooper et al., who reported that IL-12p40 mRNA expression was significantly reduced in the lungs of old mice following aerosol infection

with *M. tuberculosis* (7). In their study Cooper et al. examined later times after infection than we examined, and in our study IL-12p40 mRNA expression did increase in the lungs of young mice at later times after infection (data not shown). Our data therefore conclusively demonstrate not only that the Th1 cytokines necessary for nonspecific CD8-T-cell production of IFN- γ in vitro are present in the lungs of old mice soon after

aerosol challenge with *M. tuberculosis* but also that the levels are elevated compared the levels in young mice. It is currently not clear whether the cell population responsible for IL-12 and IL-18 production, which we presume to be macrophages (15, 36), is larger in the lungs of old mice or whether individual macrophages from old mice are each capable of producing more cytokine upon infection with *M. tuberculosis*. Either model could account for the increased production of IL-12 and IL-18 that we observed in vivo.

Our in vitro data demonstrate that IL-2 or IL-15 can enhance the IL-12/IL-18-driven IFN- γ production by CD8 T cells, yet we found no evidence of an increase in mRNA for either of these cytokines. Whereas the capacity of CD4 T cells to produce IL-2 reportedly is impaired in old mice (22), there is very little information regarding the age-associated production of IL-15. However, studies have shown that IL-15 can drive in vivo proliferation of memory CD8 T cells (43), and in our model we observed increased proliferation of CD8 T cells in the lungs of old mice compared to the proliferation in young mice during infection with *M. tuberculosis*. Similarly, in the absence of IFN-I signaling, which is required for IL-15-driven proliferation (43), CD8 T cells failed to expand in the lungs of old mice during *M. tuberculosis* infection (39). The combined data suggest that IL-15-mediated proliferation can account for the expansion of CD8 T cells in the lungs of old mice. Our inability to detect an increase in the level of IL-15 mRNA in vivo during infection with *M. tuberculosis* may therefore indicate that IL-15 signaling can occur in the absence of newly synthesized cytokine and perhaps is facilitated by increased expression of the IL-15R β chain of CD8 T cells from old mice. An alternative point of view is that neither IL-2 nor IL-15 is produced in vivo in response to infection with *M. tuberculosis* and that the transient early resistance that we observed in old mice is mediated entirely by IL-12 and IL-18. Tough et al. have shown that in vivo delivery of IL-12 or IL-18 can also induce bystander proliferation of memory CD8 T cells in young mice (35). Since IL-12 and IL-18 were readily detected in the lungs of old mice, it is equally possible that Th1 cytokines produced in response to *M. tuberculosis* infection were responsible for the expansion of the CD8-T-cell pool that we observed in vivo. In combination with our in vitro data, these proposed models have direct applications for the design of immunotherapeutics for the elderly as they indicate that CD8-T-cell-mediated immunity could be further enhanced or extended by in vivo delivery of IL-2 or IL-15.

In agreement with the reports of Berg et al. (4), we consistently found that a minor population of CD8 T cells from the lungs of young mice was also capable of responding to IL-12 and IL-18 in vitro. The data suggest that resident pulmonary CD8 T cells from young mice should also be able to produce IFN- γ in response to infection with *M. tuberculosis*. Where young and old mice differ significantly, however, is in the number of memory CD8 T cells in the lungs, in the increased expression of receptors for Th1 cytokines on CD8 T cells, and in the increased local production of Th1 cytokines, which appears to amplify the early IFN- γ production in the lungs of old mice. Nonetheless, our studies using old mice may have identified an area of CD8-T-cell immunity that has not been investigated yet in young mice. The ability to nonspecifically increase the number of IFN- γ -producing CD8 T cells in the

FIG. 7. Model for early control of mycobacterial growth in the lungs of old mice. Following aerosol infection with *M. tuberculosis*, production of IL-12 and IL-18 is increased compared to the production in young mice, which stimulates memory CD8 T cells to nonspecifically produce IFN- γ and leads to macrophage activation and early control of mycobacterial CFU. CD8 T cells from the lungs of old mice proliferate more rapidly in response to infection, further amplifying the CD8-mediated effector response and control.

lungs of young mice and subsequently enhance early control of *M. tuberculosis* infection could provide an additional protective immune mechanism against this pulmonary pathogen.

In summary, we found that following infection with *M. tuberculosis* old mice are capable of producing significantly more IL-12 and IL-18 in their lungs than young mice produce, demonstrating that, when there is an encounter with *M. tuberculosis*, innate immune responses in the lung are enhanced in old mice. Furthermore, CD8 T cells isolated from the lungs of naïve old mice were predominantly memory T cells that express receptors for the Th1 cytokines IL-18, IFN- γ , and IL-15. In vitro, we found that a significant proportion of CD8 T cells nonspecifically produced IFN- γ when they were cultured in the presence of IL-12 and IL-18 and that this response could be further enhanced by addition of IL-2 or IL-15. We therefore propose that the early control of *M. tuberculosis* in the lungs of old mice is initiated by increased production of IL-12 and IL-18, which in turn drives the production of IFN- γ by resident memory CD8 T cells and ultimately leads to transient control of the mycobacterial load in the lungs of old mice (Fig. 7). The early resistance to *M. tuberculosis* infection seen in old mice appears to be mediated by two independent but synergistic mechanisms. First, the increased production of Th1 cytokines in the lungs of old mice creates an environment that stimulates memory CD8 T cells. Second, old mice have a large resident population of memory CD8 T cells in their lungs that are receptive to Th1 cytokines and can be nonspecifically stimulated to secrete IFN- γ . Furthermore, we also have in vivo evidence that pulmonary CD8 T cells from old mice proliferate earlier and more abundantly than their counterparts from young mice proliferate, possibly in response to IL-15. These data suggest that resident CD8 T cells that can be stimulated to produce IFN- γ not only are more numerous in the lungs of old mice but also proliferate in the lungs following *M. tuberculosis*

infection, further amplifying the mechanism that facilitates the early control of *M. tuberculosis* in the lungs. These combined events result in a reduction in the bacterial load in the lungs of old mice. Therefore, we identified a novel mechanism in the lungs of old mice that at least transiently enhances the overall immune response to a respiratory pathogen.

Because of the increased susceptibility of the elderly to numerous respiratory pathogens and the projected doubling of the elderly population by the year 2025, it is imperative that we identify alternative strategies for protecting the elderly from the morbidity and mortality that is associated with poor immune control of infectious diseases. In order to accomplish this, we need a much more comprehensive understanding of how the aging immune system responds to pathogens. We identified a population of CD8 T cells present in the lungs of naïve old mice that, through a novel mechanism, transiently enhances the overall immune response to a respiratory pathogen. Further characterization of this mechanism could lead to enhancement and design of vaccines or postexposure therapies for the elderly.

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