Mononuclear Phagocyte-Derived Interleukin-10 Suppresses the Innate Pulmonary Granuloma Cytokine Response in Aged Mice

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Granulomas are sequestration responses observed in a wide variety of clinical conditions, including mycobacterial infection. We previously reported impaired adaptive, Th1 cell-mediated pulmonary granuloma formation in response to bead-immobilized *Mycobacterium bovis*-purified protein derivative in aged mice. To reveal determinants of age-related immune deficits, the present study examined the effect of aging on early innate stage pulmonary granuloma formation. Aged mice formed more neutrophil-rich innate granulomas with augmented CXCL2 expression followed by a pattern of rapid decay of tumor necrosis factor- α , interleukin (IL)-6, CCL3, and CXCL2. This was associated with enhanced IL-10 expression. Blockade of IL-10 signaling with anti-IL-10 receptor antibody reversed the age-related decay. Intracellular flow cytometric analysis revealed that CD11b⁺Gr-1^{+/-} mononuclear phagocytes were the primary leukocyte sources of IL-10 in lungs, and their numbers were increased in aged mice. When exposed to purified protein derivative *in vitro*, young and old CD11b⁺Gr-1^{+/-} mononuclear phagocytes from blood or lung had comparable IL-10 expression, suggesting *in vivo* signals in the aged environment enhanced the number of IL-10-producing cells in the aged lung. Our findings reveal a novel mechanism of age-associated IL-10 mediated pulmonary immune suppression with the potential to alter downstream adaptive immunity. (Am J Pathol 2007, 171:829–837; DOI: 10.2353/ajpath.2007.061122)

other environmental antigens. Thus, respiratory exposures represent a major challenge for the aging immune system, and the aged lung is known to display altered immunoinflammatory responses. Influenza and pneumococcal pulmonary infections have higher fatality rates in older than in younger people.¹ It is unclear if aging leads to increased pathogen exposure because barrier and mucociliary clearance function may be compromised in apparently healthy seniors.² Reactivation of *Mycobacterium tuberculosis* infection is a well-known phenomenon in aged populations. Other phenomena such as increased numbers of neutrophils in bronchoalveolar lavage have been described in normal elderly humans, suggesting chronic subclinical inflammation,³ but the mechanism for this is undetermined. Little is known about the effect of aging on innate immune responses in the lung, and studies of the effect of aging on adaptive immune responses *in vivo* are primarily limited to responses to systemic rather than organ-targeted antigen challenge.^{4–10}

Innate immunity is recognized as a critical stage of the immune response, providing initial host resistance to pathogens and determining the nature of subsequent immune events, thereby ultimately affecting host morbidity and mortality.^{11,12} To provide a systematic approach to the study of innate immune responses in the lung, we developed clinically relevant models of synchronized pulmonary granuloma formation in the mouse using pathogen antigen-coated agarose beads.¹³ Granulomas are innate responses of mononuclear phagocytes that can be modified by superimposed adaptive immune mechanisms. These focal inflammatory lesions represent a primitive sequestration response observed in a wide variety of conditions, including *M. tuberculosis* infection. Granulomas of the anamnestic, hypersensitivity type, involve antigen (Ag)-specific T-cell participation. Aging is associated with increased susceptibility to and reactivation of tuberculosis.^{14,15} It has

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Daily, the lung is exposed to more than 10,000 liters of air with potential hazardous chemicals, microorganisms, and

been shown that aged mice have a decreased capacity to generate effector T cells upon *M. tuberculosis* infection.^{14,15} We have previously reported that aging is associated with impaired T-cell-mediated pulmonary granuloma responses using antigen-bead models.¹⁶ Alteration in T-cell cytokine production was associated with abrogated inflammation in both Th1 and Th2 types of pulmonary granuloma responses.¹⁶ However, it was not clear whether the age-associated changes were attributable to intrinsic defects in naïve T cells or defects in T-cell priming by the innate immune system. In this study, we examined the effect of aging on the innate pulmonary granuloma response to mycobacterial purified protein derivative (PPD).

Based on reports of age-associated, functional deficits in innate effector cells, we hypothesized that aging leads to diminished innate immune responses to PPD in the lung. Surprisingly, naïve aged mice responded vigorously to the PPD bead challenge in the lung, as judged by lesion sizes. However, aged mice developed more neutrophil-rich lesions, and the cytokine response diminished prematurely. In contrast, interleukin (IL)-10 was augmented and blockade of IL-10 signaling reversed the age-related defect in the cytokine response. Intracellular flow cytometric analysis revealed that CD11b⁺Gr-1^{+/−} cells of the mononuclear phagocyte system were the major cellular sources of IL-10 in the lung, and aging was associated with increased numbers of the IL-10-producing cells. Importantly, when exposed to PPD *in vitro*, no age-associated increases in the number of IL-10-producing cells were found in either naïve lung cells or peripheral blood monocytes, suggesting that age-related tissue environmental factors are responsible for the increase in the number of immune suppressive IL-10-producing cells in the aged lung.

Materials and Methods

Mice

Male C57BL/6 mice of indicated ages were obtained from the National Institute of Aging contract colony at Harlan Laboratories (Indianapolis, IN). Mice were maintained under specific pathogen-free conditions and provided food and water *ad libitum*. Necropsy examinations were performed at the time of mouse sacrifice to exclude individuals with organ failure or neoplasm. The University of Michigan Committee on Use and Care of Animals approved all animal studies.

Mouse Models

Innate pulmonary granuloma responses were elicited in naïve mice of different age groups by intravenous injection of 6000 Sepharose 4B beads coupled to *M. bovis* PPD (Department of Agriculture, Veterinary Division, Ames, IA) as previously described.¹³

Antibodies and Treatment Protocol

Monoclonal anti-IL-10 receptor blocking antibody (clone 1B1.3a) was obtained commercially (BD Pharmingen,

San Jose, CA). For IL-10 receptor-blocking experiments, mice were injected intraperitoneally with 200 μ g of blocking antibody dissolved in 0.4 ml of phosphate-buffered saline immediately after the PPD bead injection. Young and aged mice injected with PPD beads and vehicle were used as controls.

Isolation of CD11b-Positive Cells

CD11b⁺ cells were isolated using mouse CD11b (Mac-1) MicroBead columns (Miltenyi Biotec Inc., Auburn, CA) according to the manufacturer's instructions. Enrichment of CD11b⁺ cells was confirmed by postisolation flow cytometric analysis, which demonstrated 99% purity.

Single Cell Preparation and Flow Cytometry

Single-cell suspensions were prepared from lung as described previously.¹³ Briefly, after perfusion with ice-cold RPMI 1640 medium (JRH Biosciences, Lenexa, KS), lungs were excised, placed in cold RPMI 1640 medium, and then homogenized in a Waring blender. Homogenates were incubated in complete medium containing collagenase consisting of RPMI 1640 medium, 10% fetal bovine serum (Intergen, Purchase, NY), 10 mmol/L glutamine, 100 μ g/ml streptomycin, 100 U/ml penicillin, and 1000 U/ml type IV collagenase (Sigma Chemical Co., St. Louis, MO). The digest was sieved through a stainless steel mesh (no. 100) and then washed four times by centrifugation in RPMI 1640 medium. Total cell yields were determined by standard hemocytometric counting.

Fluorochrome-labeled monoclonal antibodies used for flow cytometry included anti-CD11b/Mac-1 (M1/70), anti-CD40 (3/23), anti-Ly-6G and Ly-6C (Gr-1, clone RB6-8C5), anti-CD11c (HL3), anti-I-A^b (11-5.2), (all from BD Pharmingen), and anti-F4/80 (A3-1) (from Serotec, Raleigh, NC). All isotype controls and anti-CD16/CD32 (2.4G2) were also from BD Pharmingen. After blocking with anti-CD16/CD32 for 5 minutes, cells were stained with fluorescent-labeled antibodies or isotype control antibodies in 2% fetal bovine serum-phosphate-buffered saline buffer. For intracellular cytokine staining, single-cell suspensions were incubated at 37°C in a humidified 5% CO₂ atmosphere for 3 hours in complete medium in the presence of brefeldin A (1 μ g/ml) (BD Pharmingen) with or without PPD (5 μ g/ml).

Peripheral blood leukocytes from young and aged mice were isolated from heparinized blood after red cell lysis using a mouse erythrocyte lysing kit (R&D Systems, Minneapolis, MN) followed by three washes by centrifugation with final suspension in complete medium for culture. The leukocytes were incubated for 3 hours in the presence of brefeldin A with or without PPD. Cells were harvested and stained for expression of CD11b and Gr-1. Intracellular cytokine staining for IL-10 was performed using a Cytofix/Cytoperm kit (BD Pharmingen) according to the manufacturer's directions. A FACScan flow cytometer with CellQuest software (BD Pharmingen) was used for data acquisition and analysis. In the absence of brefeldin A, IL-10 was not

detectable indicating *de novo* synthesis. Data were acquired from individual mice in each experiment and then means and variations determined.

Morphometry

Individually excised lung lobes were inflated and fixed with 10% buffered formalin for morphometric analysis. Granuloma area was measured blindly from formalin-inflated lungs that were paraffin-embedded, sectioned, and then stained with hematoxylin and eosin (H&E). Granuloma area was measured by computerized morphometry as previously described.¹⁷ A minimum of 20 lesions was measured per lung. Only granulomas with full cross sections of the bead nidus were measured.

RNA Purification and Real-Time Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analysis

RNA was isolated, reverse-transcribed, and used for real-time PCR analyses as previously described.¹⁸ Analysis of the transcripts was performed by real-time PCR using the ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA) as previously described.¹⁷ The comparative threshold cycle (CT) method recommended by the manufacturer was adopted. GAPDH was used as the endogenous reference. All primer-probe sets were purchased commercially (Applied Biosystems). The thermal cycling condition was programmed according to the manufacturer's instructions. Transcript levels were expressed as arbitrary units and calculated as previously described.¹⁹ As it is not appropriate to compare data across experiments because of the relative nature of this method, data presented in each individual graph were generated in the same PCR run and expressed in the same arbitrary units.

Statistical Analysis

The Student's *t*-test was used for direct comparison to a parallel control group and for multigroup analyses a one-way analysis of variance was performed. When *P* values were less than 0.05, means were considered to be significantly different.

Results

Naïve Aged Mice Display Altered PPD Bead-Elicited, Innate Granuloma Formation

To determine the effect of aging on pathogen recognition and granuloma formation in the lung, we used a previously characterized innate pulmonary granuloma model¹³ and compared responses in mice at 6, 18, and 24 months of age. Naïve mice of the three different age groups were challenged by intravenous injection of Sepharose beads that were covalently coupled to *M.*

bovis PPD. The injected PPD beads embolize to the pulmonary vasculature and elicit focal reactions that are half of the area reported for the anamnestic PPD bead response but induce a more complex cytokine/chemokine profile than nonpathogen beads, indicating the reactions involve innate pathogen recognition.¹³ Innate PPD bead lesions form throughout a 3-day period, resolve, and ultimately establish a PPD-specific Th1 adaptive memory response.¹³ The nascent PPD bead lesions are initially composed of mononuclear cells and neutrophils, and then by day 3, the lesions are compact collections of mostly mononuclear cells. On histological examination, the overall sizes of 3-day innate granulomas were not significantly affected by aging (Figure 1, A and B). This is unlike the secondary T-cell-mediated PPD bead-elicited granulomatous response, which is abrogated in old mice.¹⁶ However, lesions from aged mice were aberrant, containing more polymorphonuclear leukocytes. Specifi-

Figure 1. Aged mice mount altered innate cellular responses to *M. bovis* PPD bead challenge. C57BL/6 mice of 6, 18, and 24 months of age were injected intravenously with Sepharose beads coupled to *M. bovis* PPD on day 0. On day 3, lungs were harvested and analyzed for histological appearance in H&E-stained sections of paraffin-embedded samples. **A:** Histological appearance of day 3 innate PPD bead lung granulomas in 6-, 18-, and 24-month-old mice. Note the increased component of polymorphonuclear leukocytes. **B:** Mean cross-sectional areas of the day 3 innate PPD bead granulomas measured by computer-assisted microscopy. Bars are means $\pm$ SD and were generated from six individual animals. The dashed line represents the average area of the bead. **C:** Flow cytometric time-course analysis of the proportion of neutrophils (CD11b⁺Gr1^{hi}) expressed as percentage of total CD45⁺CD11b⁺ cells in PPD bead-challenged lungs. Bars are means $\pm$ SD derived from six individual mice per time point. **P* < 0.01 (analysis of variance) shows significant effect of aging on day 3. Original magnifications: $\times 100$ (A, top); $\times 400$ (A, bottom).

cally, polymorphonuclear leukocytes tended to accumulate faster in old mice, and the usual mononuclear cell-rich population that dominated by day 3 in young mice was replaced by a persistent population of neutrophils in 18- and 24-month-old mice (Figure 1C).

Cytokine/Chemokine Responses Decay Prematurely in Aged Mice during Innate Granuloma Formation

We next examined the effect of aging on chemokine/cytokine profiles induced during innate granuloma formation. Because activated T cells begin to populate the periphery by 4 days after antigen challenge in both young and aged mice (data not shown), we limited our analysis to the first 2 days to minimize contribution of adaptive immunity. As shown in Figure 2, A and B, IL-6, tumor necrosis factor (TNF)- α , IL-1 β , CCL3, and CCL4 transcripts were expressed in lungs after PPD bead challenge in both young and aged mice. Although, CCL3, IL-6, and TNF- α transcripts were induced at all ages, they decayed prematurely in aged mice (Figure 2, A and B; day 2). In contrast, IL-1 β , CCL2, and CCL7 expression patterns were unaffected (Figure 2, A and C).

In view of the increased neutrophil accumulation in innate granulomas of old mice, we specifically assessed transcript expression of CXCL ligands with known neutrophil chemotactic properties, CXCL1, CXCL2, and CXCL5. As shown in Figure 2D, of these chemokines, CXCL2, formerly known as macrophage inflammatory protein-2, was augmented fourfold on day 1 in 24-month-old mice as compared with 6-month-old mice. However, by day 2, there was a rapid decay of CXCL2 transcripts in the old group such that levels

were significantly lower than in young mice. Transiently augmented CXCL2 production might contribute to the enhanced neutrophil accumulation in old mice, but it does not fully explain their greater proportion in day 3 lesions, which may in fact reflect impaired monocyte recruitment because of decreased CCL3 production or slower clearance. In summary, an innate proinflammatory cytokine/chemokine response to PPD bead challenge is initiated in old mice and is sufficient to elicit a leukocyte response; however, portions of the response are altered and diminish prematurely.

IL-10 Expression Is Augmented in Aged Mice during Innate Granuloma Formation

Our previous studies of young mice have shown that a number of other genes are induced during innate PPD bead granuloma formation at low but detectable levels.¹³ To assess effects on these and other genes, we compared the expression of GM-CSF, IL-10, IL-12, CXCL9, CXCL10, CXCL11, inducible nitric-oxide synthase, and COX2 in young and old mice. Among these, only IL-10 expression was significantly altered by aging. As shown in Figure 3, compared with young mice, the expression levels for IL-10 were augmented by more than fivefold in 24-month-old mice on day 1 and by more than twofold in both 18- and 24-month-old mice on day 2.

Blockade of IL-10 Signaling Reverses Impaired Cytokine/Chemokine Expression in Aged Mice

IL-10 is known to inhibit a broad spectrum of inflammatory responses, including macrophage activation and cy-

Figure 2. Cytokine and chemokine responses decay prematurely in aged mice during innate pulmonary granuloma formation. C57BL/6 mice of 6, 18, and 24 months of age were injected intravenously with PPD beads on day 0. On days 1 and 2 after the PPD bead challenge, lungs were harvested for mRNA analysis by real-time RT-PCR. Naïve unchallenged lung samples are included (day 0) for comparison. Means and standard deviations of the relative mRNA expression levels of the indicated cytokines and chemokines were derived from three separate experiments with a total of six individual mice per bar. * $P < 0.05$ (analysis of variance) compared with the 6-month group. **A:** Cytokine transcripts; **B:** macrophage inflammatory chemokine transcripts; **C:** monocyte chemotactic protein transcripts; and **D:** neutrophil chemotactic protein transcripts. Expression levels are not directly comparable among different genes because of the relative nature of the method.

Figure 3. Aged mice display enhanced IL-10 expression during PPD bead-elicited innate granuloma formation. C57BL/6 mice of 6, 18, and 24 months of age were injected intravenously with PPD beads on day 0 and analyzed by real-time RT-PCR as in Figure 2. Naïve unchallenged lung samples are included (day 0) for comparison. Means and standard deviations of the relative mRNA expression levels of the indicated cytokines and chemokines were derived from three separate experiment with a total of six individual mice per bar. * $P < 0.05$ (analysis of variance) compared with the 6-month group.

Figure 4. Blockade of IL-10 signaling restores cytokine expression in aged mice to that of young mice. Groups of aged C57BL/6 mice (22 months) were injected intraperitoneally with monoclonal anti-IL-10 receptor (IL-10R)-blocking antibody or vehicle immediately after intravenous PPD bead injection on day 0. A parallel group of young C57BL/6 mice were injected intravenously with PPD beads to serve as a positive control. Lungs were harvested for mRNA analysis by real-time RT-PCR on day 2. Means and standard deviations of the relative mRNA expression levels of the indicated cytokines and chemokines levels were derived from two separate experiments with four individual mice in each group. **A:** age-affected cytokine and chemokine transcripts; **B:** neutrophil chemotactin, CXCL2, transcripts. * $P < 0.05$ compared with vehicle-treated aged mice.

tokine/chemokine production.^{20,21} Based on this, we hypothesized that enhanced IL-10 production in aged mice may be responsible for the accelerated decay of cytokine/chemokine expression in aged mice. If this was the case, then IL-10 blockade might restore cytokine production to the level of young mice. To test this possibility, we injected aged mice intraperitoneally with IL-10 receptor (IL-10R)-blocking monoclonal antibody (clone 1B1.3a) immediately after the PPD bead injection. The time of injection was selected to ensure blockade of receptors before the rapid induction of IL-10 after PPD bead challenge. Control aged mice injected with PPD beads and vehicle showed significantly lower levels of expression of TNF- α , IL-6, and CCL3 than young mice as we previously observed (Figure 4A). In contrast, old mice treated with IL-10R-blocking antibody displayed restoration of chemokine/cytokine transcript expression to levels comparable with that of young mice (Figure 4A). Interestingly IL-10R blocking did not significantly alter the expression of IL-10, suggesting the increased IL-10 expression in aged mice was not driven by IL-10. With regard to the neutrophil chemotactin CXCL2, as shown in Figure 4B, IL-10 receptor blockade augmented CXCL2 transcript expression in old mice by 10-fold on day 2, exceeding the levels of untreated young mice. Thus, CXCL2 seemed to be highly sensitive to IL-10-mediated suppression on day 2. These findings argued that the rapid decay of cytokine/chemokine expression after day 1 in the PPD bead-challenged aged mice was mediated by endogenous IL-10.

Cells of the Mononuclear Phagocyte System Are a Major Source of IL-10 and Increase during the Innate Pulmonary PPD Bead Granuloma Response in Aged Mice

The above findings were consistent with the known role of IL-10 in tempering inflammatory mediator production.²⁰ We next wished to determine the source and the etiology of its augmented expression in aged mice. In mice, potential

IL-10-producing cells include CD4⁺ T cells, $\gamma\delta$ T cells, B cells, mast cells, macrophages, and B-1 cells.^{20,21} Constitutive IL-10 production has also been reported in lung epithelial cells.²² The innate IL-10 response to pathogen challenge in the lung has not been studied at the single cell level in aged mice. To identify the cellular sources of IL-10 in the lung during innate granuloma formation, we examined lung cells from PPD bead-challenged 6- and 22- to 24-month-old mice because these groups showed the greatest difference in IL-10 transcript expression. The cells were incubated for 3 hours without stimulation in the presence of brefeldin A to allow intracellular accumulation of spontaneously produced cytokines.²³ The intracellular accumulation step is necessary for subsequent intracellular cytokine detection by specific antibodies. The cells were then analyzed by flow cytometry. In preparations from both young and old mice, IL-10-producing cells were initially identified as a subset of CD11b^{hi} cells among total lung cells (Figure 5A). This excluded lymphocytes that were CD11b-negative or weakly CD11b-positive (data not shown). Further analysis showed that IL-10 was expressed by a subset of mononuclear phagocytes (CD11b^{hi}Gr-1^{+/−}) but not neutrophils (CD11b^{hi}Gr-1^{hi}) (Figure 5B).

The proportion and the total number of the IL-10-producing cells in lungs with 2-day innate PPD bead granulomas were also determined using the IL-10 intracellular flow cytometry approach as described above. Compared with young mice, there was a significant increase in the proportion and total number of the IL-10-producing cells in the lungs of the aged mice (Figure 5C). Because average cell yields from lungs were similar for young and old mice, this indicated that the frequency of IL-10⁺CD11b⁺ mononuclear cells increased with age. On the other hand, the levels of IL-10 expression by individual IL-10⁺ cells were similar in young and aged mice, as indicated by the mean fluorescent intensity measurement (Figure 5C). The data suggested that increased numbers of IL-10-producing mononuclear phagocytes were responsible for the augmented levels of IL-10 expression observed in the lungs of aged mice.

Constitutive IL-10 mRNA Expression by CD11b⁺ Cells in Unchallenged Lungs Increases with Age

Because constitutive IL-10 expression by the lung has been proposed as an important regulatory mechanism,²² we wished to determine whether aging also affected constitutive IL-10 production. In our analyses of unchallenged lungs, IL-10 transcript expression was low when compared with challenged lungs, which likely made it difficult to detect differences in constitutive expression at the whole lung level. Because IL-10-producing cells were primarily in the CD11b⁺ cell subpopulation, we compared IL-10 mRNA expression levels in magnetic cell sorting bead-isolated CD11b⁺ lung cells from unchallenged, young and aged mice. In three separate experiments, higher levels of IL-10 expression were detected in the CD11b⁺-enriched cells from aged as compared young mouse lungs (Figure 6). Thus, using a more sen-

Figure 5. Numbers of IL-10-producing CD11b⁺Gr1^{lo} mononuclear cells increase in aged mice during innate pulmonary PPD bead granuloma formation. Young (4 months) and aged (22 months) C57BL/6 mice were injected intravenously with PPD beads on day 0. Lungs were harvested on day 2. Single-cell suspensions from digested whole lung tissues were incubated for 3 hours in complete medium in the presence of brefeldin A without stimulation to allow intracellular accumulation of spontaneous production of cytokines. The cells were then stained for expression of CD11b, Gr-1, and intracellular IL-10 and analyzed by flow cytometry. **A:** Scattergram demonstrating IL-10 expression by CD11b⁺ cells in the lung. **B:** Scattergram demonstrating that IL-10 is not expressed by Gr-1^{hi} (neutrophils) but by a subset of Gr-1^{lo} (mononuclear phagocytes) among gated CD11b⁺ lung cells. **C** shows the proportion and number of IL-10-producing cells in the lung and the mean fluorescent intensity (MFI) of IL-10⁺ cells in young versus aged mice. **P* < 0.05 compared with the young group. Data are pooled from two independent experiments (*n* = 5).

Figure 6. Numbers of CD11b⁺ cells constitutively producing IL-10 in the lung increases with age. Lungs from naïve young (4 months) and aged (22 months) C57BL/6 mice were harvested and cells dispersed, and CD11b⁺ cells were enriched using magnetic cell sorting anti-CD11b beads. The enriched cells were analyzed for the expression of IL-10 mRNA using real-time RT-PCR. In each of the three independent experiments, IL-10 was expressed at twofold to fivefold higher levels in aged mice (solid black bars) than young mice (open bars). To obtain sufficient cells, bars represent pooled cells from two mice in each experiment.

sitive direct analysis of enriched CD11b⁺ cells, we detected enhanced constitutive IL-10 production in unchallenged lungs of old mice.

The Ex Vivo IL-10 Response to PPD by Blood and Lung CD11b⁺Gr1^{lo} Mononuclear Cells Does Not Change with Age

To determine whether there was an intrinsic enhancement of IL-10 induction among mononuclear cells from aged mice, we examined antigen-elicited responses of blood and lung cells *ex vivo*. Tissue mononuclear phagocytes are derived from circulating monocytes, and in aged mice their IL-10 expression might be altered before tissue infiltration. To test this, isolated peripheral blood leukocytes from young and aged mice were incubated for 3 hours in the presence of brefeldin A with or without PPD. Because brefeldin A blocks protein secretion, this system removes regulatory effects of secreted paracrine or autocrine protein signals.²³ The cells were then analyzed by intracellular flow cytometry. As shown in Figure 7A, very few IL-10-producing cells were found without PPD stimulation. Thus, IL-10 production was not constitutively expressed among circulating monocytes likely because of being in a quiescent state before migration into peripheral tissues. However, in the presence of PPD, cultured cells rapidly and dramatically increased numbers of IL-10-producing cells (Figure 7, A and B). Similar to the lung, the IL-10-expressing population was in the CD11b⁺Gr1^{lo} mononuclear cell fraction. Moreover, the numbers of IL-10-producing cells were similar in young and old PPD-stimulated blood cell cultures, demonstrating that blood precursors of the mononuclear phagocyte system in aged mice do not differ from young mice in their intrinsic capacity to produce IL-10.

We similarly examined lung cells from unchallenged young and aged mice. After thorough washing, dispersed lung cells were incubated for 3 hours in the presence of brefeldin A with or without PPD and then subjected to flow cytometric analysis as described above. Again, IL-10-pro-

Figure 7. Blood monocytes and lung mononuclear phagocytes of young and aged mice display similar *ex vivo* IL-10 responses to PPD challenge. Pooled peripheral blood leukocytes from naïve young (4 months) and aged (22 months) C57BL/6 mice were collected and washed by centrifugation before culture. Individual lungs from naïve young (4 months) and aged (22 months) C57BL/6 mice were harvested, and single-cell suspensions from digested whole lung tissues were washed by centrifugation before culture. Blood and lung preparations were incubated for 3 hours in complete medium in the presence of brefeldin A with or without PPD (5 μ g/ml). Cells were then stained for expression of CD11b, Gr-1, and intracellular IL-10 and then analyzed by flow cytometry. **A:** Scattergrams comparing IL-10-producing CD11b⁺Gr-1^{lo} cells in control and PPD-stimulated blood leukocyte cultures of young and aged mice. **B:** Bar graph showing that the frequency of IL-10-producing cells in the blood does not change with age. **C:** Scattergrams comparing IL-10-producing CD11b⁺Gr-1^{lo} cells in control and PPD-stimulated lung cell cultures of young and aged mice. **D:** Bar graphs showing the proportion and absolute number of IL-10-producing cells in the lung cells in young versus aged mice. * $P < 0.05$ compared with the young group. Two independent experiments with a total $n = 5$.

ducing cells were restricted to the CD11b⁺Gr-1^{+/−} mononuclear phagocyte population (Figure 7C). As previously noted, compared with young mice, a greater number of CD11b⁺Gr-1^{+/−} cells from aged mice were constitutively producing IL-10 when cultured in medium alone (Figure 7D). In PPD-stimulated cultures, the number of CD11b⁺Gr-1^{+/−}, IL-10⁺ cells increased by threefold to fivefold. As with peripheral blood monocytes, IL-10-producing cells increased to similar numbers and frequency regardless of age (Figure 7, C and D). Numbers of TNF- α ⁺ cells were also comparable in the stimulated populations (data not shown). These studies indicated that neither lung mononuclear phagocytes nor blood monocytes from aged mice have an intrinsically enhanced capacity to generate IL-10-producing cells in response to PPD, but rather their altered activity was dependent on *in vivo* conditions.

Discussion

This study revealed a novel mechanism of age-related, IL-10-mediated immune regulation during innate mycobacterial Ag-elicited pulmonary granuloma formation. Compared with young mice, aged mice displayed subtle alterations in the cellular composition of innate granulo-

mas manifested as enhanced neutrophil relative to mononuclear cell recruitment. Subsequent transcript analyses revealed distorted cytokine/chemokine expression profiles marked by a rapid decay of several mediators IL-6, TNF- α , and CCL3 accompanied by a transient increase in the neutrophil chemotactin CXCL2 and sustained augmentation of IL-10 expression in aged mice.

With regard to our finding of increased neutrophils in the aged mouse lung response, it is noteworthy that disproportionate neutrophil recruitment has been reported in the lungs of aged humans.³ Our finding of a transient enhancement of CXCL2 might provide some explanation, but this is probably not the sole cause. In separate studies, we have demonstrated impaired neutrophil clearance in the aged mouse lung (data not shown), which may be related to a changing immunoregulatory environment and altered mononuclear phagocyte function.

As a known immunoregulatory molecule, we performed a detailed analysis of IL-10 in the aged lung, identifying its source and functional relationships. Constitutive expression of IL-10 has been reported in a number of cell types in lungs of rats and humans by immunohistochemical methods.²⁴ We likewise detected constitutive IL-10 expression in mouse lungs localizing mainly to CD11b⁺Gr-1^{lo} mononuclear phagocytes, and aging was associated with increased numbers of these cells. The CD11b⁺Gr-1^{lo} population was found to be an important source of IL-10. We can be reasonably confident that these cells are sources of IL-10 because, unlike immunohistochemistry, intracellular cytokine staining detects cells synthesizing IL-10 and excludes cells with bound or endocytosed target protein. It should be noted that the mononuclear phagocyte population identified here as CD11b⁺Gr-1^{lo} was interstitial and did not include alveolar macrophages, which are large, highly autofluorescent, and CD11b[−] (our unpublished data).²⁵ Unlike alveolar macrophages, interstitial mononuclear phagocytes in the lung are much less accessible in humans, and consequently less is known about them. To our knowledge, there have been no studies conducted with regard to the effect of aging on these CD11b⁺ interstitial pulmonary mononuclear phagocytes. IL-10, likely derived from CD11b⁺Gr-1^{lo} cells, was responsible for the rapid decay of proinflammatory mediators because blocking IL-10 signaling restored TNF- α , IL-6, CCL3, and CXCL2 expression levels.

Our study is the first to evaluate the effect of aging on lung macrophage cytokine responses at the single cell level. Intracellular flow cytometry allows the analysis of cytokine responses with minimal *in vitro* manipulations. It is well known that cells of the mononuclear phagocyte system are extremely sensitive to *in vitro* environmental cues.²⁶ Throughout our study, we attempted to minimize *in vitro* manipulation by limiting the duration of *ex vivo* stimulation to 3 hours in the presence of a blocker of cellular secretory pathways, brefeldin A. Because brefeldin A blocks cytokine secretion, this system minimizes autocrine and paracrine effects by cytokines produced secondary to PPD recognition and signaling. In contrast, most reported studies of age-associated changes in

macrophages involve longer term *in vitro* culture and rely on cytokine measurement in supernatants.

Our findings support the notion of heterogeneous populations or subsets of mononuclear phagocytes as defined in terms of cytokine responses to pathogen-associated molecular patterns. IL-10-producing cells represent a small percentage of the total macrophage population in the lung. The same was true for TNF- α -, IL-6-, and IL-12-producing populations (data not shown). We found that lung interstitial macrophages but not peripheral blood monocytes expressed IL-10 constitutively, suggesting that signals in the environment of the aged lung direct these cells to become IL-10 producers after migration from the blood. Our blood monocyte analysis argues against preferential recruitment of precommitted IL-10-producing cells or intrinsic augmentation of IL-10-producing capacity in aged mice. Thus, age-related changes in macrophages might be attributable to changes in the balance of macrophage differentiation into various functional subsets.

Our data are consistent with the hypothesis presented by Stout and Suttles²⁷ that age-associated dysfunction of macrophages is the result of their adaptation to changes in tissue environments. Intrinsic age-acquired macrophage dysfunction is controversial. For example, TLR expression and altered signal transduction has been reported in some but not all studies.^{28–30} Altered gene expression appeared to be associated with impaired intracellular signaling. However, those studies showing age-associated impairments of mitogen-activated protein kinases (MAPKs) have been disputed by more recent studies.³⁰ Peritoneal and splenic macrophages have been studied most often in animal models. These cells reportedly displayed age-related impairments in production of proinflammatory cytokines/chemokines, including IL-6, TNF- α , CCL3, and CCL4, as well as increased production of IL-10 in culture supernatant when stimulated with TLR ligands.^{28–30} These results have been interpreted as reflecting an intrinsic age-acquired macrophage dysfunction. However, TLR ligand-stimulated macrophage culture supernatants are rich in immune mediators. Because paracrine and autocrine regulatory effects were not excluded in these studies, it is unclear if age-related changes were attributable to impaired TLR signaling or regulation by cytokines such as IL-10. Indeed, Chelvarajan and colleagues³¹ reported that age-related impairments of proinflammatory cytokines/chemokine production was reversed by IL-10 neutralization in lipopolysaccharide-stimulated macrophage cultures.

We found that aging was associated with an increased proportion and absolute number of IL-10-producing mononuclear phagocytes in the lung under steady state conditions and in response to mycobacterial antigen challenge. However, age-related differences were not found when lung cells were stimulated *in vitro* with PPD in the presence of a protein secretion-blocking agent. Furthermore, in the very early phase of innate granuloma formation, aged mice responded normally to PPD bead challenge and only subsequently showed impairments. These findings argue against the existence of intrinsic macrophage defects with regard to PPD recognition and signaling in aged mice as suggested by previous stud-

ies.^{28–30} Based on these considerations, we would propose that aging does not lead to intrinsic dysfunction of mononuclear phagocytes but rather creates a different regulatory environment, resulting in altered macrophage activation/maturation.

This alternative maturation seems to extend to dendritic cells because we have found impaired CD40 costimulatory expression among dendritic cells from lymph nodes draining challenged lungs, which was associated with impaired Th1 cell differentiation. Moreover, IL-10R blockade completely reversed these impairments (B.-C. Chiu, V.R. Stolberg, and S.W. Chensue, manuscript in preparation). These findings provide a mechanism for abrogated adaptive Th1-mediated granuloma formation observed in aged mice.¹⁶

The persisting question is what environmental signal or signals increase the incidence of IL-10-producing macrophage subsets in aged mice? One possibility is that macrophage activation/maturation status reflects changes in the homeostatic cytokine milieu because of changes in the overall balance of regulatory signals. In this vein, aged mice display increased natural T regulatory cell activity (B.-C. Chiu, V.R. Stolberg, and S.W. Chensue, manuscript submitted).

Altered IL-10 regulatory balance in the lung has potential clinical implications. IL-10-mediated suppression of pulmonary innate immune response has been described in postseptic human patients and animal models.³² In sepsis, increased production of IL-10 in the lung may be an endogenous protective response to the overabundant and often lethal systemic production of proinflammatory cytokines. Macrophage deactivation is an unfortunate consequence of IL-10 production in the lung, leading to impaired bacterial clearance.³² However, without IL-10, animals do not survive sepsis.³³ It is well known that aging is associated with various inflammatory conditions and increased serum levels of proinflammatory mediators.^{34,35} It is conceivable that more macrophages are committed to produce IL-10 in the lung as part of a hard-wired counter response to increased systemic proinflammatory mediators because of chronic inflammatory conditions.

In summary, our data demonstrate increased numbers of IL-10-producing CD11b⁺Gr1^{lo} cells in the lungs of aged mice, likely as the result of age-related changes in tissue regulatory environments rather than acquired dysfunctional innate recognition and signaling. Moreover, mononuclear phagocyte-derived IL-10 may serve as a potential target for restoring age-impaired pulmonary immunity during chronic infection. It will be important to determine whether this IL-10-producing cell population contributes to age-associated susceptibility to and reactivation of tuberculosis.

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