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Previously, we reported that IL-10-producing mononuclear phagocytes increase in lungs of aged mice, causing impaired innate cytokine expression. Since dendritic cells (DCs) contribute to innate NK cell and adaptive T cell immunity, we tested the hypothesis that age-related IL-10 might influence DC function with effects on NK and T cell activation. The results showed that DC recruitment to sites of lung inflammation was normal in aged mice (>20 mo). However, IFN-γ-producing NK cells in LPS-challenged lungs were decreased in aged as compared with young mice, which was associated with increased IL-10+CD11b+Gr-1lowCD11c+ cells consistent with mononuclear phagocytes. In vivo or in vitro blockade of IL-10 signaling restored IFN-γ-producing NK cells. This restoration was reversed by IL-12 neutralization, indicating that IL-10 suppressed sources of IL-12 in aged mice. To probe DC function in adaptive immunity, we transferred young naive OVA-specific TCR transgenic T cells to old mice. Following challenge with OVA plus LPS, Ag presentation in the context of MHC-I and MHC-II occurred with similar kinetics and intensity in draining lymph nodes of young and old recipients as measured by proliferation. Despite this, aged hosts displayed impaired induction of IFN-γ+CD4+, but not IFN-γ+CD8+, effector T cells. Blockade of IL-10 signaling reversed age-associated defects. These studies indicate that the innate IL-12/IFN-γ axis is not intrinsically defective in lungs of aged mice, but is rather suppressed by enhanced production of mononuclear phagocyte-derived IL-10. Our data identify a novel mechanism of age-associated immune deficiency.

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Abbreviations used in this paper: PAMP, pathogen-associated molecular pattern; DC, dendritic cell; MLN, mediastinal lymph node; MPS, mononuclear phagocyte system; PPD, purified protein derivative.

In addition to limiting inflammatory responses, IL-10 is known to influence DC maturation. DCs that differentiate in the presence of IL-10 become poor APCs and preferentially induce anergy or IL-10-producing CD4 T cells (9–11). Thus, age-related increased IL-10 expression in the lung is likely to have a broad spectrum of effects.

Macrophage/dendritic cell (DC)-restricted bone marrow precursors give rise to both peripheral macrophages and DCs via blood monocyte intermediates (12, 13). These cells belong to a network that is referred to as the mononuclear phagocyte system (MPS). In peripheral tissues, cells of the MPS are principal sensors of PAMPs. Exposure to PAMPs induces rapid activation, migration, and functional differentiation of these cells. Activated cells of the MPS can remain in peripheral tissues and function as immune regulatory as well as immune effector cells (14, 15). A small subset of these cells become DCs and subsequently migrate to T cell areas of regional lymph nodes where they specialize in activating T cells (reviewed in Refs. 16–18). In peripheral tissues, Ags are phagocytosed by immature DCs, then subsequently processed into peptides, which are loaded onto MHC class I and II molecules for presentation on the surface membrane. In addition to Ag processing, immature DCs also respond to inflammatory mediators in peripheral tissues and reprogram their gene expression accordingly. Migration to the T cell zone of draining lymph nodes results from maturation-associated reprogramming of chemokine receptor expression. The T cell zone DCs play an important role in Th cell differentiation by providing CD4 T cells with high-affinity TCR ligands, costimulatory molecules, and cytokines (19).

At present, no molecular marker is available to specifically and directly monitor DC function. Consequently, little is known about the effect of aging on DC function in vivo. To circumvent this problem, we evaluated DC function indirectly by comparing downstream events known to be influenced by DC activity in young and aged mice. We first compared IFN-γ production by NK
cells during the innate response to LPS in vivo and in vitro. Interaction with pathogen products induces DCs to produce low levels of IL-12, which stimulates NK cells to secrete IFN-γ. The NK cell-derived IFN-γ then activates DCs to produce greater quantities of IL-12 when they encounter pathogens (20). This reciprocal stimulation forms a positive amplification loop that is often referred to as the innate IL-12/IFN-γ axis. NK cells are the only cell type that expresses high levels of STAT4 under steady-state conditions (21). This makes NK cells unique in their ability to produce IFN-γ rapidly in response to IL-12 (22, 23). We also compared proliferation of adoptively transferred naive TCR transgenic T cells to assess DC Ag-presenting function in mediastinal lymph nodes (MLNs). This indicator was chosen based on the fact that MHC class I- and class II-restricted Ag presentation by DCs are both necessary and sufficient for inducing proliferation of naive Ag-specific CD8 and CD4 T cells, respectively (24). Next, IFN-γ expression by activated donor TCR transgenic T cells was assessed as a measure of T effector cell maturation. This is a major downstream event that depends on IL-12 production by DCs. It has been estimated that IL-12 is responsible for up to 85–90% of the IFN-γ response by CD4 T cells, whereas the contribution by Notch is only responsible for 10–15% of this response (25). Our results demonstrate impaired NK cell activation and IFN-γ-producing CD4+ T effector cell differentiation in the aged mice that could be related to IL-10-mediated suppression of the innate IL-12/IFN-γ axis.

Materials and Methods

Mice

Male C57BL/6 mice 4–5 and 20–24 mo of age were obtained from the National Institute on 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 neoplasms. C57BL/6 mice expressing CD90.1 (B6.PL-Thy1a/CyJ) and Tg(OT-II)425Cbn TCR transgenic mice on a C57BL/6 background (OT-II) were purchased from The Jackson Laboratory. CD4+ T cells from the OT-II mice are specific for OVA peptide of amino acids 323–339 (EK-LTEWTSSNVMEER) in the context of I-Ab (26). OT-II TCR transgenic mice expressing CD90.1 on C57BL/6 background were bred in house using male OT-II and female B6.PL-Thy1a/CyJ mice. C57BL/6-Tg(OT-I)-RAG1 mice (OT-I) were purchased from Taconic Farms. The OT-I line is homozygous for a transgene that encodes a TCR specific for chicken OVA 257–264 presented by the MHC class I molecule H-2Kb. OT-I TCR transgenic mice expressing CD90.1 on C57BL/6 background were bred in-house using male OT-I and female B6.PL-Thy1a/CyJ mice. The University of Michigan Committee on Use and Care of Animals approved all animal studies.

Mouse models

For intranasal Ag challenge, mice were anesthetized by isoflurane inhalation, and 12 μg of OVA (grade V, from Sigma-Aldrich) and 3 μg of LPS (Sigma-Aldrich) in 15 μl of PBS were deposited intranasally. For intratracheal Ag challenge, mice were anesthetized with ketamine (dosed at 90 mg/kg; Hospira) and xylazine (dosed at 10 mg/kg; Ben Venue Laboratories) injected i.p. A
Figure 2. IL-10 production by mononuclear phagocytes in LPS-challenged lung increases with age. Young (4–5-mo-old) and aged (>20-mo-old) C57BL/6 mice were challenged with intratracheal administration of 1 μg of LPS in 15 μl of PBS. One day after the challenge, cells from the lung and MLN were stained for expression of CD11b, Gr-1, CD3e, CD19, CD11c, and intracellular IL-10 and analyzed by flow cytometry. A, Gated CD11b+ cells of the lung are shown to demonstrate that IL-10 was expressed by a subset of CD11b+Gr-1low cells. B, Gated CD3e, CD19, and autofluorescence-negative cells of the lung are shown to demonstrate that IL-10 was expressed by a subset of CD11c+ cells. Event counts shown in the quadrants are expressed as percentage of total events collected. No IL-10+ cells were found in CD3e+ and CD19+ cells (data not shown). No IL-10+ cells were found in the MLN (data not shown). Data are representative of three independent experiments. C, Numbers of total IL-10+ mononuclear phagocytes in the lung are presented as means ± SD derived from six individual mice. The number of IL-10+ mononuclear phagocytes in the lung was increased significantly in both naïve and challenged aged mice.

In vivo MHC-I- and MHC-II-restriction Ag presentation assay

CD4+ and CD8+ T cells were enriched from spleens of OT-II and OT-I transgenic mice by using CD4 and CD8 T cell-negative enrichment kits (Miltenyi Biotec), respectively. For CFSE labeling, cells were resuspended at a concentration of 107 cells/ml in PBS (Sigma-Aldrich) containing 0.01% BSA (Sigma-Aldrich). CFSE (Invitrogen) was added to the single-cell suspension at a final concentration of 5 μM and incubated for 10 min at 37°C. After incubation, the cells were washed once with complete RPMI 1640 (Sigma-Aldrich) and once with PBS. The cells were then checked for viability and fluorescent labeling using a fluorescence microscope. Five million viable TCR-transgenic T cells were resuspended in PBS and transferred i.v. into each syngeneic recipient mouse. Cells were recovered from lymphoid tissues of the recipient mice 3 or 6 days after transfer and analyzed by flow cytometry.

Figure 3. IFN-γ production by NK cells in LPS-challenged lung is impaired in aged mice. Young (4–5-mo-old) and aged (>20-mo-old) C57BL/6 mice were given intratracheal administration of 1 μg of LPS in 15 μl of PBS. One day after challenge, cells from the lung and MLN were stained for expression of CD3e, CD19, NK1.1, and intracellular IFN-γ and analyzed by flow cytometry. A, Gated CD3e, CD19, and autofluorescence-negative cells of the lung are shown to demonstrate that IFN-γ was expressed by a subset of NK1.1+CD3e- cells in young mice. Many fewer IFN-γ-producing cells were found in aged mice. Event counts shown in the quadrants are expressed as percentage of gated events. No IFN-γ+ cells were found among CD3e+ and CD19+ cells in the lungs of both young and aged mice (data not shown). No IFN-γ+ cells were found in the MLN (data not shown). Data are representative of two independent experiments. B, Total numbers of NK cells (NK1.1+CD3e-) and IFN-γ+ NK cells in the lung. Bars are means ± SD derived from four individual mice. The number of IFN-γ+ NK cells in aged mice was significantly lower than in young mice.

Single cell preparation and flow cytometry

Single-cell suspensions were prepared from lungs as described previously (27). Briefly, after perfusion with cold RPMI 1640, lungs were excised, placed in cold RPMI 1640 medium, and then homogenized for 15 s in a Waring blender. Homogenates were incubated with digestion medium containing RPMI 1640, 10% FBS (Atlanta Biologicals), and 250 U/ml type IV collagenase (Sigma-Aldrich) for 10 min at 37°C. The digest was sieved through a 40-μm cell strainer (BD Biosciences) and washed twice by centrifugation in RPMI 1640. Single-cell suspensions were also prepared from freshly harvested spleens and lymph nodes by mechanical dissociation as previously described (27). Total cell yields were determined by standard hemocytometric counting.

mAbs used included FITC-, PE- and PE-Cy5-conjugated anti-CD4 (GK1.5), anti-CD8a (53-6.7), anti-CD62L (MEL-14), anti-IFN-γ (R46A2 and XMG1.2), anti-IL-4 (11B11 and BVD6-24G2), anti-IL-10 (JES5-16E3), anti-IL-12p40 (C15.6 and C17.8), anti-CD11b/Mac-1 (M1/70), anti-CD40 (3/23), anti-Ly-6G and Ly-6C (Gr-1, clone RB6-8C5), anti-CD11c (HL3 and N418), anti-IA-Aβ (11-5.2), anti-F4/80 (A3-1), anti-CD90.1 (HS51), and anti-CD90.2 (53-2.1) (from BD Pharmingen, Serotec, or eBioscience). All isotype controls and anti-CD16/CD32 (2.4G2) were from BD Pharmingen. After blocking with anti-CD16/CD32 (2.4G2) from BD Pharmingen. After blocking with anti-CD16/CD32 for 5 min, cells were stained with fluorescent-labeled Abs or isotype control Abs in 2%}

Figure 4. Blockade of IL-10 signaling mobilizes IFN-γ-producing NK cells in LPS-challenged lung in aged mice. Aged (>20-mo-old) C57BL/6 mice were treated with i.p. administration of 400 μg of monoclonal anti-IL-10R-blocking Ab (clone 1B1.3a) or control rat IgG in 0.5 ml PBS 3–4 h before LPS challenge. Mice were then challenged and analyzed as in Fig. 3. A, Gated CD3e, CD19, and autofluorescence-negative cells in the lung are shown to demonstrate that IFN-γ was expressed by a subset of NK1.1+CD3e- cells. Event counts shown in the quadrants are expressed as percentage of gated events. Data are representative of two independent experiments. B, Total numbers of NK cells (NK1.1+CD3e-) and IFN-γ+ NK cells in the lung. Bars are means ± SD derived from four individual mice. The number of IFN-γ+ NK cells in anti-IL-10R-treated mice was significantly greater than in control-treated mice.
were emobilized to the lungs of mice at 6, 18, and 24 mo of age. This experimental approach has been used successfully to mobilize and visualize CD11c+ myeloid DCs to sites of inflammation in the lung (28–30). Although CD11c does not specifically define DCs, in the mouse it serves as the best available surrogate marker, and Ag bead-associated CD11c+ cells have been shown to display many properties of DCs, including morphology and the ability to prime naive T cells upon adoptive transfer (28–30). As previously reported (8), the extent of cellular recruitment induced by Ag-coated beads was not significantly different among age groups (data not shown). We then conducted flow cytometry analysis to determine the proportion of myeloid DCs in the lung. As shown in Fig. 1A, myeloid DCs in the lung were identified by expression of CD11c+ cell populations. Bars are means ± SD derived from two independent experiments (n = 4). The number of NK1.1+ IFN-γ cells in the control culture was significantly lower in aged mice.

Statistical analysis

Student’s t test was used for direct comparison to a parallel control group with p < 0.05 considered to indicate significance.

Results

DCs are recruited normally to sites of inflammation in the lung of aged mice

We first compared the recruitment of myeloid DCs in the lungs of young and aged mice. Beads coupled to Mycobacterium bovis PPD were emobilized to the lungs of mice at 6, 18, and 24 mo of age. This experimental approach has been used successfully to mobilize and visualize CD11c+ myeloid DCs to sites of inflammation in the lung (28–30). Although CD11c does not specifically define DCs, in the mouse it serves as the best available surrogate marker, and Ag bead-associated CD11c+ cells have been shown to display many properties of DCs, including morphology and the ability to prime naive T cells upon adoptive transfer (28–30). As previously reported (8), the extent of cellular recruitment induced by Ag-coated beads was not significantly different among age groups (data not shown). We then conducted flow cytometry analysis to determine the proportion of myeloid DCs in the lung. As shown in Fig. 1A, myeloid DCs in the lung were identified by expression of CD11b, CD11c, and MHC-II, as previously described (28, 29, 31). In all three age groups, there was a rapid 3- to 4-fold increase in the proportion of myeloid DCs in the lung, and the increase was maintained throughout the study period, demonstrating that myeloid DCs are among the leukocytes recruited to the site of

FBS-PBS buffer. A FACScan flow cyrometer with CellQuest software (BD Biosciences) was used for data acquisition and analysis.

Intracellular flow cytometry analysis of lung and MLN cells was performed as described previously (8). Briefly, single-cell suspensions were incubated for 3 h in complete medium in the presence of breflidin A (1 µg/ml) (BD Pharmingen). In some cases, cells were stimulated in culture before adding breflidin A. Cells were harvested and stained for surface Ag expression before staining for intracellular cytokines. Intracellular cytokine staining was performed using a Cytofix/Cytoperm kit (BD Pharmingen) according to the manufacturer’s directions.

Student’s t test was used for direct comparison to a parallel control group with p < 0.05 considered to indicate significance.

Results

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inflammation (Fig. 1A). However, at all the time points examined, both the proportions and total numbers of DCs in the lungs of different age groups were not significantly different (Fig. 1A). We next conducted immunohistochemical analysis to detect CD11c⁺ DCs in situ. Beads that were coupled to various types of Ags were used. In all cases, we found abundant CD11c⁺ cells at sites of bead-induced inflammation in both young and aged mice including OVA-coupled beads, which induce reduced levels of inflammation compared with pathogen-associated Ags (28). DCs were detected immunohistochemically as a cuff of cells around beads in both young and aged mice (Fig. 1B). As described previously and as shown in Fig. 1B, although CD11c⁺ cells represent only a very small percentage of the total lung cells, they concentrate at sites of Ag bead deposition (28–30). These data suggest that recruitment of myeloid DCs to sites of inflammation in the lung is not significantly impaired by aging.

The number of IL-10-producing mononuclear phagocytes in LPS-challenged lungs increases with age

To determine the effect of aging on IL-10 expression in the lung, we compared LPS-challenged lungs of young and aged mice. Innate cytokine responses are known to be rapid and transient. For innate cytokine analysis, mice were challenged with a single intratracheal administration of LPS to control the timing and dose of the challenge for analysis of early activation events. Aged mice responded vigorously to intratracheal LPS challenge and, consistent with our previous findings in PPD bead-challenged lungs (8), IL-10 was expressed by a subset of CD11b⁺Gr-1⁺/⁻...
mononuclear phagocytes in LPS-challenged lungs (Fig. 2A). Additionally, the data showed that IL-10 was expressed by CD11c<sup>−</sup> and not by CD11c<sup>+</sup> cells in the LPS-challenged lungs (Fig. 2B). Upon quantification, the total number of mononuclear phagocytes (CD11b<sup>+</sup>Gr-1<sup>+/−</sup>) in naive or challenged lungs did not significantly change with age (data not shown). However, the baseline number of IL-10-producing mononuclear phagocytes was 2-fold greater in aged than in young mice and increased nearly 3-fold upon LPS challenge (Fig. 2C). In contrast to the lung, IL-10-producing cells were not detected in the draining MLN (data not shown). These data demonstrate that although the total number of recruited mononuclear phagocytes in LPS-challenged lungs does not change with age, the proportion of IL-10-producing mononuclear phagocytes increases, potentially altering the milieu of innate maturation signals in the lung.

**Blockade of IL-10 signaling mobilizes IFN-γ-producing NK cells in LPS-challenged lung in aged mice**

DCs are an important source of IL-12 that can activate NK cells (20). Since IL-12 is a functional heterodimer and shares subunits with other cytokines, it cannot be monitored by cytokine staining. To determine the potential effect of aging on IL-12 activity in the lung, we used an indirect approach comparing IFN-γ production by NK cells in LPS-challenged lungs of young and aged mice because IL-12 is necessary for NK cell activation in response to LPS challenge (32, 33). As shown in Fig. 3, although the total number of NK cells did not change with age, the number of IFN-γ-producing NK cells was significantly decreased in aged mice as compared with young mice. Furthermore, blockade of IL-10 signaling in vivo completely restored the number of IFN-γ-producing NK cells in LPS-challenged lungs of aged mice (Fig. 4). No IFN-γ producers were detected among CD3<sup>+</sup> (T cell) or CD19<sup>+</sup> (B cell) populations (data not shown). No IFN-γ producers were found in the MLN in both young and aged mice (data not shown). These data demonstrate that IFN-γ production by NK cells is notably impaired in LPS-challenged lung of aged mice. Blockade of IL-10 signaling effectively mobilized IFN-γ-producing NK cells in aged mice.

The innate IL-12/IFN-γ axis is not intrinsically defective but is suppressed by IL-10

To determine whether NK cells from aged mice were intrinsically defective in IFN-γ production, we analyzed NK cell responses in vitro. Mature peripheral NK cells are terminally differentiated innate effector cells that produce IFN-γ and kill target cells immediately upon activation (reviewed in Ref. 34). These cells are primarily found in the blood, the red pulp of the spleen, and in sinusoidal regions of the liver. We used the spleen as a source of mature NK cells. As shown in Fig. 5A, splenocytes from aged mice produced significantly less IFN-γ in overnight culture supernatants than did those from young mice. However, similar to the in vivo lung analysis, blockade of IL-10 signaling dramatically increased the level of IFN-γ production in aged mice. As expected, blockade of IL-10 signaling also increased IFN-γ production in young mice (Fig. 5B).

To identify sources of IFN-γ among splenocytes, aged mice were stimulated overnight with LPS in culture in the presence of anti-IL-10R and subjected to flow cytometric analysis. Brefeldin A was added in the final 3 h of the culture to allow intracellular accumulation of cytokines. The cells were stained for surface expression of CD3e, CD19, MHC-II, CD11c, NK1.1, and CD8α and intracellular expression of IFN-γ or an isotype control. IFN-γ<sup>+</sup> cells were readily detectable. Nearly all IFN-γ<sup>+</sup> cells were NK1.1<sup>+</sup> (Fig. 5C, upper panel) but CD3e<sup>−</sup>CD19<sup>−</sup> and MHC-II<sup>−</sup> (Fig. 5D, upper and lower panels) and CD11c<sup>−</sup> (Fig. 5E, upper panel). The NK1.1<sup>+</sup>IFN-γ<sup>+</sup> cells were either CD8α<sup>+</sup> or CD8α<sup>−</sup> subtypes (Fig. 5C, lower panel). Quantitatively, NK1.1<sup>+</sup> cells comprised >90% of the IFN-γ<sup>+</sup> cells in anti-IL-10R-treated cultures (Fig. 5F). These data demonstrated that NK cells were the major source of innate IFN-γ production and that blockade of IL-10 signaling augments production.

It is known that LPS-elicited IL-10 production by spleen-derived mononuclear phagocytes increases with age (3–8). In accord with this, we consistently found that mononuclear phagocytes were primary sources of IL-10, and the number of IL-10-producing mononuclear phagocytes increased significantly with age (data not shown). In young mice, IL-10-mediated suppression of NK cell-derived IFN-γ...
has been shown to be due to indirect suppression of IL-12 (20). To test if this was the case in aged mice, we compared splenocytes stimulated overnight with LPS in the presence or absence of IL-10R-blocking Ab (anti-IL-10R). Few IFN-γ+ cells were found in stimulated old mouse cell cultures treated with control Ab (Fig. 5G). However, IFN-γ+ NK cells were readily detectable when IL-10R-blocking Ab was added (Fig. 5G). Note that although DCs are reported sources of innate IFN-γ (35), blockade of IL-10 signaling specifically induced IFN-γ among NK cells under these conditions (Fig. 5G). Also note that IL-12p40 was expressed exclusively by CD11c+ cells, consistent with DCs being the primary source of IL-12 (data not shown). These data demonstrate that IFN-γ expression by NK cells is exquisitely sensitive to IL-10-mediated suppression. When IL-10R-blocking Ab was added with IL-12-neutralizing Ab, no IFN-γ+ cells were found (Fig. 5G), demonstrating that IL-10-mediated suppression of IFN-γ production by NK cells was likely mediated via regulation of IL-12.

Finally, we compared IFN-γ production by NK cells in response to LPS and IL-12 stimulation in vitro. Splenocytes from aged mice were stimulated for 16 h with LPS in the presence or absence of anti-IL-10R or with recombinant IL-12 in culture. Brefeldin A was added in the final 4 h of the culture. Because the number of IFN-γ+ NK cells decayed by 24 h in both young and aged spleens, we performed the analysis at 16 h. Cells were harvested and analyzed by flow cytometry. Again, NK cells were found to be the main source of IFN-γ+ producers in both young and aged mice (data not shown). As shown in Fig. 5H, there were significantly lower numbers of IFN-γ+ NK cells in aged splenocyte cultures stimulated with LPS alone. However, in the presence of anti-IL-10R, the number of IFN-γ+ NK cells in both young and aged mice increased dramatically, with no significant difference between young and aged mice. Importantly, recombinant IL-12 alone overcame the defect, with large numbers of IFN-γ+ NK cells generated in both young and aged mice with no significant difference between groups (Fig. 5H). Taken together, the data suggest that neither NK cells nor the innate IL-12/IFN-γ axis in aged mice is intrinsically defective, but they are rather suppressed by mononuclear phagocyte-derived IL-10 in aged mice in response to LPS challenge.

Airway Ags are presented in draining lymph nodes of young and aged mice with similar kinetics and intensity

Based on the fact that engagement of TCRs by high-affinity MHC-peptide complexes is required for T cell proliferation, in vivo bioassays have been developed to detect MHC class I- and class II-restricted Ag presentation in secondary lymphoid tissues of the
mouse by using naive TCR transgenic CD8 and CD4 T cells, respectively (24, 36). This approach has been successfully used to evaluate DC functions in vivo by many investigators as an alternative to direct measurement of the expression of Ag-specific MHC-peptide complexes, which is currently not possible. After i.v. injection, naive TCR transgenic T cells quickly enter the T cell area of the secondary lymphoid tissues where they proliferate upon encounter with high-affinity MHC-peptide complexes expressed by Ag-presenting DCs.

In the mouse, it is generally accepted that adaptive immunity to pulmonary Ags develops primarily in draining MLN (37). In young mice, Ag-loaded T cell zone myeloid DCs appear in the draining MLN within hours of Ag deposition in the lung (38). Small amounts of inhaled aerosolized protein Ag can induce rapid T cell proliferation in the MLN (36). To determine the effect of aging on MHC class II-restricted presentation of airway Ags, young (4–5 mo) and aged (>20 mo) C57BL/6 mice were given intranasal administration of OVA plus LPS in 15 µl of PBS on days 0, 1, and 2. The mice were then injected i.v. with CFSE-labeled naive young OT-II cells isolated from young, naive, OT-II TCR transgenic, CD90.1 congenic mice at various time points after the OVA exposure to determine the kinetics of MHC-II-restricted Ag presentation. Three days after the OT-II cell injection, the donor cells (CD90.1+) were recovered from the spleen and MLN of the recipients and analyzed by flow cytometry. Proliferation of the OT-II cells was measured by their expression by activated naive OT-II T cells, not CD8 effector T cells, is impaired by aging

To further evaluate the innate IL-12/IFN-γ axis in vivo in aged mice, we compared IFN-γ expression by activated naive OT-II and OT-I cells in the MLN of young and aged mice because IL-12 production is required for Th1 differentiation (25). Mice were given intranasal administration of OVA plus LPS in 15 µl of PBS on days 0, 1, and 2. Naive, young OT-I cells, or OT-I transgenic cells were transferred on day 0 and recovered from the MLN on day 6. Although the total number of OT-II cells recovered from the MLN was not different between the young and aged mice, the proportion of IFN-γ-producing OT-II cells was 5-fold lower in the aged hosts than in the young hosts (Fig. 8A). In contrast, when OT-I cells were transferred and analyzed, the proportion of IFN-γ-producing OT-I cells was not affected by the age of the hosts (Fig. 8B). These data suggest that, as compared with young mice, the host environment of aged mice is less conducive to Th1 than Tc1 effector cell development in response to airway Ag challenge.

FIGURE 9. Blockade of IL-10 signaling rescues priming of IFN-γ-producing young OT-II cells in the MLN in response to intranasal LPS challenge in aged mice. Aged (>20-mo-old) C57BL/6 mice were treated with i.p. administration of 400 µg of monoclonal anti-IL-10R-blocking Ab (clone 1B1.3a) or nonspecific rat IgG in 0.5 ml saline 3–4 h before intranasal LPS plus OVA challenge. The mice were injected i.v. with young, naive OT-II T cells expressing CD90.1 on day 0 and given intranasal administration of 12 µg OVA with 3 µg of LPS in 15 µl of PBS on days 0, 1, and 2. On day 6, cells from the MLN were harvested and cultured for 3 h with OVA323–339 (OT-II) peptide in the presence of brefeldin A. The cells were stained for expression of CD90.1, intracellular IFN-γ, and CD4 and analyzed by flow cytometry. CD90.1+ CD4+ (OT-II) cells and IFN-γ+ OT-II cells are identified among total and gated CD90.1+ MLN cells, respectively. Data are representative of two independent experiments. Bars represent means ± SD derived from four individual mice. The number of IFN-γ+ OT-II cells increased significantly with anti-IL-10R treatment.

Priming of IFN-γ-producing CD4, but not CD8 effector T cells, is impaired by aging
Blockade of IL-10 signaling enhances suppression of IFN-γ-producing young OT-II cells in aged mice

To test if Th1 cell priming was suppressed by IL-10, we treated aged mice with i.p. administration of anti-IL-10R-blocking Ab (anti-CD210, clone 1B1.3a, BD Pharmingen) before LPS plus OVA challenge. Control mice were treated with nonspecific rat IgG. The mice were then given intranasal administration of OVA plus LPS in 15 μl of PBS on days 0, 1, and 2. Naive OT-II cells were transferred on day 0 and recovered from the MLN on day 6. The total number of OT-II cells recovered from the MLN was not significantly different between the anti-IL-10R-treated and IgG-treated mice. In contrast, the proportion of IFN-γ-producing OT-II cells was significantly increased by anti-IL-10R treatment (Fig. 9). These data demonstrate that blockade of IL-10 signaling effectively enhanced Th1 cell priming by DCs in response to intranasal LPS challenge, but further studies will be required to determine precise molecular mechanisms.

Discussion

Despite the important role of DCs in both immunity and tolerance, little is known about the effect of aging on DC function in vivo. To our knowledge, this study represents the first comprehensive evaluation of the innate immune environment in the respiratory system of aged mice. We used a well-characterized model of innate inflammation to assess DC recruitment. Local DC-related function in the lung was evaluated indirectly in vivo by measuring the activation of endogenous NK cells. Finally, regional DC functions in the MLN were evaluated indirectly in vivo by measuring the proliferation and differentiation of adoptively transferred transgenic T cells in the MLN. We demonstrated that aging does not impair DC accumulation at sites of inflammation in the lung, but the data suggest that increased production of IL-10 by mononuclear phagocytes leads to enhanced suppression of the innate IL-12/IFN-γ axis in lungs of aged as compared with young mice. It was found that lung-challenged aged mice displayed local impairment of NK cell activation, and although they did not show impairment of Ag processing and DC migration to the draining lymph node as judged by both the intensity and kinetics of MHC-restricted Ag presentation, they failed to adequately induce the expression of IFN-γ by Ag-specific CD4 T cells. Importantly, our data showed that the innate IL-12/IFN-γ axis was not intrinsically defective in aged mice, but rather its function was likely suppressed by mononuclear phagocyte-derived IL-10. Although excessive production of IL-10 by mononuclear phagocytes in aged mice is well documented (3–8), our study is the first to demonstrate that blockade of IL-10 signaling in aged mice can reverse deficiency in innate IFN-γ production and thus boost Th1 cell priming.

For optimal responses to pathogens that infect peripheral tissues, DC localization at the site of infection is essential for optimal Ag capture and DC maturation. These events are thought to be regulated by inflammatory mediators including adhesion molecules, chemokines, and cytokines. However, DC recruitment is likely to be mediated by multiple redundant pathways (28). It is therefore not surprising that aging does not significantly impair DC recruitment. DCs were defined originally in T cell areas of secondary lymphoid tissues in mice (39, 40). It is now clear that these cells are critical to activation of naïve T cells in secondary lymphoid tissues (41). In peripheral tissues, cells with similar morphology and surface Ag profile are often referred to as DCs. However, while these cells process Ags locally, they must migrate to lymph nodes since peripheral tissue DCs do not activate naïve T cells because naïve T cells are excluded from peripheral tissues. There is convincing evidence that a major peripheral tissue function of DCs is to regulate NK cell activation (reviewed in Refs. 42, 43). Moreover, a decline of inducible NK cell function in aged mice is recognized but the mechanism remains unclear (44). Our findings suggest that IL-10-CD11c- macrophages regulate NK activation signals produced by lung DCs. Although lung DCs (CD11b+ CD11c+ MHC-II+) share many phenotypic properties with other mononuclear phagocytes, including the expression of CD11b, F4/80, and MHC-II (14), there are clearly unique functions associated with CD11c expression. For example, we found that IL-12p40 is produced exclusively by CD11c+ DCs in the lung (data not shown), whereas IL-10 is produced by CD11c- mononuclear phagocytes.

In the absence of severe infection, increased IL-10 production may benefit aged individuals by limiting tissue damage. Consistent with this notion, it has been shown that -1082G homozygous genotype, which confers high IL-10 production, significantly increases lifespan in humans. Despite enhanced IL-10 production, aged mice are hypersensitive to LPS-induced lethality and cytokine production (45). The 50% lethal dose of LPS in aged mice is 6-fold lower than that in young mice (45). Interestingly, if IL-10 signaling is blocked, we have found that sublethal systemic doses of LPS become lethal in old mice but not young mice, suggesting that increased IL-10 production is critical and protective in old mice (B. C. Chiu, unpublished data). However, with some infectious conditions, the protective role of IL-10 may be a liability. Studies of Cooper et al. showed that in Mycobacterium tuberculosis-infected lung of aged mice, IL-12 expression was severely compromised (46). Treatment of M. tuberculosis-infected aged mice with exogenous IL-12 during the innate phase increased their immunity to the infection (46). Our data suggest that blockade of IL-10 signaling may enhance immunity against infection in aged individuals. Much like the balance between tissue regeneration and risk of developing neoplasm, IL-10-mediated immune suppression likely represents an evolutionary compromise.

Examples of age-associated enhanced responses include transiently enhanced innate resistance to M. tuberculosis (47) and enhanced resistance to systemically administered Listeria monocytogenes (48). The enhanced innate resistance to M. tuberculosis in aged mice was mediated by IFN-γ-producing Ag-nonspecific CD8 T cells (47, 49). However, IFN-γ production by these unconventional CD8 T cells is likely to be driven by cytokines other than IL-12 because IL-12 production is significantly impaired in IL-12/IL-23-deficient mice despite enhanced IL-10 production (46). The innate resistance to systemic L. monocytogenes infection is mediated by neutrophils (50). Although neutrophil response is subject to IL-10-mediated regulation, it is enhanced in aged mice despite enhanced IL-10 production (8). It is not yet clear why the neutrophil response in aged mice is enhanced. Taken together, these data suggest that the innate IL-12/IFN-γ axis may be more sensitive to IL-10-mediated suppression than other innate responses.

Mononuclear phagocyte-derived IL-10, while recognized for several years, has received relatively little in vivo analysis with regard to it potential regulatory impact. In contrast to T cells, mononuclear phagocytes produce IL-10 only transiently. In our in vitro culture studies, we found that IL-10 was produced only in the first 6 h of LPS stimulation (data not shown). Despite low levels of expression at the whole organ level, innate cytokines are known to have profound influence on immune responses. Because NK cells, DCs, and other mononuclear phagocytes colocalize at sites of inflammation, IL-10 delivered in an organ microenvironment at an appropriate time is likely sufficient to produce significant regulatory effects.
A key remaining question is what causes the age-associated increase in the number of IL-10-producing mononuclear phagocytes. Cells of the MPS in other sites such as peritoneal cavity and spleen also show an age-related increase in IL-10 production in culture supernatant when stimulated with TLR ligands (3–6). These results have been interpreted as reflecting an intrinsic age-acquired macrophage dysfunction. Altered gene expression appeared to be associated with impaired intracellular signaling. However, those studies showing age-associated impairments of mitogen-activated protein kinases have been disputed by more recent studies (5).

In the secondary lymphoid organs, the potent ability to induce naïve T cell proliferation and gene expression makes DCs unique. Most previous studies compared tissue resident DCs in young and aged individuals or focused on in vitro-generated DCs. The relationship between these cells to DCs responding to inflammation is not known. Linton et al. analyzed in vivo Ag presentation in the spleen using TCR transgenic T cells following systemic challenge (51). That study found reduced transgenic T cell expansion and differentiation in aged hosts as compared with young hosts. The defect appeared to be associated with impaired migration of both T cells and DCs to the T cell zones of the spleen. The age-associated increase in IL-10 production following systemic pathogen infection is well known (3–6, 45). Therefore, IL-10-mediated suppression of DC maturation may contribute to impaired localization of the DCs in the spleen. However, in our study we did not find reduced transgenic T cell expansion in lymph nodes of aged mice. Thus, the nature of the DC maturation defect may vary depending on the organ system and the type of Ag challenge. It will be interesting to test if blockade of IL-10 signaling can reverse age-associated defects in Ag presentation and T cell priming under other conditions. IL-10 is known to inhibit LPS-induced up-regulation of MHC-II and costimulatory molecules on monocytes and DCs (reviewed in Ref. 9). It may be surprising that, with increased IL-10 production, aging had little effect on both MHC-II- and MHC-I-restricted Ag presentation as measured by T cell proliferation. However, T cell proliferation requires minimal up-regulation of MHC and costimulatory molecules (17, 36). Indeed, substantial levels of T cell proliferation precede IL-10-induced T cell tolerance (11).

In contrast to CD4 T cells, CD8 T cells produce IFN-γ following primary activation (52). Furthermore, TCR-dependent IFN-γ production is unaltered in STAT4- and T-bet-deficient CD8 T cells compared with wild-type controls (53, 54). These data suggest that the innate IL-12/IFN-γ response is not involved in regulating IFN-γ gene expression by CD8 T cells, which may explain why the age-associated defect in the innate IL-12/IFN-γ response did not affect development of IFN-γ-producing CD8 T cells. Consistent with our findings, previous studies using TCR transgenic CD8 T cells found no age-associated defects in CD8 T cell response in mice challenged with tumor cells (55).

Aging is associated with increased susceptibility to respiratory infections. The mechanism is not well understood. In mouse models of infection, age-related changes in host response are widespread and complex. Additionally, the kinetics of the response differ with primary viral (56) and bacterial infection (47) in aged mice. Clearly, simpler models are needed to dissect mechanisms. LPS strongly stimulates cells of the mononuclear phagocyte system. Models of LPS challenge are well suited for mechanistic studies because they are short-lived and without complicated kinetics. Our data suggest that the age-associated increase in IL-10 production by mononuclear phagocytes in the lung may have broad suppressive effects ranging from impairing local innate NK effector function to reduced CD4 Th1 cell priming. Targeting IL-10 may improve not only effector function but also vaccine efficacy in elderly humans.

Disclosures

The authors have no financial conflicts of interest.

References


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