The Role of Toll-like Receptors in Age-Associated Lung Diseases

Maria Volkova,1 Yitao Zhang,1 Albert C. Shaw,2 and Patty J. Lee1

1Department of Internal Medicine and Section of Pulmonary and Critical Care Medicine and 2Section of Infectious Diseases, Yale University School of Medicine, New Haven, Connecticut.

Address correspondence to Patty J. Lee, MD, Department of Internal Medicine and Section of Pulmonary and Critical Care Medicine, Yale University School of Medicine, 333 Cedar Street, PO Box 208057, New Haven, CT 06520. Email: patty.lee@yale.edu

The aging lung is faced with unique challenges. The lungs are the only internal organ with a direct interface with both the internal and the external environments and as a consequence are constantly sampling diverse, potentially harmful elements. Therefore, the lungs have evolved a sophisticated, multilayered detection system to distinguish low-level, nonharmful signals from those that are toxic. A family of innate immune receptors, Toll-like receptors (TLRs), appears to serve such a function. Initially described as pattern-recognition receptors that recognize and protect against microbes, TLRs can also respond to diverse, nonmicrobial signals. The role of Toll-like receptors in noninfectious, age-related chronic lung disease is poorly understood. This review presents our current understanding of the biology of age-related lung diseases with a focus on the role of Toll-like receptors in idiopathic pulmonary fibrosis, chronic obstructive pulmonary disease, and late-onset asthma.

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AGING is associated with a range of lung diseases. The basis of age-related lung pathology is poorly understood. Older individuals have accumulated exposures to environmental insults such as pollutants, allergens, and infections that can injure the lung and adversely impact lung repair responses. Aging is also associated with increased proinflammatory cytokine production (1) and the lungs manifest some degree of inflammation even in otherwise healthy, older individuals (2). Age-related deteriorations in immunity, termed “immunosenescence,” are presumably responsible for the increased susceptibility to infections and neoplasm in older individuals (3). Little is known about the impact of immunosenescence on lung diseases. Immunity is generally referred to as innate or adaptive immunity, with the former characterized by rapid, less-specific immune responses to infectious agents and the latter characterized by delayed, more specific responses that generate immunologic memory. The Toll-like receptors (TLRs) are pattern recognition receptors that link innate and adaptive immunity. This review will explore the role of TLRs on common, age-related lung diseases.

TLRs are pattern-recognition receptors that also include nucleotide oligomerization domain (Nod)–like receptors (NLRs) and retinoic acid–inducible gene I–like receptors (Table 1). These receptors are frontline defense against infections and trigger intracellular signaling pathways of innate immunity and inflammation. TLRs are associated with membranes either at the cell surface or in endosomal compartments; they are expressed in a wide range of immune cells in the lung, such as alveolar macrophages and dendritic cells, as well as in the pulmonary epithelium and endothelium (4). TLRs recognize not only a range of microbial products termed pathogen-associated molecular patterns but also some endogenous ligands mostly released by dying or necrotic cells and associated with inflammation, which have been termed danger-associated molecular patterns. To date, 13 TLRs have been identified in mice and 10 in humans. TLR activation leads to proinflammatory cytokine response via nuclear factor-κB–dependent pathways and the upregulation of type I interferons and interferon-dependent genes (5).

NLRs are cytoplasmic receptors, in contrast to the membrane-associated TLRs (6). NLRs act as scaffolding proteins that assemble signaling platforms and trigger nuclear factor-κB and mitogen-activated protein kinase–mediated activation of inflammatory caspases. There are more than 20 NLR family members, and they recognize primarily bacterial structures (7,8). NLR1 and NLR2 are the best-characterized members of the NLR subfamily.
Both of them recognize a different subunit of bacterial proteoglycans (7,9). Interestingly, both NLR1 and NLR2 are highly expressed in epithelial cells, suggesting that similar to TLR4 they could mediate the cross-talk between epithelial cells and other immune cells in the airway. NLR2 plays an important role in chronic obstructive pulmonary disease (COPD)–induced mortality due to bacterial pathogens; impaired NLR2 expression deteriorates lung epithelial barrier function and impairs microorganism clearance (10). Retinoic acid–inducible gene I–like receptors are important in viral sensing and clearance, as reviewed elsewhere in this issue (11). We will focus on the impact of aging on TLRs and implications for the pathogenesis of age-related lung diseases such as idiopathic pulmonary fibrosis (IPF), COPD, and late-onset asthma (Table 1).

**TLRs and Aging**

One of the best-understood TLRs is TLR4, which is involved in recognition of the lipopolysaccharide (LPS) moiety of gram-negative bacteria, heat-shock proteins, hyaluronic acid, β-defensin-2, oxidized low-density lipoprotein, fibronectin, and amyloid peptide (12). One of the first attempts to characterize TLR changes with aging in mice was performed by Renshaw and colleagues (13) who compared macrophage TLR gene expression between young (2–3 month) and old (18–24 month) C57BL/6 mice. They found not only decreased TLR expression but also functional reduction in downstream TLR targets. However, other studies in C57BL/6 mice carried out using gene expression microarray analysis revealed relatively preserved gene expression for several TLR family members but decreased levels of TLR signaling intermediates. Decreases in signaling intermediates included components of the nuclear factor-kB cascade as well as a paradoxical increase in p38 mitogen-activated protein kinase expression and LPS-induced phosphorylation in macrophages from aged mice (14,15). In aged Balb/c mice, TLR2 and TLR4 expression in splenic macrophages appeared preserved, but impaired cytokine production and decreases in TLR-induced total and phosphorylated p38 protein were noted (16). Beyond potential effects of genetic background, these studies in aged mice suggest impairment in TLR signal transduction, as well as potential positive and negative regulatory roles for p38 mitogen-activated protein kinase.

The largest study (159 participants) to date in human monocytes revealed an age-associated decreased in TLR1/2-induced production of tumor necrosis factor α and interleukin-6 that was strongly associated with decreased TLR1, but not TLR2 surface expression, and a decrease in p38 phosphorylation (17,18). Of note, decreased TLR8-induced IL-6 production was also observed in monocytes from older, compared with young individuals. In this study, whereas a small but statistically significant decrease in surface TLR4 expression in monocytes from older individuals was observed, no change in LPS-induced cytokine production was found; however, these results do not exclude age-associated alterations under different conditions (eg, threshold signaling effects elucidated with differing concentrations of TLR agonists, as detailed subsequently). A TLR1/2 functional defect in cells from older individuals was recently observed in a second study of human monocytes that evaluated developmental subsets stratified by CD14 and CD16 expression (19).

Age-associated alterations in TLR function in human dendritic cells have also been found. In the largest of these studies (n = 104), decreased TLR-induced cytokine production (as assessed via intracellular cytokine staining) was observed for virtually all TLRs assessed (TLR1/2, TLR2/6, TLR3, TLR5, and TLR8) in myeloid dendritic cells (mDCs)—which express a wide range of TLRs and are critical for the generation of Th1 responses) and plasmacytoid dendritic cells (pDCs—which express a more limited range of TLRs—mainly TLR7 and TLR9—and are particularly adept at producing type I interferons in response to viral infection, 20). Such age-associated TLR functional defects were also observed for pDCs in a smaller study of TLR function (n = 37), and mirror age-associated alterations observed for murine pDCs (21). Human mDC function appeared preserved; however, mDCs from older or young individuals were pooled for analysis, potentially obscuring age-associated differences (22).

Taken together, these results suggest that immunosenescence affects the innate immune system and TLR function, in particular for both monocyte and dendritic cell populations in humans. On the other hand, there is evidence for a paradoxically heightened proinflammatory environment in the context of human aging, with elevated levels of cytokines and acute phase reactants associated with functional decline—termed the “inflamm-aging” hypothesis (23). In this regard, Panda and colleagues (20) found substantially elevated levels of basal intracellular cytokine production in the absence of TLR stimulation in both mDCs and pDCs from older, but not young, individuals—suggesting a dysregulation of cytokine production that may not be able to be further augmented by additional exogenous TLR engagement. In other contexts, TLR-induced cytokine may be elevated in cells from aging individuals. For example, TLR4- and TLR8-dependent as well as self-DNA–induced TNF-α and IL-6 production were increased in monocyte-derived dendritic cells from older, compared with young, individuals (24,25). Moreover, expression of certain TLRs, such as TLR5, appear increased in macrophages isolated via adhesion to plastic in older, compared with young, individuals. Conceivably, these age-associated increases in TLR-induced cytokine production could reflect differentiation or activation of cell lineages in inflammatory environments and, combined with dysregulation of cytokine production, could contribute to an increased proinflammatory environment while overall TLR responsiveness to infectious agents or vaccines remains blunted in older individuals.
Our laboratory found that TLR4 in peripheral blood monocytes from older individuals were less responsive to LPS compared with that of younger individuals. There appeared to be a threshold effect in which older people’s monocytes treated with increasing doses of LPS produced decreasing levels of IL-6 compared with the robust responses of monocytes from younger individuals (P. J. Lee, MD, unpublished data, 2012). In combination with evidence by MacRedmond et al. (26) that cigarette smoke and severe COPD are associated with depressed TLR4 function in people, an intriguing theory emerges in which age- and cigarette-smoke–induced impairments in TLR4 responsiveness underlie the pathogenesis of age-related lung diseases such as COPD.

**CHRONIC OBSTRUCTIVE PULMONARY DISEASE**

COPD is currently the fourth leading cause of death in the United States, and as the population ages, will reach epidemic proportions within the next decade (27). COPD is most commonly diagnosed in the seventh and eighth decades of life and is characterized by chronic airflow obstruction associated with bronchopulmonary inflammation, thought to be mainly driven by macrophages, CD8+ lymphocytes, neutrophils, and dendritic cells (2,28,29). Apart from important preventive measures, such as smoking cessation, specific treatments do not exist. Acute exacerbations are associated with worsening symptoms, lung function decline, and increased mortality (30). Respiratory infections as well as cigarette smoking are the major causes of acute exacerbations and are thought to play a role in the development or progression of the disease (31,32). The risk of developing COPD increases with aging. Notably, most lungs more than the age of 35 years show histologic evidence of emphysema, a lung-destructive process, and a major manifestation of COPD (33). Alveolar lung cells of COPD patients exhibit increased cell death, proliferation, and turnover when compared with asymptomatic smokers’ and nonsmokers’ lungs (34). Another biomarker of aging is telomeres, which represent the regions at the end of chromosomes that protect DNA against degradation and remodeling. Telomeres shorten with age (35). COPD patients had shorter telomeres in alveolar type II epithelial and endothelial cells as well as circulating leukocytes, compared with control patients (36,37). Moreover, patients’ telomere length positively correlated with the extent of airflow obstruction and inversely correlated with a lung-specific inflammatory marker, surfactant protein D (38).

The most commonly used animal model for COPD development has been cigarette smoke, but genetic mouse models of accelerated aging show emphysema in the lungs (39). Homozygous mutant Klotho mice, which have a shorter lifespan and develop age-related disorders, show emphysema in their lungs despite normal lung development (40). Histone deacetylase protein sirtuin (SIRT1), an anti-inflammatory and anti-aging molecule, was decreased in rat lungs in response to cigarette smoke (41). Moreover, these changes promoted expression of inflammatory cytokines that were attributed to posttranslational oxidative modification in the lungs of COPD patients (42).

The role of TLR4 in cigarette smoke-induced COPD has yet to be determined. Acute exposure to cigarette smoke has been reported to activate TLR4 in mice and human cells with subsequent inflammatory cytokines release as well as neutrophil recruitment to the lung (43,44). von Scheel and colleagues (45) recently demonstrated that TLR4 expression levels do not differ between smokers with and without COPD. Others report that the responsiveness of TLR4 to its ligand, LPS, decreased with age or smoking, two processes that predispose to emphysema. Furthermore, severe COPD (forced expiratory volume in 1 second was <1) was associated with greater reductions in TLR4 expression and function (26). In addition, there is evidence that several TLRs are depressed with aging or smoking, supporting our contention that TLR4 is part of a larger, more complex system that is critical to lung health and disease, the significance of which is only recently being explored.

Using genetic mouse models, our laboratory has shown that specific TLRs, especially TLR4, are necessary to maintain lung integrity as the animal ages. Whereas much is known about the role of TLRs in host responses to infection, virtually nothing is known about their role in the aging lung. We found that mice deficient in TLR4 exhibit age-related lung enlargement that resembles pulmonary emphysema in patients, both histologically and functionally (46). Our data indicate that TLR4 deficiency leads to increased reactive oxygen species generation, collectively referred to as oxidant stress, via the upregulation of a novel NADPH oxidase, Nox3. We were able to rescue the animals from TLR4 deficiency-induced emphysema with oral administration of NADPH oxidase inhibitors. More recently, we also identified a poorly understood cathepsin, Cathepsin E, as a candidate mediator of the increased lung destruction observed in the setting of TLR4 deficiency and Nox3 induction.

In addition, we expanded our studies to determine the role of alternative TLRs and have identified that a deficiency in TLR1, but not TLR2, 3, 5, or 9, also leads to emphysema (P. J. Lee, MD, unpublished data, 2012). We found that although alternative TLR pathways are involved in maintaining lung integrity, the mechanisms are distinct. This suggests that there are redundant TLR pathways responsible for lung homeostasis but distinct effector pathways, ligands, and cell types are involved. Even within TLR4 signaling, different cell types may regulate distinct downstream pathways. TLR function may serve as a biologic marker for disease severity or, as our mouse data suggest, determine susceptibility to disease. As TLR4 function declines with age or cigarette smoke exposure in humans, we believe that the synergistic or additive effects of age and smoking on TLR4 function in susceptible individuals may explain the pathogenesis and temporal characteristics of COPD.
Idiopathic Pulmonary Fibrosis

IPF is lethal, progressive, fibrotic process of unknown cause that is limited to the lung (47). IPF is a relatively rare disease with symptoms that typically occur at age from 50 to 70 years. It is difficult to predict from onset how long a patient will maintain pulmonary function or who will experience acute exacerbations. IPF has generally been considered a chronic, insidious, inflammatory disease, and consequently, research in this disorder has primarily focused on its inflammatory component and, more recently, on its fibroproliferative aspect. A microarray analysis of lung samples from patients with IPF shows clear differences in expression among 2000 genes, most of them related to tissue remodeling, including extracellular matrix, basement membrane components, and myofibroblast or smooth muscle cell–associated as well as epithelial cell–related genes (48,49).

Some understanding of the mechanisms underlying IPF has been gleaned from studies of familial pulmonary fibrosis. For example, genome analysis revealed mutations in telomerase, an enzyme that protects telomeres from damage and whose activity declines with age (50).

Epithelial cells from IPF patients also show endoplasmic reticulum and apoptosis stress markers (51). Cytoprotective responses of the endoplasmic reticulum decline with age, leading to oxidative stress and protein misfolding (52). The process by which cells discard misfolded proteins, autophagy, also declines with age thereby allowing mutated protein aggregates to accumulate in older individuals. Autophagy has recently been recognized as a component of the innate immune response (53,54). Loss of autophagy allows mitochondria-generated oxidants to spontaneously activate the NLR inflammasome complex leading to inappropriate inflammation and cell death (55). TLR4 activation has also been shown to induce autophagic signaling in macrophages (56). Conversely, autophagic signaling has been shown to modulate cytosolic TLR signaling by regulating the delivery of ligands (57,58). Therefore, TLRs may serve to limit the inflammatory response and help to restore cellular homeostasis through the activation of autophagic pathways.

Table 1. List of Abbreviations

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<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>COPD</td>
<td>Chronic obstructive pulmonary disease—characterized by irreversible or partially irreversible chronic airflow obstruction and abnormal sputum production; previously used interchangeably with emphysema and “chronic bronchitis”</td>
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<tr>
<td>IPF</td>
<td>Idiopathic pulmonary fibrosis—a process of progressive, irreversible lung parenchymal scarring</td>
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<tr>
<td>TLR</td>
<td>Toll-like receptors—TLRs recognize pathogen- and danger-associated patterns that result in induction of immune and inflammatory genes (75)</td>
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<tr>
<td>NLR</td>
<td>Nucleotide oligomerization domain (Nod)–like receptors—cytoplasmic proteins that can induce inflammatory- or apoptosis-related responses (75)</td>
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<td>RLR</td>
<td>Retinoic acid–inducible gene I (RIG-I)–like receptors—cytoplasmic RNA helicases are the major viral sensors that initiate antiviral immune responses in cells (75)</td>
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<tr>
<td>mDC</td>
<td>Myeloid dendritic cells—antigen-presenting cells that potentially originate from monocytes. Major stimulator of T cells (76)</td>
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<tr>
<td>pDC</td>
<td>Plasmacytoid dendritic cells—antigen-presenting cells that potentially originate from plasma cells or B cells. Produce high amount of interferon α (76)</td>
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Figure 1. Schema depicts proposed biologic mechanisms of aging and reported interactions between components of the innate immune system and age-related lung diseases. TLR, Toll-like receptor; NLR, nucleotide oligomerization domain (Nod)–like receptor; Sirt, sirtuin; ROS, reactive oxygen species; IPF, idiopathic pulmonary fibrosis; and COPD, chronic obstructive pulmonary disease.
Several animal models have been used to study IPF and, thus far, intratracheal bleomycin is the best-characterized murine model in use (59). Bleomycin has been shown to induce lung injury and fibrosis in a wide variety of experimental animals. A disadvantage of this model system is its variability in the development of fibrosis and it does not resemble all the physiological and morphological changes of IPF in people (57,60). Reports indicate TLR2 is a promising target for the development of therapeutic agents against IPF. Clearance of hyaluronan—a major component of extracellular matrix—is crucial for resolving lung inflammation via its receptor CD44 (61). Jiang and colleagues (62) showed that in cells lacking CD44, the interactions among TLR2, hyaluronan, and TLR4 regulate both the innate inflammatory response as well as epithelial cell integrity. This data suggest that endogenous polymeric matrix fragment–TLR interactions are extremely important in lung injury and repair.

ASTHMA

Although asthma is often considered to be a childhood disorder, the symptoms and clinical manifestations can occasionally make their initial appearance in older individuals. Asthma is clinically characterized by airway Th2 inflammation, reversible airflow obstruction, and bronchial hyperresponsiveness. In older asthmatics, sensitivity to indoor allergens is believed to be more prominently associated with the presence of asthma than in younger asthmatics (63,64). Unfortunately, asthma is largely underdiagnosed in the older individuals. The reasons may be that older individuals may underestimate the severity or significance of their symptoms, attribute symptoms to other causes such as aging, COPD, or congestive cardiac failure, or dismiss symptoms entirely (65). COPD is an important comorbidity frequently leading to the misdiagnosis of asthma in older people (66). Similar to COPD, a role for TLRs has been invoked in asthma. Higher TLR2 expression was detected in peripheral blood neutrophils as well as macrophages from middle-aged asthma patients (62,67). Moreover, TLR2 expression increased with asthma severity. These changes may lead to increased sensitivity to endogenous TLR2 ligands, such as hyaluronan fragments, and contribute to chronic lung inflammation and airway remodeling in asthma. Specific TLR polymorphisms have also been linked to susceptibility to asthma (68). Interestingly, the TLR4 Asp499Gly polymorphism, which is associated with hyporesponsiveness to endotoxins, has been associated with an increased risk of asthma in children as well as in COPD (69,70).

Viral infections are a common cause of morbidity and mortality in all the aforementioned chronic lung diseases. The role of aging on the innate immune responses to viral infection is reviewed elsewhere in this edition (11). A special mention should be made regarding the role of TLRs in the decreased vaccine responsiveness observed in elderly individuals. Notably, the extent of TLR-induced expression of B7 costimulatory proteins such as CD80 and CD86 in monocytes was substantially decreased for all TLRs assessed (TLR1/2, TLR2/6, TLR4, TLR5, TLR8) and was strongly associated with antibody response to influenza vaccine (71). In addition, TLR-induced cytokine production in mDCs and pDCs was also strongly associated with influenza vaccine antibody response (20). These results provide evidence for the close relationship between innate immune system engagement and the adaptive immune responses elicited by the influenza vaccine. Moreover, these studies suggest that augmentation of TLR signaling in the context of vaccination could represent a means for improving immunization responses in older individuals.

CONCLUSIONS

An overarching theme in aging as well as age-related lung disease is excessive oxidant generation and dysregulated inflammation, which are both linked. The “free radical theory of aging” is one of the major hypotheses that have been supported by a number of in vitro and in vivo studies (72,73). According to this theory, the normal process of aging is accelerated by oxidative stress, leading to organ dysfunction and failure. Many age-related chronic diseases demonstrate persistent chronic inflammation both locally and systemically (74). However, organ or site-restricted inflammation could become a systemic one as a consequence of chronicity and the presence of favorable genetic or epigenetic factors. An example is COPD, which may start as a lung-specific process but eventually extends systemically.

Age-related disorders in the lung likely result from multiple hits (Figure 1). First, immunosenescence can predispose older individuals to increased lung infections. Second, immunosenescence coupled with lung injury, such as cigarette smoke, leads to a synergistic impairment in specific immune pathways, such as TLR4, ultimately leading to the development of a noninfectious, age-related lung process such as COPD. The causes and consequences of the loss of TLR4 function are illustrative of the aforementioned process. It is beyond the scope of this review to detail the lung infection and cancer aspects of aging but, clearly, a common theme of immune responses, oxidants, and inflammation course through a range of lung processes. The specific contributions of each of the components of the immune system to age-related lung diseases remain unclear. Nonetheless, the current evidence is sufficiently compelling to justify further intense investigations.

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