

New immune pathways from chronic post-viral lung disease

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To better understand the immunopathogenesis of chronic inflammatory lung disease, we established a mouse model of disease that develops after respiratory viral infection. The disease that develops in this model is similar to chronic obstructive lung disease in humans. Using this model we have characterized two distinct phases in the chronic disease process. The first phase appears at three weeks after viral infection and depends on type I interferon-dependent expression and then subsequent activation of the high-affinity IgE receptor (FcεRI) on conventional lung dendritic cells, which in turn recruit IL-13-producing CD4⁺ T cells to the lower airways. The second phase becomes maximal at seven weeks after infection and depends on invariant natural killer T (iNKT) cells and lung macrophages. Cellular cross-talk relies on interactions between the semi-invariant Vα14Jα18 T-cell receptor on lung iNKT cells and CD1d on macrophages as well as iNKT cell-derived IL-13 and IL-13 receptor on macrophages. These interactions drive macrophages to a pattern of alternative activation and overproduction of IL-13. This innate immune axis is also activated in patients with chronic obstructive lung disease, as evidenced by increased numbers of iNKT cells and IL-13-producing alternatively activated macrophages marked by chitinase 1 production. Together the findings identify two new immune pathways responsible for early and late phases of chronic inflammatory lung disease in experimental and clinical settings. These findings extend our understanding of the complex mechanisms that underlie chronic obstructive lung disease and provide useful targets for diagnosis and therapy of this common disorder.

Keywords: dendritic cell; natural killer T cell; NKT; alternatively activated macrophage; interleukin-13; chronic obstructive lung disease

Introduction

Chronic inflammation is critical to the pathogenesis of many common diseases and is generally attributed to persistent activation of the immune system. This type of long-term immune activation is often linked to aberrations of the adaptive immune system, involving antigen-specific antibodies or effector T-cell responses that react against endogenous stimuli.^{1,2} It is possible, however, that components of the innate immune system also contribute to the development of chronic inflammatory disease.³ Thus, precisely how the immune system drives chronic inflammatory disease still needs to be fully defined. In that context, lung disease offers a useful paradigm for investigating mechanisms underlying

chronic inflammation as chronic obstructive lung disease represents one of the most common types of chronic illness that affects humans. In particular, asthma affects approximately 10% of the population, and chronic obstructive pulmonary disease (COPD) is the fourth leading cause of death in the United States and worldwide.⁴ Although asthma and COPD are regarded as separate clinical entities, it is evident that overlapping airway pathology can occur in both diseases.⁵ It is even possible that patients diagnosed initially with asthma may be at risk for developing COPD later in life.⁶ Thus, asthma and COPD may constitute discrete manifestations of a disease continuum resulting from a common pathological process that should be defined in molecular, rather than clinical, terms.

Previous studies have identified genetic and environmental factors that contribute to the development of chronic obstructive lung disease. Of special interest to this review, several clinical studies have proposed a role for respiratory viral infection in precipitating as well as contributing to the pathogenesis of chronic obstructive lung disease. Perhaps the strongest disease association for virus-induced lung disease exists for respiratory syncytial virus (RSV),⁷ a member of the *Paramyxoviridae* family that commonly infects the bronchial airways of infants and adults, causing severe bronchiolitis in a subpopulation of individuals.⁸ A number of prospective case-controlled studies provide evidence that severe RSV-induced bronchiolitis in early infancy constitutes a significant risk factor for the subsequent development of childhood asthma.^{9–13} Putative etiological associations have also been made for other respiratory viruses, but most of these (especially human metapneumovirus [hMPV], rhinovirus [RV], human parainfluenza virus [hPIV], adenovirus, and coronavirus) are more likely associated with exacerbation of existing disease.¹⁴ Furthermore, a number of clinical studies suggest that respiratory viral infections are the major trigger for exacerbations of asthma and COPD.^{15–21} This association has been confirmed experimentally using mouse models that have demonstrated that viral infection can cause features of inflammatory lower airway disease in susceptible strains of mice.^{22,23} However, these observations did not fully develop a causal relationship between respiratory viral infection and chronic airway disease or define the precise mechanism by which viral infections contribute to the development and persistence of this type of disease.

The purpose of this review is to summarize recent findings obtained from our laboratory that detail how the immune response to respiratory viral infection drives the development of chronic obstructive lung diseases, particularly asthma and COPD. The review is organized into five subsequent sections that address (1) immune pathways that are conventionally associated with chronic obstructive lung disease; (2) animal models that can be used to study the basis for chronic obstructive lung disease; (3) the identification of a distinct adaptive immune response that mediates the initial phase of chronic lung disease after viral infection; and (4) the identification of a novel innate immune pathway that mediates the second persistent phase of chronic lung

disease after viral infection. The final section provides a summary and perspective on this field of research.

Traditional pathways for chronic obstructive lung disease

Respiratory tolerance, and mucosal immunity in general, is maintained by a complex system of host defense mechanisms aimed at eliminating aeropathogens while tolerating innocuous airborne antigens and commensal microorganisms. Presumably, this immune balance of reaction versus tolerance may become permanently skewed and thereby progress to chronic inflammation. A scheme that is commonly used to describe inflammation in the lung (and other sites) is based on a model of immune polarity for the T helper (Th) cell system. Within this system, CD4⁺ Th cell-dependent responses are classified into Th type 1 (Th1) or type 2 (Th2). Th1 cells characteristically mediate delayed-type hypersensitivity reactions and selectively produce interleukin (IL)-2 and interferon (IFN)- γ . This type of response is also found during respiratory viral infection. In contrast, Th2 cells promote B-cell-dependent humoral immunity, IgE class switching, eosinophil recruitment, mast cell proliferation, alternative activation of macrophages, and selective production of IL-4, IL-5, and IL-13 that are all typical of an infection with an extracellular parasite and are commonly found during an allergic response.²⁴ Indeed, this type of response can be detected in allergen-challenged mice as well as allergic asthmatics,^{5,25–27} and Th2 cytokine production is often a prominent feature of asthma.^{28–31} For example, IL-13 is increased in the bronchoalveolar lavage fluids of allergen-provoked asthmatic subjects³⁰ and *IL-13* mRNA and protein expression has been detected in bronchial biopsies obtained from allergic and nonallergic asthmatic patients.^{32,33} In addition, polymorphisms at the *IL-13* gene locus have been shown to confer an enhanced risk of asthma and COPD.^{34–36} Increased IL-13 expression is also found in some but not all studies of subjects with COPD.^{37–39} This discrepancy is likely a result of the heterogeneity of disease within and between individuals and is a challenge for current clinical trials of anti-IL-13 therapy in subjects with asthma.⁴⁰

Some investigators have proposed that Th2-biased immune deviation may also influence the

response to viral infection. For example, RSV infection may elicit Th2 cytokine production in allergen-sensitized mice.^{41–43} Whether RSV infection can drive persistent production of IL-13 remains uncertain. Moreover, allergic inflammation is not characteristic of all forms of asthma or COPD. For example, bronchial biopsy specimens obtained from subjects with severe asthma and COPD often contain increased numbers of CD8⁺ T cells, macrophages, and neutrophils.^{44,45} Moreover, subjects with even moderate asthma exhibit activation of IFN-signaling proteins, indicative of a Th1-type response.⁴⁶ Furthermore, studies in mice using adoptive transfer protocols indicate that pro-inflammatory cells, such as Th1 CD4⁺ and CD8⁺ T-cell subsets, can potentiate allergic airway disease.^{47,48} Concerning as well is the observation that many forms of airway disease that are “Th2-polarized” are in fact precipitated by exposure to nonallergic stimuli (such as respiratory viruses) that would ordinarily elicit a Th1 response. Lastly, the Th1 versus Th2 paradigm has been further challenged by the discovery of Th17 (IL-17-producing) and T regulatory or Th3 (IL-10- and transforming growth factor [TGF]- β -producing) subsets of T cells, which appear to influence allergic inflammation of the airways.^{49,50} Thus, the immune response underlying chronic airway disease does not strictly adhere to the Th2 paradigm as aspects of both Th1 and Th2 immune responses appear to drive disease.^{51–53} Because the Th1 versus Th2 model of adaptive immunity did not fully explain the subsequent development and maintenance of chronic inflammatory disease, we questioned whether other aspects of immunity and inflammation might also be critical for the pathogenesis of chronic airway disease following viral infection. Accordingly, we sought to establish an experimental model that would enable us to precisely assess the role of the innate and adaptive systems in the development and maintenance of chronic obstructive lung disease.

Animal models of chronic airway disease

Animal models of inflammatory airway disease have generally relied on allergen-driven responses that are meant to be representative of allergic asthma. The most common approach for these models involves sensitization and subsequent airway chal-

lenge using chicken ovalbumin, but others have also applied allergens derived from insects, e.g., house dust mite and cockroach, as well as infectious agents, e.g., *Schistosoma mansoni*, *Nippostrongylus brasiliensis*, and *Aspergillus fumigatus*. Although these models do not fully replicate the features of asthma in humans, they serve to identify cellular and molecular determinants of allergen-induced inflammation.^{54–58} A critical drawback to acute allergen challenge models is the transient nature of the airway disease. Allergen-driven models of airway disease therefore fundamentally limit study of the immune mechanisms that contribute to persistent inflammation found in chronic obstructive lung disease in humans. In addition, these models are unlikely to account for the nonallergic component of lung disease found after viral infection or other inflammatory stimuli, such as cigarette smoke.

Thus, to better understand how the immune response drives chronic airway disease, we developed a new model of chronic airway disease that develops after viral infection. The rationale for developing this type of model is rooted in epidemiological evidence linking acute viral infection with chronic airway disease.^{9–13,15–21} Because infection with RSV is most strongly linked to the development of asthma, investigators have often used RSV as the etiological agent for study. In fact, RSV infection alone or in combination with allergen challenge has now been studied as a model for airway inflammation in guinea pigs, ferrets, rats, and mice.^{59–63} On the basis of similar associations with exacerbations of asthma, others have also studied human rhinovirus (RV) and metapneumovirus (hMPV). The limitation of these models is that human pathogens often replicate poorly in other species. In the case of RSV, an extremely high inoculum of virus must be administered in order to cause disease in mice, resulting in an all-or-none pattern of illness.⁶⁴ Accordingly, we have advanced a model based on a natural mouse pathogen known as Sendai virus (SeV), a parainfluenza virus closely related to other human paramyxoviruses (e.g., RSV, hMPV, and hPIV). In contrast to RSV, SeV replicates at high efficiency in the lower airway of mice, resulting in acute inflammation of the small airways that maintains high fidelity to viral bronchiolitis in humans. Administration of a modest SeV inoculum (e.g., 1×10^5 plaque-forming cells) to young immunocompetent mice (e.g., 6–10 weeks of age)

elicits a top-down pattern of infection that is limited to airway epithelial cells and neighboring macrophages.^{65–67} This illness is therefore remarkably similar to the one observed in severe RSV infections in humans. It appears likely that this pattern of infection is necessary for the subsequent development of chronic lung disease in this model and perhaps in humans as well.

Our assessment of SeV-induced pathology in different inbred strains of mice led to the finding that progression from acute infection to chronic disease occurred singularly in the context of the C57BL/6J (B6) genetic background. Infection of this strain with SeV triggers an acute antiviral response that is followed by a delayed but permanent switch to chronic airway disease manifested by mucous cell metaplasia (with increased lung levels of *Muc5ac* mRNA and *Muc5ac* immunostaining), immune cell infiltration (with increased numbers of macrophages and neutrophils), and airway hyper-reactivity (signified by increased pulmonary resistance in response to inhaled methacholine).^{23,68,69} This chronic airway disease becomes apparent at 3 weeks after viral inoculation, reaches maximal levels at 7 weeks after inoculation, and persists indefinitely.^{23,37,68,69} Induction of chronic disease requires live replicating virus because administration of UV-inactivated virus or MDA5/Tlr3-agonist polyinosinic : polycytidylic acid will not cause subsequent disease (Ref. 37 and unpublished observations).

The conventional dendritic cell–T-cell axis in airway disease

Our analysis of the viral model in B6 mice provided the opportunity to assess gene-targeted and transgenic mice available on this genetic background. Initial analysis of our model using mice with a targeted deletion of intercellular adhesion molecule 1 (ICAM-1), a widely-expressed adhesion molecule, revealed that acute disease could be genetically segregated from chronic disease.²³ In particular, *ICAM-1*^{-/-} mice failed to develop a robust inflammatory response and concomitant airway hyper-reactivity at 3 weeks after viral infection. Nonetheless, *ICAM-1*^{-/-} mice remained susceptible to the development of chronic inflammatory lung disease. These observations thereby established the concept that separate pathogenic mechanisms could account for acute

versus chronic inflammatory disease, laying the conceptual framework for our model of immune-based chronic lung disease.

A significant breakthrough in our understanding of virus-induced lung disease came with the discovery that the acute and chronic disease that developed after viral infection required the production of IL-13 in the lung.⁶⁸ Using a combination of high-speed fluorescence-activated cell sorter (FACS) and real-time PCR analysis, we were able to identify CD4⁺ T cells as the chief cellular source of IL-13 production at 3 weeks after viral infection.³⁷ This finding raised the possibility that elements of an adaptive immune response that are characteristically involved in allergen-induced airway disease might also contribute to virus-induced airway disease. To address this prospect, we pursued the underlying mechanism for CD4⁺ T-cell production of IL-13 in our model and in doing so uncovered a novel immune axis that links the antiviral response to lung disease.⁷⁰ In brief, we found that IL-13 production by CD4⁺ T cells following acute SeV infection is dependent upon induction of type I IFN production. Type I IFN drives expression of high-affinity IgE receptor FcεRI on the surface of conventional dendritic cells (cDCs). Subsequent activation of cDCs through FcεRI cross-linking then drives the production of CCL28 that mediates recruitment of IL-13-producing CD4⁺ T cells to the lung. These findings establish a novel mechanism whereby the antiviral adaptive immune response, which is characteristically pro-inflammatory or Th1 in character, can be transformed into an allergic Th2 response marked by the chronic production of IL-13 by CD4⁺ T cells. The next section provides some of the pertinent evidence to support this mechanism.

The antiviral immune response induces expression of FcεRI on resident cDCs

We approached our study of the viral model by considering that respiratory viral infections are known to drive antiviral IgE production and consequent immune cell expression of FcεRI in humans.^{71–73} We found that total serum IgE and virus-specific IgE were increased by 7 days and were still increased at 3 weeks after inoculation with SeV. The question as to how IgE might be involved in mediating chronic disease became the focus of further study.

What was known at the time of our studies was that the high-affinity IgE receptor was detectable on mast cells, basophils, and eosinophils in mice and humans⁷⁴ where it is expressed as a tetrameric complex consisting of one α (Fc ϵ RI α), one β (Fc ϵ RI β), and two γ (Fc ϵ RI γ) chains.⁷⁵ However, in humans surface expression of Fc ϵ RI was additionally described for peripheral blood cDCs, plasmacytoid DCs (pDCs), monocytes, and Langerhan cells, although in these leukocyte subsets expression is restricted to a trimeric form (Fc ϵ RI $\alpha\gamma\gamma$) that lacks the β chain.^{76–78} Given this extended profile of Fc ϵ RI expression in humans, we questioned whether we could detect induced expression of this receptor complex on mouse lung cDCs following viral infection. Using a combination of FACS-based forward/side scatter properties and high CD11c expression levels to identify lung cDCs (obtained following repeated bronchoalveolar lavage to remove alveolar macrophages), we observed that SeV infection leads to rapid migration of lung cDCs to the draining lymph nodes in a CCR5–CCL5-dependent manner.⁷⁹ Furthermore, we found that the majority of the cDCs remaining in the lung after acute infection matured and differentiated, as marked by upregulation of CD86 and major histocompatibility complex (MHC)-II surface expression.

As the function of this resident population of cDCs was uncertain, we sought to further characterize them. Real-time PCR analysis of purified lung cDCs revealed that Fc ϵ RI α expression was induced within 3 days and remained stable for 2–3 weeks after viral inoculation. In addition, the level of Fc ϵ RI α expression on lung cDCs was comparable to levels on c-kit⁺ lung mast cells.⁷⁰ Immunoprecipitation, immunoblotting, and real-time PCR assays revealed that the trimeric form of the Fc ϵ RI receptor alone was expressed in lung cDCs, consistent with the pattern of expression on human antigen-presenting cells. Furthermore, virus-induced expression of Fc ϵ RI α was largely restricted to cDCs residing in the lung, and not in the spleen or draining lymph nodes, thus spatially restricting downstream effects of cDC-specific Fc ϵ RI α expression to events occurring in the lung parenchyma.

To determine whether Fc ϵ RI expression was consequential to the host in the course of the acute antiviral immune response, we compared several immune parameters in wild-type and Fc ϵ RI α ^{-/-}

mice. To this end, we observed that Fc ϵ RI α ^{-/-} mice exhibited comparable acute morbidity and identical patterns of viral replication and clearance (as determined by plaque-forming and real-time PCR assays). Furthermore, comparative analysis of costimulatory molecule expression on resident cDCs derived from wild-type or Fc ϵ RI α ^{-/-} mice revealed no differences in the levels of MHC-II, CD80, or CD86, thereby indicating that the basic antigen-presenting functions of cDCs from Fc ϵ RI α ^{-/-} mice were intact. We also found that Fc ϵ RI α -deficient mice generated comparable numbers of antiviral cytotoxic T cells in response to virus, as determined by SeV-specific tetramer analysis of lung immune cells. Therefore, while viral infection induces expression of Fc ϵ RI on the surface of cDCs, this expression pattern does not influence the acute immune response to virus.

Type I IFN receptor regulates cDC expression of Fc ϵ RI

We next assessed the mechanism responsible for induction of Fc ϵ RI α gene expression in lung cDCs after viral infection. We recognized the influence of serum IgE level on the expression of the high-affinity IgE receptor on mast cells in allergy.⁸⁰ We therefore reasoned that SeV-specific IgE could drive Fc ϵ RI expression on lung cDCs after SeV infection as well. However, we found that total serum IgE and SeV-specific IgE did not appreciably increase until 7 days after infection. As the onset of expression of Fc ϵ RI α on lung cDCs predates the induction of IgE expression, factors other than IgE must drive Fc ϵ RI α expression in our model. Indeed, even IgE-deficient mice exhibit the same increase in Fc ϵ RI α expression on lung cDCs as wild-type mice after viral infection.⁸¹

We therefore focused our attention on the role of type I interferon signaling in regulating Fc ϵ RI expression as these events occur in concert after viral infection. We directly examined whether type I interferon signaling was necessary for induction of Fc ϵ RI α expression on lung cDCs using type I IFN receptor (IFNAR)-deficient (IFNAR^{-/-}) mice. We found that lung cDCs from IFNAR^{-/-} mice did not manifest induction of Fc ϵ RI α expression after SeV infection. Adoptive transfer using either IFNAR^{-/-} or wild-type purified cDCs established that IFNAR expression on

cDCs was not necessary for induction of *FcεRIα* expression on lung cDCs. This finding suggested that IFN- α/β functions through additional cellular intermediates and so we expanded our assessment to include additional adoptive transfer studies that identified neutrophils as a critical link between upstream type I interferon signaling and downstream cDC activation.⁸²

FcεRI on lung cDCs drives Ccl28-dependent recruitment of Th2 cells

We reasoned that FcεRI activation on lung cDCs could be linked to increase in IL-13-producing T cells in the lung after viral infection. To assess this possibility, we applied a co-culture system using lung cDCs and CD4⁺ T cells. In this system, we found that that antigen-dependent interaction of lung cDCs and T cells can drive T-cell proliferation and production of IL-13; however, cross-linking FcεRI on the surface of cDCs did not influence this process. This finding led us to examine whether FcεRI signaling might elicit additional responses that could account for local IL-13 production in the lung, e.g., through recruitment of IL-producing T cells to the lung. Indeed, we were able to demonstrate that cell supernatants derived from cross-linked cDCs were capable of stimulating the migration of CD4⁺ T cells in a chemotaxis assay system. Ligand desensitization and blocking antibody assays revealed that CCL28-CCR10 interactions were responsible for mediating this process. Of relevance to this finding, increased expression of CCL28 is associated with both human asthma and mouse models of allergic airway disease.^{83–85} This immune axis therefore provides an additional mechanism for CCL28 production and action in the setting of inflammatory airway disease.

To extend our findings *in vivo*, we re-evaluated the viral mouse model and found that lung levels of *Ccl28* mRNA were significantly increased in wild-type mice after viral infection. Furthermore, the increase in *Ccl28* gene expression after infection was blocked in *FcεRI α ^{-/-}* mice, suggesting that FcεRI-mediated signals drove this process. We also found that the relative numbers of IL-13-producing CD4⁺ T cells were significantly reduced in SeV-infected *FcεRI α ^{-/-}* mice compared to wild-type mice. In addition, *FcεRI α ^{-/-}* mice demonstrated a marked decrease in the number of Muc5ac-expressing ep-

ithelial cells and total lung *Muc5ac* mRNA levels at 3 weeks after viral infection. To confirm that FcεRI expression on cDCs is necessary for lung disease, we performed adoptive cell-transfer experiments using cDCs derived from wild-type or *FcεRI α ^{-/-}* mice transferred into *FcεRI α ^{-/-}* recipients. We found that reconstitution with wild-type but not *FcεRI α ^{-/-}* cDCs restored post-viral mucous cell metaplasia in *FcεRI α ^{-/-}*-recipient mice. The significance of FcεRI-dependent CCL28-mediated disease was verified using mice that were treated with anti-CCL28 blocking mAb. In these experiments, mice treated with anti-CCL28 mAb were prevented from developing mucous cell metaplasia relative to control IgG2b-treated mice. Together these findings establish a direct role for FcεRI in mediating the airway disease that develops after viral infection. These findings support the proposal that mucous cell metaplasia after viral infection depends on an adaptive immune response that begins with IFN-mediated expression of FcεRI on resident cDCs and continues to activation of FcεRI α , presumably through SeV-specific IgE, to transduce signals in these cDCs to drive production of Ccl28 and subsequent recruitment of IL-13-producing CD4⁺ T cells to the lungs. This model of disease is relevant to a clinical understanding of post-viral inflammatory airway disease, a condition that is marked by airway hyper-reactivity and is sometimes associated with elements of atopy, including elevated levels of serum IgE.⁸⁶ This condition often resolves by several weeks after viral infection (in humans and in the mouse model), but it is possible that a chronic inflammatory condition might develop in the face of ongoing infectious or allergic stimuli.

The natural killer T cell–macrophage axis in airway disease

Our discovery of the cDC–T-cell axis helps explain how the adaptive immune response can drive inflammatory disease in the 3-week period after viral infection. However, mice deficient in *FcεRI α ^{-/-}* still develop chronic disease that peaks at 7 weeks after infection (unpublished observations, L.A. Benoit, D. Byers, M.H. Grayson, and M.J. Holtzman), thus suggesting that this adaptive immune axis does not mediate the later phase of chronic disease. We therefore speculated that components of the innate immune system might drive the continued

disease process that also depends on IL-13-expressing macrophages.³⁷ When we pursued the underlying mechanism for macrophage production of IL-13 in this model, we uncovered a second novel immune pathway leading to chronic inflammatory lung disease. Unexpectedly, this pathway occurred independently of an adaptive immune response, as evidenced by disease progression in *MHC-II^{-/-}*, *Cd4^{-/-}*, and *Cd8^{-/-}* mice³⁷ as well as in B-cell-deficient (*muMT*) mice (unpublished observations). FACS-based analysis of the lung immune infiltrates in chronically affected mice revealed a substantial increase in the number of natural killer T (NKT) cells. Further assessment revealed that CD4⁻ NKT cells were directly responsible for the production of IL-13 by macrophages. Assessment of lung tissue from patients with severe asthma and COPD indicated that this innate immune response axis was also activated in humans with chronic obstructive lung disease. Collectively these findings indicate that the switch between acute antiviral immune responses to chronic inflammatory disease requires the persistent activation of a novel macrophage–NKT-cell innate immune response axis and thereby provides mechanistic insight into the pathogenesis of chronic inflammatory disease that is distinct from other reports of NKT-cell activation in asthma and asthma models.^{87–91} We present an outline of these findings in this section of the review.

Persistence disease depends on macrophage production of IL-13

As indicated above, B6 mice infected with SeV develop chronic airway disease that is first detected at 3 weeks and is maximal in severity at 7 weeks after viral inoculation. Coinciding with the disease time course, significant IL-13 production is observed at 3 weeks and is maximal at 7 weeks after inoculation. A direct causative role for IL-13 in mediating chronic airway disease was established when mice treated with an IL-13 decoy receptor (sIL-13-R α 2-Fc) and *IL-13* gene-targeted mice failed to develop mucous cell metaplasia or airway hyper-reactivity after viral infection. We detected comparable levels of remnant viral RNA in *IL-13^{-/-}* and wild-type mice, indicating that chronic lung disease depends on immune cell production of IL-13 rather than differences in viral RNA levels between mouse strains.

We next investigated the cellular sources of chronic IL-13 production in the lung after viral infection. Whereas CD4⁺ T cells contributed the highest amount of *IL-13* mRNA at 3 weeks, we found that macrophages became the predominant source of *IL-13* mRNA at 7 weeks after inoculation. In each case, the individual cellular contribution to total lung *IL-13* mRNA levels was based on an increased number of cells recruited to the lung as well as increased production of *IL-13* mRNA per cell. Secondary sources of *IL-13* mRNA included NK cells and NKT cells, although no detectable *IL-13* mRNA was found in mast cells, basophils, neutrophils, DCs, B cells, or CD8⁺ T cells. We also found that lung macrophages isolated from SeV-infected mice produced corresponding increases in IL-13 protein. In addition, immunohistochemical analysis detected IL-13⁺ cells with typical macrophage morphology and CD68 expression in alveolar, interstitial, and epithelial locations.

To determine whether IL-13-producing macrophages are required for persistent lung disease, we next examined macrophage-deficient mice. We first assessed *op/op* mice that are macrophage deficient due to a loss of function mutation in the *Csf1* (or *M-csf*) gene. Compared to wild-type mice, the *op-op* mice exhibited markedly decreased levels of *IL-13* and *Muc5ac* gene expression at 7 weeks after infection. However, there are notable immune defects in these mice that could complicate the interpretation of our results, and so we applied a clodronate-containing liposome delivery protocol to our model to selectively deplete lung macrophages.⁶⁶ Clodronate treatment was initiated after clearance of infectious virus to avoid any possible effects on the acute infection process. Using this system, we found no differences in the levels of *IL-13* or *Muc5ac* mRNA at 3 weeks after viral inoculation, consistent with a lack of IL-13 production by macrophages at this early time. However, macrophage-depleted mice demonstrated significantly decreased levels of *IL-13* and *Muc5ac* gene expression compared to control mice at 7 weeks after inoculation. Furthermore, immunohistochemical analysis of lung sections indicated that IL-13-producing macrophages were no longer found in clodronate-treated mice. We therefore concluded that IL-13⁺-producing macrophages were contributing to the chronic disease in this model.

Natural killer T-cell-mediated activation of macrophages

In addressing the issue of macrophage activation in our model, we reasoned that an additional aspect of the immune system was driving persistent IL-13 production by lung macrophage. As noted above, this type of chronic pressure is generally attributed to activation of the adaptive immune system. However, strain-based analysis revealed that mice genetically deficient in CD4⁺ T cells, CD8⁺ T cells, or B cells continued to develop mucous cell metaplasia in concert with increased *Il-13* and *Muc5ac* mRNA levels after viral infection. These findings suggested that elements unrelated to the adaptive immune system might drive persistent macrophage activation. To assess this issue more thoroughly, we performed T-cell depletion studies using antibody treatment that was initiated following clearance of infectious virus to circumvent the possibility that these primary immune deficiencies could somehow be interfering with the acute antiviral response. Similar to mice with genetic T-cell deficiencies, mice that were depleted of CD4⁺ T cells, CD8⁺ T cells, or both CD4⁺ and CD8⁺ T cells demonstrated increased numbers of IL-13-producing macrophages, significant induction of *Il-13* and *Muc5ac* gene expression, and mucous cell metaplasia after viral infection. These findings indicated that additional components outside of the adaptive immune system must contribute to the chronic disease that develops after viral infection.

This unexpected finding in animals deficient in elements of the adaptive immune system us to consider whether innate immune cells could be responsible for chronic macrophage activation. This possibility was supported when we discovered that NKT cells were recruited to the lung (on the basis of increased numbers of cells) and activated (on the basis of increased *Il-13* mRNA production) at 7 weeks after SeV infection. Analysis of whole lung NKT-cell populations indicated that CD4⁻ predominated over CD4⁺ cells and that both populations of NKT cells produced *Il-13* without a corresponding increase in *Il-4* mRNA. Although the relative contribution of NKT cells to the overall levels of *Il-13* mRNA in the lung was relatively small compared to macrophages, the persistent production of *Il-13* mRNA by CD4⁻ NKT cells might still regulate downstream macrophage activation and pro-

duction of IL-13. In fact, mice that were genetically deficient in NKT cells as a result of *Cd1d* or *J α 18* gene targeting^{92,93} were largely protected from the usual increase in IL-13-expressing macrophages as well as *Il-13* and *Muc5ac* mRNA and airway hyper-reactivity at 7 weeks after viral inoculation. There was no influence of NKT-cell deficiency at 3 weeks after inoculation, consistent with the time course for macrophage production of IL-13 in the viral model. These findings support a role for invariant NKT (iNKT) cells in the chronic activation of macrophage production of IL-13 after viral infection.

Direct and indirect mechanisms of NKT-cell-mediated activation of macrophages

NKT cells could promote macrophage activation through recruitment (similar to the role of cDCs in the first phase of disease) as well as activation of macrophages in the lung. Evidence for NKT-cell-dependent recruitment of macrophages was substantiated by the observation that *Cd1d*^{-/-} mice exhibited decreased numbers of lung macrophages relative to wild-type mice at 7 weeks after viral inoculation. Moreover, purified lung CD4⁻ NKT cells released significant levels of monocyte/macrophage chemotaxins Ccl3 and, to a lesser extent, Ccl5, after *in vitro* stimulation with phorbol 12-myristate 13-acetate (PMA)-ionomycin. Similarly, this cell population produced increased levels of *Ccl3* mRNA when isolated from mice at 7 weeks after inoculation compared to uninfected control mice. Increased *Ccl3* levels were localized to the CD4⁻ NKT compartment, consistent with the increased activity of CD4⁻ NKT cells in chronic lung disease after viral infection.

To determine whether NKT cells might directly stimulate macrophage production of IL-13, we established a co-culture system using purified populations of NKT cells and macrophages. Cells used in this assay system were principally derived from the lungs because NKT cells and macrophages isolated from other compartments proved less capable of inducing IL-13 production. In addition, the cells were derived from uninfected mice to avoid downregulation of T-cell receptor (TCR) function and background production of IL-13. Using this co-culture system, we found that direct NKT-cell-macrophage interaction resulted in production

of *Il-13* mRNA and IL-13 protein by adherent macrophages within 24 h of co-culture. The NKT-cell effect on macrophage production of IL-13 was relatively specific for lung NKT cells as naive CD4⁺ T cells failed to induce IL-13 production by macrophages. Furthermore, NKT-cell-induced production of IL-13 by macrophages was significantly inhibited by treatment with an anti-CD1d-blocking mAb and markedly increased by addition of α -GalCer, a synthetic CD1d ligand. These results indicated that NKT-cell-dependent activation of macrophages required interaction of V α 14-J α 18 invariant TCR on the NKT cell with CD1d on the macrophage. We also found that NKT cells from *Il-13*^{-/-} mice were no longer capable of driving macrophage production of IL-13 in the co-culture system. This finding established a new role for IL-13–IL-13 receptor (IL-13R) signaling in controlling the NKT-cell–macrophage interaction. Co-culture analysis also indicated that NK1.1⁺ NKT cells were primarily responsible for driving IL-13 production by macrophages (unpublished observations). Analysis of liver NKT cells indicated that these cells were less effective than lung NKT cells for macrophage activation, requiring the addition of α -GalCer to stimulate macrophage production of IL-13. Nonetheless, preparations of either CD4⁺ or CD4⁻ liver NKT cells were equally capable of activating lung macrophages to produce IL-13 following addition of α -GalCer. Thus, while CD4⁺ NKT cells have been the focus for studies of the response to allergens in the lung,⁸⁸ it appears that CD4 expression is not necessary for NKT cells to drive macrophage-dependent disease after viral infection. Instead, the NKT-cell–macrophage pathway is primarily dependent on invariant iTCR–CD1d and IL-13–IL-13R interactions as well as NK1.1 expression.

Positive feedback for IL-13 production

We further characterized the downstream events in the NKT-cell–macrophage–IL-13 immune axis to better define whether this pathway could upregulate expression of additional cytokines and/or cytokine receptors. Oligonucleotide microarray analysis of lung mRNA identified *Il-13 receptor α* (*Il-13ra1*) as the predominant cytokine-related gene that was significantly upregulated after viral infection. Increased *Il-13ra1* expression no longer developed in *Cd1d*^{-/-} mice, suggesting that NKT cells stimulate

an increase in *Il-13ra1* mRNA. The increase in lung *Il-13ra1* mRNA expression after viral infection was relatively small, further suggesting that increased expression of *Il-13ra1* might be restricted to a subpopulation of cells. In fact, *Il-13ra1* mRNA expression was restricted mainly to lung macrophages, and this expression was significantly decreased in macrophages isolated from *Cd1d*^{-/-} mice. These observations suggested that the interaction of IL-13 produced by NKT cells with IL-13R on the surface of the macrophage could serve to amplify macrophage production of IL-13. Indeed, we found that blockade of IL-13 signaling by delivery of sIL-13R α 2-Fc caused a marked decrease in the levels of IL-13-producing macrophages, which in turn caused a decrease in total *Il-13* mRNA levels in the lung. Together these findings suggest a positive feedback loop whereby IL-13 signaling induces expression of IL-13R and IL-13 by macrophages in an NKT-cell-dependent manner.

Alternative activation of macrophage

Further analysis of whole genome microarrays also revealed a pattern of gene expression that is characteristic of an alternative pathway for activation of macrophages.^{94,95} We found especially prominent upregulation of genes encoding chitinase-like proteins (*Chi3l3/4*), resistin-like molecules (*Rtn1a* or *Fizz1*), arginase (*Arg1*), matrix metalloproteinases (*Mmp12*), and arachidonate 12-lipoxygenase (*Alox12e*) at 7 weeks after viral inoculation. Our results were corroborated by real-time PCR analysis, which further revealed that expression of these transcripts was completely blocked in *Il-13*^{-/-} mice and largely blocked in *Cd1d*^{-/-} and *Ja18*^{-/-} mice. Immunostaining and FACS analysis indicated that expression of Chi3l3/4 proteins were principally restricted to CD68⁺ macrophages. Previous work established that alternative activation of macrophages may occur in the setting of parasitic infection or allergy,²⁴ but our results provide the initial evidence that this type of response also occurs in response to viral infection. The biological consequences of alternative activation remain uncertain. There is some evidence that arachidonate 12-lipoxygenase and arginase 1 may promote while resistin-like molecule α may protect against allergic airway inflammation.^{96,97} However, the role of these gene products as well as chitinase

and chitinase-like proteins for the development of chronic lung disease after viral infection in experimental and clinical settings still needs to be defined. Nonetheless, as developed in the next section, these proteins can still be useful biomarkers of the NKT-cell–alternatively activated macrophage immune axis in humans.

NKT cell–macrophage pathway in humans

Excessive mucus production is a common feature of acute respiratory infection as well as chronic obstructive lung disease. Autopsies performed on asthmatic patients who died in *status asthmaticus* demonstrate postmortem findings of mucus plugging and inspissation in approximately 75% of cases. Similarly, occlusion of the small airways by mucus-containing inflammatory exudates is associated with early death in patients with COPD.⁹⁸ Given the involvement of NKT cells and macrophages in the development of mucous cell metaplasia after viral infection, we sought to extend our analysis of this immune axis to patients with chronic lung disease that is also characterized by excess mucus production.

Although our analysis of human samples is at an early stage, we have obtained significant evidence that the NKT-cell–macrophage immune axis is activated in severe asthma and COPD in humans. In particular we have found that bronchoalveolar lavage samples derived from severe asthmatics contain an increased number of IL-13⁺CD68⁺ macrophages compared to samples obtained from healthy control subjects. In addition, we observed that lung samples obtained from patients with severe COPD exhibit significant mucous cell metaplasia and a corresponding increase in *IL-13* mRNA and protein relative to control lungs. IL-13⁺ cells in the lungs of COPD subjects were identified as lung macrophages on the basis of typical cell morphology and CD68 immunostaining. The increase in IL-13⁺CD68⁺ macrophages is accompanied by an increased number of invariant V α 24-TCR NKT cells in lung samples from COPD subjects relative to non-COPD controls. These findings are remarkably similar to our findings in the mouse model of chronic lung disease after viral infection.

Despite the high-fidelity of our mouse-to-man observations, we also recently demonstrated that mouse models of chronic obstructive lung disease

may serve to guide but may not fully predict human lung behavior.⁹⁹ For example, as noted above, *Chit3l3/4* is the chitinase and chitinase-like family member that is most inducible during the lung disease that develops after viral infection or allergen challenge in the mouse model. However, there is no homologue for *Chi3l3/4* in the human genome. Thus, the mouse model cannot predict which chitinase or chitinase-like family member may be equivalently increased in humans with corresponding lung disease. This circumstance, therefore, requires a comprehensive analysis of this family in humans with chronic lung disease. When this type of analysis was performed, we found instead that chitinase 1 is selectively increased in subjects with severe COPD. Similar to the mouse model, chitinase 1 expression was localized to lung macrophages. In addition, the increase in chitinase levels in the lung was reflected by an increase in circulating levels that could be detected in plasma samples from COPD subjects. In these subjects, the plasma levels of chitinase 1 were correlated significantly with disease severity. This level thereby provides a means to noninvasively track the status of alternatively activated macrophages in chronic lung disease and better differentiate molecular phenotypes in heterogeneous patient populations. In addition, these observations provide further evidence that the innate iNKT-cell–alternatively activated macrophage immune axis may constitute a significant pathway for chronic obstructive lung disease. Additional work is needed to define the functional role of this pathway in complex lung diseases and in other chronic inflammatory diseases that may also be linked to the antiviral response.

Summary

A new mouse model has provided evidence of two distinct immune pathways that drive chronic lung disease in this model and in humans (summarized in Fig. 1). The disease that develops in this model is manifest by mucous cell metaplasia and airway hyper-reactivity that are characteristic features of asthma and COPD in humans. The first phase of chronic disease develops at 3 weeks after viral infection and depends on type I IFN-dependent expression of the high-affinity IgE receptor (Fc ϵ R1) on lung cDCs. Engagement of the Fc ϵ R1 causes the release of CCL28 to drive the recruitment of

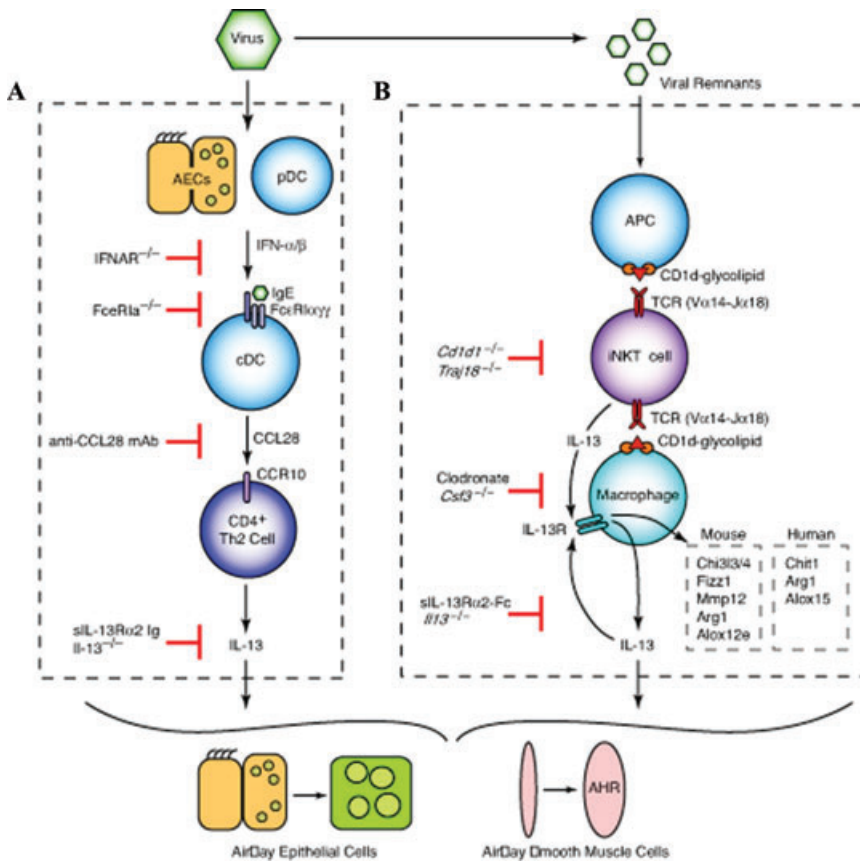


Figure 1. Cellular and molecular scheme for immune pathways leading to chronic lung disease after viral infection. (A) For the first phase of chronic disease, viruses cause airway epithelial cell (AEC) and plasmacytoid dendritic cell (pDC) production of type I interferon (IFN). Subsequent type I IFN receptor (IFNAR) signaling leads to upregulation of FcεRI expression on resident lung conventional (c)DCs. In turn, FcεRI activation by viral antigen and antiviral IgE leads to production of CCL28 and recruitment of CCR10-expressing IL-13-producing T helper (Th)2 cells to the lung. The actions of IL-13 drive differentiation of airway epithelial cell precursors toward mucous cells (mucous cell metaplasia) and airway smooth muscle cells to become more reactive to contractile agonists (airway hyper-reactivity [AHR]). (B) For the second phase of chronic disease, viral remnants activate antigen-presenting cells (APCs) and thereby facilitate CD1d-dependent antigen presentation and consequent activation of invariant CD4⁺ natural killer T (NKT) cells. NKT cells then interact directly with lung macrophages via IL-13 production and binding to the IL-13 receptor (IL-13R) as well as contact between invariant Va14 T-cell receptor (TCR) and glycolipid-loaded CD1d. This interaction leads to increased expression of IL-13R and production of IL-13 that drives a positive feedback loop to amplify IL-13 production and alternative activation of macrophages, including *Chi3l3/4*, *Fizz1*, *Mmp12*, *Arg1*, and *Alox12e* gene expression in mice and *Chit1*, *Arg1*, and *Alox15* in humans.

IL-13-producing Th2 cells to the airways, resulting in local action of IL-13 on airway epithelial cells to produce mucous cells and on airway smooth muscle cells to promote hyper-reactivity. This mechanism fits with the conventional proposal that the adaptive immune system is responsible for chronic inflammatory disease but raises the novel concept that an

antiviral (Th1) response can be closely coupled to an allergic (Th2) response. Thus, the findings challenge the existing paradigm for separation of Th1 and Th2 responses, at least in the setting of post-viral disease.

The second phase of chronic disease that we have described for this mouse model is fully manifest at 7 weeks after viral infection and depends

instead on an innate immune axis involving subsets of NKT cells and macrophages in the lung. These two cell types are specially programmed to allow for interactions between the semi-invariant TCR on the NKT cell and CD1d on the macrophage as well as NKT-cell-derived, IL-13 derived, and IL-13R on the macrophage. Together these ligand–receptor interactions drive macrophages to a pattern of alternative activation and overproduction of IL-13. This model thereby provides for how the innate immune system can be solely responsible for mediating a chronic inflammatory disease. The relevance of this NKT-cell–macrophage immune axis is underscored by our additional findings of increased numbers of iNKT cells and IL-13-producing alternatively activated macrophages in the lungs of subjects with chronic obstructive lung disease. Moreover, the mouse model guided the identification of a biomarker of this innate immune pathway, i.e., macrophage-derived chitinase 1. Initial results indicate that plasma levels of chitinase 1 correlate with disease severity in a subset of subjects with severe COPD. These findings reinforce the role of this new NKT-cell–macrophage pathway in the development of chronic obstructive lung disease and provide a new line of investigation for linking acute viral infection to chronic inflammatory disease.

In that regard, we recognize that the present insights into new immune pathways also raise substantial questions that need to be addressed to better define the basis for chronic inflammatory disease. Perhaps the most immediate issue raised by these studies is the need to identify the mechanism for persistent activation of NKT cells after viral infection and in chronic disease. One possible explanation is that remnant virus may provide ongoing stimulation to NKT cells, perhaps through activation of the retinoic acid inducible gene protein 1-like or Toll-like receptor family members. In support of this possibility, we find evidence of viral persistence of SeV that is similar to reports of RSV in animal models.^{37,100,101} Additional work must be directed at the possibility that viral remnants contained in cDCs, macrophages, or other cell types might drive chronic NKT-cell activation after viral infection. A second question relates to the role of alternative activation of macrophages in chronic inflammatory disease. In that regard, our data and that of others suggest both pro-inflammatory and anti-inflammatory actions of

alternatively activated macrophages may be found under experimental and clinical conditions.^{96,97,102} At present, we view these macrophage products as potentially useful biomarkers of immune-mediated disease, but further definition of macrophage function is needed if the same products are pursued as targets for therapeutic intervention. Thus, the role of alternatively activated macrophages in the propagation of chronic airway disease also requires further assessment. The insights obtained from the present high-fidelity model of post-viral disease should serve to guide these future studies.

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Conflict of interest

The authors declare no conflicts of interest.

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