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Immune Pathways for Translating Viral Infection into Chronic Airway Disease¹

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Abstract

To better understand the immune basis for chronic inflammatory lung disease, we analyzed a mouse model of lung disease that develops after respiratory viral infection. The disease that develops in this model is similar to asthma and chronic obstructive pulmonary disease (COPD) in humans and is manifested after the inciting virus has been cleared to trace levels. The model thereby mimics the relationship of paramyxoviral infection to the development of childhood asthma in humans. When the acute lung disease appears in this model (at 3 weeks after viral inoculation), it depends on an immune axis that is initiated by expression and activation of the high-affinity IgE receptor (FcεRI) on conventional lung dendritic cells (cDCs) to recruit interleukin (IL)-13-producing CD4⁺ T cells to the lower airways. However, when the chronic lung disease develops fully (at 7 weeks after inoculation), it is driven instead by an innate immune axis that relies on invariant natural killer T (iNKT) cells that are programmed to activate macrophages to produce IL-13. The interaction between iNKT cells and macrophages depends on contact between the semi-invariant Vα14Jα18-TCR on lung iNKT cells and the oligomeric MHC-like protein CD1d on macrophages as well as NKT cell production of IL-13 that binds to the IL-13 receptor (IL-13R) on the macrophage. This innate immune axis is also activated in the lungs of humans with severe asthma or COPD based on detection of increased numbers of iNKT cells and alternatively activated IL-13-producing macrophages in the lung. Together, the findings identify an adaptive immune response that mediates acute disease and an innate immune response that drives chronic inflammatory lung disease in experimental and clinical settings.

1. INTRODUCTION

One of the major tasks of current medical research is to define the pathogenesis of chronic inflammatory disease. In that regard, chronic inflammatory diseases of the lung such as asthma and chronic obstructive pulmonary disease (COPD) represent one of the most common types of chronic illness that affects humans. A critical step towards defining the molecular mechanisms underlying these illnesses came with formal recognition of the role of immunity and inflammation. Since then, evidence of excessive airway inflammation in concert with lung disease has led to a widening search for the types of inflammatory cells and mediators that might be responsible for abnormal airway function. Cell types implicated

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in the development of airway inflammation include immune cells as well as parenchymal lung cells. Cell-cell interactions have been attributed to classes of mediators that include lipids, proteases, peptides, glycoproteins, glycolipids, and cytokines. The leading scheme for integrating this information has been based on the classification of the adaptive immune system, and especially the responses of T helper (Th) cells. In this scheme, CD4⁺ T cell-dependent responses are classified into T helper 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 , whereas Th2 cells promote B-cell dependent humoral immunity and selectively produce IL-4, IL-5, and IL-13. For the most part, Th2-based reactions constitute the fundamental response to allergen inhalation and thus account for the overproduction of Th2-derived cytokines that is characteristic of allergic asthma (Poston *et al.*, 1992; Ying *et al.*, 1995). By extension, the same reactions may underlie the inflammation and consequent airway hyperreactivity (AHR) and mucus overproduction that are also characteristic of the airway disease found in COPD. The Th1 versus Th2 paradigm has become more complicated by the recognition of Th17 (IL-17-producing) and Treg (IL-10- and TGF- β -producing) subsets of T cells, and these subsets may also contribute to inflammatory airway disease at least in part by increasing the Th2 response (Wang and Liu, 2008; Wilson *et al.*, 2008).

However, some research findings appear to challenge the Th2 hypothesis for asthma and related airway diseases. For example, studies in mice using adoptive transfer with Th1 and Th2 cells indicate that Th1 cells may also be necessary for initiating the allergic response (Castro *et al.*, 2000; Randolph *et al.*, 1999). Moreover, it is unclear how a Th2-polarized response accounts for airway disease that is also triggered by exposure to nonallergic stimuli, especially infection with respiratory viruses, that would ordinarily elicit a Th1 response. Indeed, the broader issue of the relationship between acute infection, especially due to viruses (including respiratory viruses), and the subsequent development of chronic inflammatory disease (including lung disease) remains uncertain. Perhaps by analogy to the allergic response, the link between infection and the subsequent development of chronic inflammatory disease has been attributed to alterations in the adaptive immune system (Fig. 5.1). However, the exact mechanism of how the immune system might direct a switch from an acute response to infection to a chronic inflammatory disease remains unknown. Because of these uncertainties, we have questioned whether other aspects of immunity and inflammation might also be critical for the pathogenesis of airway disease after viral infection. Therefore, we aimed to develop a model that better accounted for the development of allergy and asthma and was based on a more precise appraisal of the innate and adaptive systems in the airway. Accordingly, this review will summarize how our view of the immune response to respiratory viruses has evolved, based on the identification of specialized programming for host defense and pathogenic programming in chronic disease.

The review is divided into five major sections. Section 2 summarizes the background development of a high-fidelity mouse model for chronic inflammatory lung disease. This model relies on infection with a type of respiratory virus that has been linked to the development of chronic lung disease in humans. Section 3 summarizes work on this model that provides a scheme for the development of acute disease after viral infection. This work has uncovered an immune axis that links the antiviral response that is often Th1 in character to an allergic-type response that is generally Th2 in character. Section 4 summarizes studies of the same mouse model for the development of chronic lung disease and extends these findings to studies of human subjects with airway disease. This work has defined a novel innate immune axis that relies on T cell receptor semi-invariant natural killer T (iNKT) cells that are programmed to activate macrophages to produce IL-13 as well as other products that are characteristic of an alternative pathway for macrophage activation. Section 5 summarizes this review.

2. BACKGROUND FOR THE MODEL

To understand how a specific immune response leads to chronic lung disease, it was critical to generate a representative experimental model that demonstrated a close linkage between acute viral infection and the subsequent development of chronic lung disease. To be faithful to the clinical condition, the model should also include the element of genetic susceptibility as well as virology, immunology, and pathology components that are similar to what occurs in humans. In that regard, epidemiological studies of humans indicate that respiratory syncytial virus (RSV) is the most common cause of serious respiratory illness in infancy and that this particular paramyxovirus is frequently associated with the later development of persistent asthma (Castro *et al.*, 2008; Sigurs *et al.*, 2005). Some experimental evidence suggests that a related paramyxovirus known as human metapneumovirus (hMPV) can cause chronic airway disease in mice, but mucus production was increased for only a short period of time after infection (Hamelin *et al.*, 2006). There is also a reported association between rhinovirus infection and childhood asthma (Jackson *et al.*, 2008), though in this instance, rhinoviruses appear to be associated with acute exacerbation of existing disease rather than acting as a primary cause of chronic disease. Thus, RSV appears to contain distinct ingredients to drive the chronic inflammatory lung disease that develops after the resolution of acute infection.

Based on the epidemiological findings from clinical studies, investigators have often used infection with RSV to develop a model of virus-induced chronic lung disease. In general, however, RSV (like most human pathogens) replicates poorly in the mouse lung unless the virus is first adapted using serial passage or other experimental approaches. To circumvent these issues, RSV is delivered at a high inoculum, but the resulting all-or-none pattern of illness often includes a severe alveolitis rather than the primary bronchiolitis that is typical of human disease (Graham *et al.*, 1988). To better capture this critical feature of human airway disease, we selected an infectious agent known as Sendai virus (SeV). This virus is a mouse parainfluenza-type I virus that is similar to the other paramyxoviruses (e.g., RSV, hMPV, and human parainfluenza virus) that more commonly infect humans. In contrast to infection with human pathogens such as RSV and hMPV, SeV replicates at high efficiency in the mouse lung and causes an acute virus-mediated inflammation of the small airways that is essentially indistinguishable from the RSV-mediated disease observed in humans (Shornick *et al.*, 2008; Tyner *et al.*, 2005; Walter *et al.*, 2001). In particular, the pattern of illness after SeV inoculation resembles the so-called “top-down” infection found in humans. In this case, an intermediate inoculum causes infection limited to the airway mucosa and inflammation that is largely restricted to peribronchial and bronchiolar tissues. Smaller inoculum will cause illness confined to the upper airways (i.e., sinusitis and bronchitis) whereas larger inoculums will cause disease that extends to the alveolar compartment (i.e., pneumonitis). Because chronic inflammatory disease is likely to be found in the small pulmonary airways, we suspect that a severe infection at this site is critical for the subsequent development of chronic lung disease (similar to the case in humans where severe RSV bronchiolitis is associated with chronic asthma).

Consistent with these principles, we found that the acute antiviral response to SeV infection is followed by a delayed but permanent switch to chronic airway disease in mice. This disease is characterized by overproduction of mucus [marked by mucous cell metaplasia (MCM)] and increased airway reactivity to inhaled methacholine (defined by AHR) (Patel *et al.*, 2006; Tyner *et al.*, 2006; Walter *et al.*, 2002). These disease traits of MCM and AHR are hallmark features of asthma and COPD in humans. These traits are also the primary causes of morbidity and mortality in these conditions, and therefore are primary targets for therapy. As developed in this review, the airway disease is first detected at 3 weeks after viral inoculation, reaches its maximal level at 7 weeks, and persists for at least a year later (Kim

et al., 2008; Patel *et al.*, 2006; Tyner *et al.*, 2006; Walter *et al.*, 2002). This time course is consistent with the one in humans, wherein chronic lung disease also lasts indefinitely after infection.

As noted above, another characteristic of chronic lung disease in humans that needs to be represented in the mouse model is genetic susceptibility. In that regard, we have observed that the development of chronic lung disease after SeV infection is manifested most vigorously in the C57BL/6J strain of inbred mice. By contrast, the BALBc/J strain of mice exhibits a very similar pattern of acute illness in the first week after viral inoculation, but fails to develop any significant acute disease at 3 weeks or chronic disease at 7 weeks after inoculation (Patel *et al.*, 2006). Other mouse strains (e.g., CV129, C3H/HeJ, or A/J) are so sensitive to SeV (and develop such severe alveolitis) that it is difficult to capture the top-down pattern of illness that is typical of severe RSV infection in humans. In both C57BL/6J and BALBc/J mice, the initial reverse transcriptase polymerase chain reaction (RT-PCR) analysis of virus in whole-lung homogenates indicated that SeV was completely cleared before the onset of airway disease on postinoculation week 3 (Patel *et al.*, 2006; Walter *et al.*, 2002). However, more sensitive PCR assays indicated that low levels of virus may persist for longer periods of time in each of these mouse strains (Kim *et al.*, 2008) and (E. Agapov and M. J. Holtzman, unpublished observations). The role for this remnant viral RNA in driving a chronic immune response still needs to be fully defined. This role as well as the one for host genetics will likely only be resolved after we define the type of immune response that causes the chronic disease found in this model. In that regard, the C57BL/6J strain provides a suitable genetic background for transgenic and knockout mice that could be used to define the immune mechanism for chronic inflammatory disease after viral infection.

3. cDC T CELL IMMUNE AXIS

In this section, we review our analysis of the mouse model to define how the acute airway disease develops at 3 weeks after respiratory viral infection. This pattern of illness is also frequently found in humans, wherein illness is manifested by persistent cough, sputum production, or wheezing after respiratory viral infection. Based on the short-term course of illness, we assumed that the acute illness might be mediated by the innate immune system. We reasoned that inhibition of the acute inflammatory response could be achieved by targeted disruption of airway epithelial immune-response genes. These genes form a network that is directly induced by viral replication and is dominated by an array of IFN-responsive genes (Koga *et al.*, 1999; Look *et al.*, 1998; Walter *et al.*, 2001). Among candidate genes that might mediate immune cell traffic, the intercellular adhesion molecule (ICAM)-1 is the predominant determinant for epithelial-immune cell adhesion *in vitro* (Nakajima *et al.*, 1994, 1995; Taguchi *et al.*, 1998). Indeed, we found that ICAM-1 expression was induced primarily on host airway epithelial cells (AECs) by viral infection and was necessary for full development of acute inflammation and concomitant postviral AHR. Moreover, we demonstrated that the acute disease (but not the later chronic disease) depended on the upregulation of ICAM-1 (Walter *et al.*, 2002). Therefore, this study illustrated that the acute disease could be genetically segregated from the chronic disease (using ICAM-1-null mice), and thereby served to establish a separate pathogenic mechanism for acute versus chronic inflammatory disease. However, this study did not further define the immune basis of how the acute or chronic disease developed.

The next breakthrough in defining the immune basis for the persistent AHR and MCM after viral infection came when we discovered that these disease traits depended on the production of IL-13 in the lung (Kim *et al.*, 2008; Tyner *et al.*, 2006). Using high-speed flow cytometry in combination with real-time PCR analysis, we were able to identify a major cellular source of IL-13 production as CD4⁺ T cells (Kim *et al.*, 2008). Although CD4⁺ Th2

cells are a traditional source of IL-13 production during the allergic response, it is remarkable that this same cell population is also a source of chronic IL-13 production after viral infection. This finding raised the possibility that elements of an adaptive immune response characteristic of the one that develops after allergen exposure (a Th2 response) might also mediate the disease found after viral infection (a Th1 response). When we pursued the underlying mechanism for CD4⁺ T cell production of IL-13 in this model, we discovered a novel pathway that links acute viral infection to chronic lung disease (Fig. 5.2). This pathway is initiated when viral induction of type-I IFN production drives expression of the high-affinity IgE receptor (FcεRI) on cDCs. Subsequent activation of FcεRI causes production of the chemokine CCL28 and consequent recruitment of CD4⁺ T cells that produce IL-13 (Grayson *et al.*, 2007a). As described below, the scheme is based on the findings that (1) viral infection induces type-I IFN receptor (IFNAR)-dependent expression of FcεRI on cDCs that are resident in mouse lung tissue; (2) expression of FcεRI on cDCs is followed by generation of antiviral IgE; (3) IgE-dependent activation of FcεRI on cDCs causes release of the T cell chemoattractant CCL28; (4) blockade of CCL28 inhibits MCM after viral infection; (5) loss of FcεRI (in *FceRIα*^{-/-} mice) leads to a decrease in CCL28 production, decreased recruitment of IL-13-producing CD4⁺ T cells to the lung, and inhibition of MCM after viral infection, while adoptive transfer of wild-type cDCs to *FceRIα*^{-/-} mice restores this immune cascade to wild-type conditions. The findings thereby establish a pathway that mediates chronic IL-13 production by CD4⁺ T cells after viral infection, and serves to explain how an antiviral response, that is generally Th1 in character, can drive an allergic/asthmatic response that is generally Th2 in character.

3.1. Viral infection causes FcεRIα expression on lung DCs

Respiratory viral infection is a recognized stimulus of IgE production, and IgE levels are known to regulate FcεRI expression (Rager *et al.*, 1998; Skoner *et al.*, 1995; Welliver *et al.*, 1986). We therefore asked which cell type(s) in the lung might express FcεRI during the course after viral infection. In rodents, the high-affinity IgE receptor had been identified only on mast cells, basophils, and possibly eosinophils (Dombrowicz *et al.*, 2000). However, in humans, the receptor may also be found on skin and peripheral blood cDCs, as well as bronchoalveolar lavage fluid plasmacytoid dendritic cell (pDCs), monocytes, and Langerhan cells, albeit as a trimeric form (FcεRIαγ) that lacks the FcεRIβ chain (Bieber *et al.*, 1992; Foster *et al.*, 2003; Schroeder *et al.*, 2005). We therefore questioned whether this form of the receptor might also be found on resident lung cDCs after viral infection. Using forward/side scatter characteristics and a high level of CD11c expression to identify lung cDCs, we observed that SeV infection leads to a rapid and sustained decrease in the number of lung cDCs, but at least a portion of this cell population remains in the lung and becomes more mature and differentiated (Grayson *et al.*, 2007b). Because the function of this resident population of cDCs was uncertain, we examined it in more detail.

We found that lung cDCs began to express the α-chain of the high-affinity receptor for IgE (FcεRIα) at SeV postinoculation Day 3, and this expression remained detectable for 2–3 weeks after inoculation (Grayson *et al.*, 2007a). Viral induction of FcεRIα expression was specific to cDCs resident in the lung, since there was no detectable expression of FcεRIα on cDCs isolated from draining lymph nodes or spleens of mice after SeV infection. No difference was noted in other surface molecule expression (MHC-II, B220, CD11b, CD80, CD86, or CD23) on lung cDCs from wild-type or *FceRIα*^{-/-} mice. The level of expression of FcεRIα on lung cDCs at postinoculation Day 7 was similar to levels found on c-kit⁺ lung mast cells. However, mast cells also expressed FcεRIα at baseline before inoculation. We did find a similar level of induction of FcεRIα on pDCs in the lung, but we did not detect expression of FcεRIα on lung CD4⁺ or CD8⁺ T cells, B220⁺ B cells, Mac-3⁺ macrophages, or GR-1⁺ neutrophils.

Based on work with isolated cells (particularly mast cells), mice have been reported to be obligate expressers of the classical tetrameric form (FcεRIαβγγ) of FcεRI (Blank *et al.*, 1989). To determine whether mouse lung cDCs were indeed expressing the tetrameric or trimeric (FcεRIαγγ) form of the receptor, we analyzed mouse lung cDCs for expression of each FcεRI component chain. Based on immunoprecipitation, immunoblotting, and real-time PCR assays for individual FcεRI components, we showed that mouse lung cDCs appear to express only the trimeric (FcεRIαγγ) form of FcεRI (Grayson *et al.*, 2007a). This is the same form of FcεRI as expressed on human antigen-presenting cells (APCs), although expression in mouse cDCs requires induction by a productive viral infection. This requirement may explain the failure to detect FcεRI expression on mouse DCs in previous work. The loss of *FceRIa* gene expression was not associated with an altered immune response against SeV, as evidenced by clearance of SeV. Further, development of an adaptive immune response was not impaired given the appropriate expansion of SeV-specific CD8⁺ T cells in the lung.

3.2. Type-I IFN regulates FcεRIα expression

We next explored what component of the antiviral response is responsible for induction of FcεRIα expression on lung cDCs during viral infection. Serum IgE level tightly regulates expression of the high-affinity IgE receptor in humans, so we reasoned that total IgE and, more likely, SeV-specific IgE might drive FcεRI receptor expression after viral infection in mice. However, we found that serum total IgE and SeV-specific IgE do not increase until 1 week after inoculation (Grayson *et al.*, 2007a). This time course lags behind the onset of expression of FcεRIα on lung cDCs, suggesting that the level of IgE is not driving the expression of FcεRIα under these conditions. In fact, we found that IgE-deficient (*IgE*^{-/-}) mice continued to develop the same increase in FcεRIα expression on lung cDCs as wild-type littermates (*IgE*^{+/+}) after viral infection. Therefore, IgE levels did not appear to regulate the appearance of FcεRIα on lung cDCs after viral infection in mice.

Given that initial expression of FcεRIα developed at the same time as IFN-dependent responses in this viral model, we examined whether IFN signaling was necessary for FcεRIα expression. We found that IFNAR deficient (*IFNAR*^{-/-}) mice no longer exhibited an increase in FcεRIα expression on lung cDCs during viral infection (Grayson *et al.*, 2007a). By contrast, mice that were deficient for *CD1d*, *CD4*, *CD8*, *perforin*, or *MyD88* gene expression showed no defect in *FceRIa* expression after viral infection. Thus, while IFNAR is critical for expression of FcεRIα on lung cDCs after viral infection, there is no significant role for NKT cells, CD4⁺ T cells, or CD8⁺ T cells, or for the innate antiviral perforin- or MyD88-dependent Toll-like receptor pathways in this process. We also determined that IFNAR expression on cDCs was not necessary for FcεRIα expression after viral infection. For these experiments, we purified lung cDCs from *IFNAR*^{-/-} and wild-type control mice and used carboxyfluorescein diacetate succinimidyl ester (CFSE) to discriminate from endogenous cDCs after transfer into wild-type or *IFNAR*^{-/-} recipients. We found that either *IFNAR*^{-/-} or wild-type cDC transfer into wild-type mice allowed for expression of FcεRIα on cDCs. By contrast, transfer of either genotype of cDCs into *IFNAR*^{-/-} mice did not permit expression of FcεRIα on cDCs after viral infection.

These findings imply that IFN acts through another intermediate cell to activate expression of FcεRI on lung cDCs. Using similar cell transfer experiments, we later demonstrated that neutrophils are required for mediating the IFNAR signal to cDCs after viral infection (Grayson *et al.*, 2008). Moreover, the absence of dipeptidyl peptidase I (DPPI), a lysosomal cysteine protease found in neutrophils, dampens the acute inflammatory response and the subsequent MCM that develops after viral infection in mice (Akk *et al.*, 2008). This attenuated phenotype is accompanied by a significant decrease in the accumulation of neutrophils and the local production of CXCL2, TNF, IL-1β, and IL-6 in the lung of infected *DPPI*^{-/-} mice. Adoptive transfer of DPPI-sufficient neutrophils into *DPPI*^{-/-} mice restored

the levels of CXCL2 and enhanced cytokine production on Day 4 after inoculation and the subsequent MCM at 3 weeks after inoculation. Together, these results indicate that DPPI-dependent neutrophil recruitment also contributes to the acute disease after viral infection.

3.3. FcεRI activation drives CCL28 production

Because IL-13 drives postviral MCM, and Th2 cells are a source of IL-13 in this process (Kim *et al.*, 2008; Tyner *et al.*, 2006), we reasoned that generation of Th2 cells might be linked to FcεRI engagement on cDCs. Using a cell culture system that contains CD4⁺ T cells and cDCs, we showed that antigen-specific CD4⁺ T cells produced IL-13 when cultured with antigen-pulsed cDCs regardless of whether cDCs were isolated from wild-type or *FceRIa*^{-/-} mice or from infected or uninfected mice (Grayson *et al.*, 2007a). Furthermore, cross-linking FcεRI had no effect on T cell production of IL-13 or proliferation. Thus, lung cDCs are capable of driving the development of IL-13 producing CD4⁺ Th2 cells, but this process does not require expression or activation of FcεRI.

Since we did not detect a requirement for cross-linking FcεRIα in the development of the T cell cytokine response, we next assessed whether FcεRI might be involved in the generation of T cell chemoattractants. Lung cDCs were purified from wild-type mice and were then cultured with a cross-linking antibody against FcεRIα or a control hamster IgG. Supernatants from these cultures were then used in a modified Boyden chamber assay to assess whether a functional T cell chemoattractant had been produced. Supernatants from FcεRIα cross-linked cDCs induced significantly more CD4⁺ T cell migration than did IgG control supernatants, indicating that engagement of the receptor led to production of a CD4⁺ T cell chemoattractant (Grayson *et al.*, 2007a). To identify the chemokine receptor for this T cell chemoattractant, we added individual T cell chemokines (CCL5, CCL22, and CCL27) to the upper chamber and evaluated the effect on chemotaxis. Only CCL27 (C-TACK) inhibited this T cell migration, indicating that the CD4⁺ T cells were moving in response to a CCR10 agonist. Only two known CCR10 agonists have been identified, CCL27 and CCL28 (MEC). Using blocking mAbs, we found that the chemotactic activity was entirely due to CCL28. Relevant to this finding, we noted that CCL28 was associated with both human asthma and mouse models of asthma (English *et al.*, 2006; John *et al.*, 2005; Wang *et al.*, 2000).

To establish that CCL28 expression was inducible in cDCs after a more physiological form of FcεRI activation, we loaded lung cDCs from post-inoculation Day 7 with ovalbumin (Ova)-specific IgE. We found that addition of Ova (by cross-linking IgE bound to FcεRI on the cDC) caused a mark increase in *CCL28* mRNA levels (Grayson *et al.*, 2007a). Similar results were obtained when using the anti-FcεRIα antibody to directly cross-link the receptor. To determine whether FcεRI activation also caused CCL28 expression *in vivo*, we returned to experiments with the mouse model of virus-induced lung disease. As the description of development below, we found that lung levels of CCL28 mRNA were increased after SeV infection (Grayson *et al.*, 2007a). In addition, we found a significant decrease in MCM in mice treated with an anti-CCL28 blocking mAb versus control IgG2b. This finding suggested that CCL28 is a chemotactic factor for CD4⁺ T cells producing IL-13 and that this particular chemokine is necessary for the full development of MCM after viral infection.

3.4. FcεRI on cDCs is required for acute lung disease

To verify our proposed FcεRI-driven pathway *in vivo*, we next monitored each of the downstream steps in mice that were deficient in *FceRIa* (*FceRIa*^{-/-}). Both *FceRIa*^{-/-} and wild-type control mice exhibited similar morbidity (as monitored by weight loss), development of an adaptive immune response (as evidenced by the development of SeV-

specific CD8⁺ T cells), and clearance of virus from the lung (based on SeV copy number) during the acute phase of viral infection. Despite a similar acute response to viral infection, we found that *FceRIa*^{-/-} mice exhibited decreased expression of CCL28, decreased frequency of lung CD4⁺ T cells, a decreased levels of *IL-13* and *GATA-3* mRNA in lung CD4⁺ T cells, a decrease in CCR10⁺ CD4⁺ T cells, a decreased level of *GATA-3* mRNA (typically found in Th2 cells) compared to *T-bet* mRNA (typically found in Th1 cells), and a marked decrease in the number of Muc5ac-expressing epithelial cells and total lung *Muc5ac* mRNA after viral infection (Grayson *et al.*, 2007a). These findings further supported the proposal that inhibition of MCM in *FceRIa*^{-/-} mice was based on decreased accumulation of IL-13-producing CD4⁺ T cells due to a lack of production of CCL28 by the resident FcεRIα⁺ lung cDCs. Together, these findings provide further support for the proposal that activation of FcεRIα on cDCs leads to preferential accumulation of CD4⁺ Th2 cells in the lung after SeV infection. (CD4⁺ GATA-3⁺ IL-13-producing T cells are referred to here as Th2 cells, although they do not appear to produce IL-4 under these conditions.)

To further prove that FcεRI expression on cDCs was necessary for recruitment of CD4⁺ Th2 cells to the lung and MCM after viral infection, we performed adoptive cell transfer experiments with cDCs from wild-type or *FceRIa*^{-/-} mice transferred into *FceRIa*^{-/-} recipients followed by SeV inoculation. We found that reconstitution with wild-type cDCs restored postviral MCM in *FceRIa*^{-/-} recipient mice. By contrast, transfer of *FceRIa*^{-/-} cDCs was unable to restore the development of MCM after viral infection. Furthermore, we found that CD4⁺ T cells isolated from lungs of *FceRIa*^{-/-} mice that had received wild-type cDCs expressed *IL-13* and *GATA-3* mRNA at significantly higher levels than mice that received cDCs from *FceRIa*^{-/-} mice. Together, these results indicate that FcεRI on lung cDCs is critical for Th2 cell recruitment and MCM that develops at 3 weeks after viral infection. We note that FcεRI expression returns to baseline levels by 3 weeks after viral inoculation (Grayson *et al.*, 2007a). Consistent with this finding, it appears that *FceRIa*^{-/-} mice are able to manifest chronic lung disease by 7 weeks after viral inoculation (L. A. Benoit, D. E. Byers, M. H. Grayson, and M. J. Holtzman unpublished observations). Thus, another immune pathway must mediate the chronic lung disease that develops after viral infection in the mouse model.

4. NKT CELL–MACROPHAGE IMMUNE AXIS

As summarized in the previous section, our discovery of the cDC-T cell pathway presents a relevant paradigm of how the adaptive immune response can drive acute inflammatory disease after viral infection. This finding was unexpected since the adaptive immune response is most often proposed to drive chronic inflammatory disease and accordingly to have a central role in the development of a variety of inflammatory diseases (Anderson and Bluestone, 2005; Busse and Lemanske, 2001; Herrick and Bottomly, 2003; Jones *et al.*, 2006; Seino and Taniguchi, 2006). By contrast, the innate immune system is believed to mediate the acute response to an infectious agent and is not thought to be solely responsible for a chronic inflammatory state (Mattner *et al.*, 2005). Thus, as we approached the issue of defining an underlying mechanism for the chronic inflammatory lung disease in this model, we might have predicted that chronic inflammation was most likely mediated by the adaptive rather than the innate immune response. Unexpectedly, we next learned that the innate immune response can be solely responsible for the development of chronic inflammatory disease.

In this section, we summarize our work on defining the innate immune pathway for the chronic inflammatory lung disease that develops fully at 7 weeks after respiratory viral infection. The initial insight into the immune basis for chronic lung disease in this model was the identification of lung macrophages as a significant source of IL-13 in the long-term

response that developed after viral infection. When we pursued the underlying mechanism for this observation, we uncovered a second immune pathway (summarized in Fig. 5.3) that developed independently of an adaptive immune response and depended on iNKT cells to drive macrophage production of IL-13 (Kim *et al.*, 2008). Analysis of lung tissue obtained from patients with asthma and COPD indicated that this innate immune axis was also activated in humans with chronic obstructive lung disease. Together, the findings from studies of mice and humans provided a mechanism for the transition of an acute viral infection into a chronic inflammatory disease and new mechanistic insight into the pathogenesis of chronic inflammatory disease. Here, we present an outline of these findings and point out how this novel immune pathway differs from other reports of NKT cell and macrophage activation in asthma and asthma models (Akbari *et al.*, 2003,2006;Lisbonne *et al.*, 2003;Sen *et al.*, 2005;Vijayanand *et al.*, 2007).

4.1. IL-13 requirement for chronic lung disease

After C57BL/6J mice are infected with SeV, airway disease is first detected at 3 weeks after viral infection (as developed above in the previous section), but disease does not become maximal until 7 weeks after infection (Kim *et al.*, 2008; Tyner *et al.*, 2006; Walter *et al.*, 2002). Disease is manifested by leukocyte infiltration as well as AHR (signified by increased pulmonary resistance in response to inhaled methacholine) and MCM (marked by increased lung levels of *Muc5ac* mRNA and *Muc5ac* immunostaining). Under these conditions, MCM is manifested by overexpression of the predominant airway mucin *Muc5ac* and depends on differentiation of mucous cells from airway epithelial precursor cells, that is, ciliated cells and Clara cells that are normally present in the airway epithelium (Tyner *et al.*, 2006). Despite the high levels of IL-13 production in the lung, we did not observe pulmonary emphysema or fibrosis at any time after viral infection (Kim *et al.*, 2008). These additional abnormalities can be found after overexpression of the *Il-13* gene in transgenic mouse models (Chapoval *et al.*, 2007), but the cellular pattern of IL-13 expression in these models may not reflect endogenous cytokine production that is found naturally after viral infection or perhaps other conditions.

Since IL-13 has been widely identified as a critical mediator of lung disease in experimental animal models (including the acute disease that developed after viral infection) and in human asthma, we investigated the role of IL-13 in the development of chronic disease in this model. Similar to the time course for appearance of airway disease, IL-13 production was first detected at 3 weeks and reached maximal levels at 7 weeks after viral inoculation (Kim *et al.*, 2008; Tyner *et al.*, 2006). Furthermore, mice treated with an IL-13 decoy receptor (sIL-13-R α 2-Fc) or targeted to disrupt *Il-13* gene expression (*Il-13*^{-/-}), no longer developed either MCM or AHR at any time after viral infection (Kim *et al.*, 2008). The inhibition of AHR was independent of any changes in baseline airway caliber. By viral plaque-forming assay, infectious virus is no longer detectable by 2 weeks after infection (Patel *et al.*, 2006; Walter *et al.*, 2002), and, by real-time PCR assay, SeV-specific RNA is decreased to trace levels before airway disease appears at 3 weeks after infection (Kim *et al.*, 2008). Lung levels of viral RNA were similar in *Il-13*^{-/-} and wild-type mice (Kim *et al.*, 2008). Therefore, the inhibition of chronic lung disease in *Il-13*^{-/-} mice does not appear to depend on any difference in viral replication or clearance. Nonetheless, virus-specific RNA may persist for long periods of time, and this remnant viral RNA may serve to drive a chronic immune response leading to disease. This issue will likely only be resolved after we define the type of immune response that causes the chronic disease traits found in this model.

4.2. IL-13 production by macrophages

We next investigated the cellular sources of IL-13 in the lung after viral infection. As noted above, CD4⁺ T cells (CD3⁺CD4⁺) contributed the highest amount of *Il-13* mRNA among immune cells in the lung at 3 weeks, but macrophages (Mac1⁺CD68⁺) became the predominant cellular source of *Il-13* mRNA by 7 weeks after viral inoculation (Kim *et al.*, 2008). For CD4⁺ T cells and macrophages, the contribution to 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. Other minor sources of *Il-13* mRNA included NK cells and NKT cells. However, there was no detectable *Il-13* mRNA produced by mast cells, basophils, neutrophils, DCs, B cells, or CD8⁺ T cells. We also found that lung macrophages cultured from SeV (but not SeV-UV) at 7 weeks after infection expressed *Il-13* mRNA and produced IL-13 protein. IL-13 protein release was further increased by treatment with PMA-ionomycin. Regardless of PMA-ionomycin treatment, IL-13 protein production was increased in lung macrophages compared to CD4⁺ T cells and in cells isolated from SeV-inoculated mice compared to SeV-UV-inoculated control mice. This pattern for IL-13 protein production was consistent with the analysis of *Il-13* mRNA levels. Furthermore, cells with typical macrophage morphology and CD68 expression were positive for IL-13 immunostaining in alveolar, interstitial, and epithelial locations in wild type but not in *Il-13*^{-/-} mice. In some sections, we found a marked accumulation of macrophages in the airway epithelium that was not observed in uninfected control. The high production of IL-13 per macrophage compared to other cell types (e.g., CD4⁺ T cells) may be responsible for the detection of CD68⁺IL-13⁺ but not CD4⁺IL-13⁺ cells in tissue. Together, these findings provided evidence that lung macrophages are a significant cell source of chronic IL-13 production after viral infection. Ongoing work is aimed at defining the precise subset of macrophages that manifest IL-13 production.

To determine whether macrophages are necessary for chronic lung disease, we next examined macrophage-deficient mice. We first studied *op/op* mice that are macrophage-deficient due to a loss of function mutation in the *Csf1* (also known as *M-csf*) gene. Since macrophages are required for antiviral defense (Tyner *et al.*, 2005), these mice were infected with a reduced viral inoculum. Compared to wild-type mice inoculated with the same amount of SeV, the *op/op* mice exhibit markedly decreased levels of *Il-13* and *Muc5ac* gene expression (Kim *et al.*, 2008). To preserve the ability to develop an acute immune response to viral infection, we next studied wild-type mice that were treated with clodronate-containing liposomes using a protocol that selectively depletes lung macrophages (Tyner *et al.*, 2005). Liposome treatment was begun after clearance of infectious virus to avoid any possible effect on the acute infection. Thus, there was no need to decrease the viral inoculum to preserve survival, and the usual inoculum was administered in these experiments. We found no difference between control and clodronate-liposome treated mice in the levels of *Il-13* or *Muc5ac* mRNA at 3 weeks after viral inoculation, which was consistent with a lack of IL-13 production by macrophages at this early time point (Kim *et al.*, 2008). However, macrophage-depleted mice exhibited significantly decreased levels of *Il-13* and *Muc5ac* gene expression at 7 weeks after viral inoculation compared to mice that were treated with empty liposomes. Immunostaining of lung sections indicated that clodronate-treated mice were depleted of IL-13-producing macrophages. The loss of IL-13 producing macrophages caused a decrease in *Il-13* mRNA and MCM that was in proportion to the macrophage contribution to total *Il-13* mRNA production in the lung at 7 weeks after viral inoculation.

4.3. NKT cell activation of macrophages

We reasoned that persistent pressure from the immune system was necessary to activate macrophages for prolonged periods after viral clearance. As noted above, this type of chronic pressure is generally attributed to activation of the adaptive immune response.

However, we found that MHC Class II-deficient *H2-Ab1*^{-/-} mice that lack most CD4⁺ T cells continued to develop MCM in concert with increased *Il-13* and *Muc5ac* mRNA levels after viral infection (Kim *et al.*, 2008). We found the same susceptibility to develop chronic airway disease in CD4⁺ and CD8a⁺ T cell-deficient mice. Similar to wild-type mice, these immunocompromised mice contained no detectable levels of viral RNA at baseline and only trace levels of viral RNA in the lung by 3 weeks after viral inoculation. To fully avoid interfering with the acute antiviral response, we also achieved T cell depletion using antibody treatment that (like sIL-13Rα2-Fc and clodronate treatment) was not initiated until after clearance of infectious virus. Similar to mice with a genetic T cell deficiency, mice that were antibody-depleted of CD4⁺ T cells, CD8⁺ T cells, or both CD4⁺ and CD8⁺ T cells also showed the expected increase in the number of IL-13-producing macrophages, induction of *Il-13* or *Muc5ac* gene expression, and MCM after viral infection. These findings do not exclude a role for T cells in the development of chronic lung disease after viral infection. Indeed, we find that CD4⁺ T cells are a significant source of IL-13 production at 3 weeks and 7 weeks after viral inoculation. The findings with T cell depletion do, however, suggest that other types of immune cells may also influence the chronic disease traits that develop after viral infection.

The unexpected findings in the context of T cell deficiency led us to consider whether a new type of cellular mechanism could mediate a chronic immune response. In that context, we noted that, in addition to T cells and macrophages, the NKT cell population was recruited to the lung at 7 weeks after viral inoculation. This increase was observed irrespective of whether NKT cells were detected using a α-galactosylceramide (α-GalCer) analog-loaded CD1d-tetramer or by costaining for the NK1.1 and CD3 antigens. Further analysis of the lung NKT cell population indicated that the CD4⁻ as well as CD4⁺ NKT cells were recruited into the lung. In fact, CD4⁻ NKT cells appeared in greater numbers in the lung, especially at 7 weeks after viral inoculation. The involvement of CD4⁻ NKT cells in driving chronic inflammation was consistent with our finding that treatment with a CD4-depleting mAb did not decrease *Il-13* or *Muc5ac* mRNA production.

Chronic NKT cell activation after viral infection was associated with a relatively selective increase in *Il-13* mRNA production. Thus, we found relatively little induction of *Ifn-β*, *Ifn-γ*, or *Il-4* gene expression by NKT cells (particularly the predominant CD4⁻ NKT cell population) in the setting of chronic lung disease after viral infection. This pattern of low *Il-4* mRNA production was also found for CD4⁺ T cells at 7 weeks after viral inoculation. Consistent with these findings, treatment with an anti-IL-4 mAb from postinoculation Day 12 to Week 3 (or Week 7) caused no significant change in lung *Il-13* or *Muc5ac* mRNA levels or corresponding airway reactivity or mucous cell levels. Therefore, the profile of Th2-type cytokine production after viral infection is distinct from the one after allergen challenge, where IL-4 production is more prominent and often contributes significantly to the development of disease. NKT cell contribution to overall levels of *Il-13* mRNA in the lung is relatively small compared to macrophages at 7 weeks after viral inoculation. Nonetheless, the persistent production of *Il-13* mRNA and the accumulation of NKT cells in the lung suggested that this cell population might act in a regulatory role to drive the chronic production of IL-13 by macrophages in the lung.

The majority of mouse NKT cells express the semi-invariant V_α14-J_α18 TCR chain that recognizes glycolipid antigen presented by the oligomorphic CD1d, an MHC Class-I-like protein (Bendelac *et al.*, 1995; Kowano *et al.*, 1997). To investigate the role of NKT cells in our virus-induced model of chronic lung disease, we studied mice that were deficient in NKT cells due to loss of *Cd1d* or *Ja18* gene expression (Cui *et al.*, 1997; Mattner *et al.*, 2005). We found that *Cd1d*^{-/-} mice have markedly decreased levels of IL-13-expressing macrophages in lung tissue as well as decreased levels of lung *Il-13* and *Muc5ac* mRNA

after viral infection. Moreover, the decrease in IL-13-producing macrophages as well as *Il-13* and *Muc5ac* mRNA levels in the lung were observed at 7 weeks but were not observed at 3 weeks after viral inoculation. This finding was consistent with the time course for increased macrophage production of IL-13 and with the effects of macrophage depletion in the experiments described above. Furthermore, *Cd1d*^{-/-} mice at 7 weeks after viral inoculation also had decreased AHR relative to wild-type control mice, and this decrease was independent of any changes in baseline airway caliber. We also observed similar inhibition of virus-induced *Il-13* and *Muc5ac* gene expression in the lungs of *Ja18*^{-/-} mice. In both strains, lung levels of viral RNA were similar to wild-type mice. Together, these findings indicated that NKT cells (independent of CD1d-dependent actions on NKT cells or APCs) were necessary for chronic lung disease after viral infection.

4.4. Basis of NKT cell–macrophage interaction

Based on the findings in NKT cell-deficient mice, we postulated that NKT cells might directly influence the population of IL-13-producing macrophages after viral infection. We reasoned that NKT cells could act by recruiting macrophages to the lung and activating this cell population to produce IL-13. A role for NKT cell-dependent recruitment of macrophages was substantiated when we found that the usual increase in lung macrophages found in wild-type mice was blocked in NKT cell-deficient mice at 7 weeks after viral inoculation (Kim *et al.*, 2008). In support of a mechanism for NKT cell-dependent recruitment of macrophages, we observed that purified lung CD4⁻ NKT cells released biologically relevant amounts of the monocyte/macrophage chemokines (predominantly CCL3) after stimulation with PMA-ionomycin. Furthermore, lung NKT cells isolated from lungs at 7 weeks after viral inoculation produced increased levels of macrophage chemokine mRNA compared to NKT cells from age-matched control mice without virus-induced lung disease. The predominant chemokine mRNA produced by NKT cells after viral infection was *Ccl3* (consistent with the profile for chemokine production at the protein level), and increased *Ccl3* (as well as *Ccl2* and *Ccl4*) mRNA levels were found exclusively in CD4⁻ rather than CD4⁺ NKT cells (consistent with the increased activity of CD4⁻ NKT cells in chronic lung disease after viral infection). These findings suggest that NKT cells may recruit macrophages to the lung after viral infection, but additional work is required to better define the functional role of NKT cell-derived chemokines *in vivo*.

To determine whether NKT cells might directly stimulate macrophage production of IL-13, we next established a system for coculture of purified NKT cells and macrophages (Kim *et al.*, 2008). In this system, we used NKT cells and macrophages from mice without SeV inoculation to achieve low background levels of IL-13 production. We used α -GalCer-analog-loaded CD1d-tetramer to isolate NKT cells and thereby obtained the same iNKT cell population that was targeted in *Cd1d*^{-/-} and *Ja18*^{-/-} mice. The system was also constructed so that NKT cells could be removed after cell–cell interaction to allow for monitoring IL-13 production derived only from adherent macrophages.

A role for lung NKT cell-mediated activation of macrophage IL-13 production was established when we found that lung NKT cells, when cocultured with lung macrophages, caused macrophage production of *Il-13* mRNA and release of IL-13 protein (Kim *et al.*, 2008). The NKT cell effect on macrophage production of IL-13 was relatively specific for lung NKT cells since it was not found for naïve CD4⁺ T cells. NKT cell stimulation of macrophage IL-13 production was significantly inhibited by treatment with anti-CD1d mAb, indicating that this NKT cell–macrophage immune axis required direct contact of V α 14-J α 18-TCR on the NKT cell with CD1d present on the macrophage. Furthermore, NKT cell-dependent activation of lung macrophages was increased by 10-fold in the presence of TCR-CD1d ligand α -GalCer, achieving IL-13 production levels similar to PMA-ionomycin stimulation of macrophages. NKT cell-derived IL-13 was also necessary for NKT cell-

dependent activation of macrophage IL-13 production, since activation was lost if coculture was performed with NKT cells from *Il-13*^{-/-} mice. CD4⁻ NKT cells are more abundant than CD4⁺ NKT cells in the lung, both at baseline and after SeV infection. To determine whether CD4 expression also influenced NKT cell–macrophage interaction, we studied liver NKT cells to provide a more abundant source of CD4⁺ and CD4⁻ NKT cells. We found that liver NKT cells were less effective than lung NKT cells in driving macrophage activation, in that liver NKT cells required α -GalCer ligand to stimulate macrophage production of *Il-13* mRNA. Nonetheless, pure preparations of either CD4⁺ or CD4⁻ liver NKT cells were both capable of activating lung macrophages when CD1d glycolipid ligand was added to the coculture system. Thus, while CD4⁺ NKT cells have been the focus for studies of the response to allergen in the lung (Akbari *et al.*, 2003), it appears that CD4 expression is not necessary for NKT cell capacity to drive macrophage production of IL-13. Instead, NKT cell-dependent macrophage activation appears to depend primarily on invariant TCR-CD1d and IL-13–IL-13 receptor (IL-13R) interactions.

4.5. IL-13 production and alternative pathway activation

We next further characterized the downstream events in the NKT cell–macrophage–IL-13 immune axis that we had identified. It was possible that persistent activation of this immune axis could be driven by upregulation of a cytokine or cytokine receptor. We therefore applied oligonucleotide microarrays to analyze mRNA isolated from the lungs of mice at 3 and 7 weeks after viral inoculation and examined the microarray gene expression data to identify any chronic changes in cytokine or cytokine receptor expression after viral infection. The only significant change among cytokines or cytokine receptors was induction of IL-13 receptor alpha chain (*Il-13ra1*) gene expression (Kim *et al.*, 2008). Real-time PCR analysis of whole lung samples indicated that *Il-13ra1* mRNA was upregulated in wild-type and *Il-13*^{-/-} mice but not in *Cd1d*^{-/-} mice. This finding indicated that NKT cells stimulated an increase in *Il-13ra1* mRNA.

The relatively small increase in *Il-13ra1* mRNA after viral infection in whole lung samples suggested that increased expression might be restricted to a subpopulation of lung cells such as macrophages. We therefore repeated the real-time PCR analysis of *Il-13ra1* mRNA using macrophages that were FACS-purified from whole lung samples. Relative to whole lung samples, we found that there was a much greater increase of *Il-13ra1* gene expression in lung macrophages isolated after viral infection, and this increase was also blocked in *Cd1d*^{-/-} mice. Similarly, immunohistology revealed that *Il-13ra1* was colocalized with the macrophage marker CD68 as well as with IL-13 in lung tissue sections obtained from mice at 7 weeks after inoculation. We therefore hypothesized that the interaction of IL-13 with IL-13 receptor (IL-13R) could amplify macrophage production of IL-13. Indeed, we found that blockade of IL-13 action by sIL-13R α 2-Fc caused a marked decrease in the levels of IL-13-producing macrophages, which, in turn, caused a decrease in *Il-13* mRNA levels in the lung. Together, these findings suggest a positive feedback loop in which IL-13 signaling causes increased production of IL-13 by macrophages. This feedback loop may be amplified by an increase in *Il-13ra1* mRNA expression that is driven by NKT cells. Together with the data from NKT-macrophage coculture, it appears that the IL-13 receptor on macrophages could respond to IL-13 from NKT cells or macrophages to further drive IL-13 production.

We also further characterized the nature of macrophage activation in the immune axis that we had identified. The microarray gene expression data was therefore reexamined to identify any additional chronic changes in gene expression after viral infection. Relative to mice inoculated with UV-inactivated virus, we observed a pattern of gene expression that is characteristic of an alternative pathway for activation of macrophages (Gordon, 2003; Pouladi *et al.*, 2004). The mRNAs encoding chitinase-like proteins (*Chi3l3/4* and *Fizz1*) arginase (*Arg1*), matrix metalloproteinase (*Mmp12*), and arachidonate 12-lipoxygenase

(*Alox12e*) were significantly upregulated at 7 weeks, and these same mRNAs were also upregulated but to a lesser degree at 3 weeks after viral inoculation (Kim *et al.*, 2008). Real-time PCR assays for these gene products confirmed the microarray data. Furthermore, these changes were completely blocked in *Il-13^{-/-}* mice, and they were partially inhibited in *Cd1d^{-/-}* mice after viral infection. Immunostaining for Chi3l3/4 protein indicated that the increase in the amount of this protein was localized predominantly to lung macrophages and occurred at the same time as the increases in *Chi3l3/4* mRNA levels. Moreover, FACS-purified lung macrophages exhibited upregulation of the same markers of alternative activation as whole lung samples at 7 weeks after inoculation in wild-type mice as well as downregulation of expression in NKT cell-deficient mice. The findings indicate that viruses can trigger an NKT cell–macrophage–IL-13 immune axis that, when properly amplified, can effectively drive the alternative pathway for activation of macrophages in a chronic response.

Previously, immune pathways promoting IL-13 production were viewed primarily as protection against parasitic infection (Gordon, 2003; Skold and Behar, 2003). Infection by extracellular parasites is known to stimulate a Th2 cell response with production of IL-4 and IL-13 that can in turn activate macrophages by an alternative pathway (Gordon, 2003). A similar type of response is found after allergen challenge in mice and in allergic asthma in humans (Webb *et al.*, 2001; Zhu *et al.*, 2004). By contrast, intracellular bacteria and viruses characteristically activate a Th1 response with production of IFN- γ and consequent activation of macrophages via the classical pathway. Development of this IFN-dependent pathway is proposed to downregulate the allergic response and protect against allergic airway disease (von Mutius, 2007). However, these general patterns were largely defined within the context of an acute immune response (Holtzman *et al.*, 1996). The present observations indicate that viruses can also trigger long-term activation of NKT cells and achieve chronic production of IL-13 by macrophages themselves. In combination with IL-13 receptor signaling, this innate mechanism can establish a state of persistent macrophage activation that is typical of the alternative pathway. Presumably, this mechanism evolved to mount a long-term innate immune response independently of CD4⁺ or CD8⁺ T cells and thereby enable a response to low-level endemic pathogens that are poorly presented by MHC but adequately presented by CD1d. However, in at least some genetic backgrounds, it appears that this type of innate immune activation can also lead to the development of chronic inflammatory airway disease in an experimental model. The next question was whether similar immune activation occurs in humans with chronic inflammatory airway disease.

4.6. NKT cell–macrophage pathway in humans

In that context, we extended our analysis of the NKT cell–macrophage immune axis to humans with chronic airway disease. Based on the established association of acute respiratory viral infection and the subsequent development of chronic asthma, we first investigated whether the NKT cell–macrophage–IL-13 pathway was activated in patients with asthma. We detected an increased number of macrophages that immunostained positive for IL-13 in bronchoalveolar lavage samples obtained from patients with severe asthma relative to the number in samples from healthy control subjects (Kim *et al.*, 2008). Ongoing work aims to address whether the development of IL-13-producing macrophages is specific for severe asthma relative to mild and moderate forms of the disease.

To obtain suitable samples of lung tissue for more detailed analysis, we analyzed lung tissue obtained from lung transplant recipients with severe COPD and lung donors that did not have COPD. The lung tissue obtained from COPD patients exhibited significant MCM as evidenced by an increased number of MUC5AC⁺ mucous cells and higher levels of *Muc5ac* mRNA in the lung (Kim *et al.*, 2008). Similar to our findings in the mouse model of chronic

lung disease, we also detected an increased level of *IL-13* mRNA in COPD lungs with chronic MCM. The increase in *IL-13* mRNA levels was associated with an increase in the number of cells that immunostain for IL-13 protein in COPD lungs. These IL-13⁺ cells were identified as lung macrophages based on typical morphology and positive immunostaining for CD68. Furthermore, these IL-13⁺CD68⁺ macrophages were found in increased numbers in lungs from COPD patients compared to non-COPD controls. The results from immunostaining indicate that only a subset of macrophages develop the capability for IL-13 production, so additional work is needed to better define the characteristics of this subset.

In that regard, it appears that markers of alternatively activated macrophages (AAMacs) may also be found in concert with increased production of IL-13 in humans with severe asthma or COPD. These markers include arachidonate 15-lipoxygenase, arginase 1, and chitinase and chitinase-like proteins (e.g., Chitinase 3L1 and Chitinase 1 (Chupp *et al.*, 2007; Shannon *et al.*, 1993; Siebold *et al.*, 2008; Zimmerman *et al.*, 2003) and (E. Agapov, J. Battaile, and M. J. Holtzman, unpublished observations). An initial report suggested that AMCase levels are also increased in asthma (Zhu *et al.*, 2004), but this finding was not confirmed in subsequent studies (Siebold *et al.*, 2008). These findings underscore the likelihood that genes from the mouse and human may be homologous for coding sequence without sharing regulatory elements. In addition, small samples of tissues or populations may lead to inaccurate assessment of biomarkers. This issue must be carefully addressed, since a specific immune axis (e.g., the NKT cell–macrophage axis) is likely activated in only a subset of patients with airway disease, and larger populations will need to be studied to obtain accurate molecular phenotypes for complex diseases.

Nonetheless, we have obtained additional evidence for activation of the NKT cell–macrophage system in chronic inflammatory airway disease in humans. Thus, in addition to evidence of macrophage activation, we have also begun to analyze the behavior of lung NKT cells under normal and disease conditions. In an initial analysis, we were able to detect NKT cells in lung tissue based on immunostaining for the invariant V α 24 T cell receptor chain. In concert with increased levels of mucous cells, IL-13 production, and IL-13⁺ macrophages, we also found that V α 24⁺ NKT cells were present in increased numbers in the lungs of patients with COPD compared to non-COPD controls (Kim *et al.*, 2008). Recent data indicates that increased levels of *Va24* mRNA are also detectable in lung samples from COPD patients (J. Battaile and M. J. Holtzman, unpublished observations). Together, the findings suggest that an innate NKT cell–macrophage–IL-13 immune axis may be activated in human disease conditions that are similar to the mouse model of virus-induced chronic airway disease.

Our findings for NKT cell–macrophage behavior in mice and humans are distinct from previous reports of immune abnormalities in chronic inflammatory disease in general, and lung disease in particular. For example, iNKT cells were necessary for AHR in a mouse model of allergen-induced asthma and were found in increased numbers in allergic asthma patients (Akbari *et al.*, 2003, 2006; Lisbonne *et al.*, 2003; Sen *et al.*, 2005). However, subsequent reports indicated that NKT cells were not necessary for airway inflammation in the mouse model and that the numbers of iNKT cells in BAL fluid or endobronchial biopsies were no different from normal in either asthma or COPD patients (Das *et al.*, 2006; Vijayanand *et al.*, 2007). Our findings thereby highlight the utility of a more complete assessment of the innate immune response in a mouse-to-man translational approach. When this type of analysis was done, we found that respiratory viral infection provided a more effective stimulus than acute allergen challenge for chronic activation of the immune system in an experimental mouse model. In addition, we achieved a more accurate assessment of a small subset of lymphocytes by using the relatively larger amounts of sample tissue that can be obtained in lung resection or transplantation in patients with airway disease. Together,

these resources provided for a synchronized analysis of the mouse model in combination with human patients, and this combination was essential to establish a previously unrecognized role for CD4⁺ NKT cells and IL-13-producing macrophages in the inflammatory process that drives chronic disease. The results thereby provide the first evidence that the innate immune system, which was formerly thought to act only acutely and transiently, can instead manifest a persistent response that leads to chronic inflammatory disease.

5. SUMMARY

In summary, there is a critical clinical need for better understanding and treatment of chronic inflammatory lung diseases such as asthma and COPD. To address this issue, we analyzed new mouse and cell-based models of chronic inflammatory lung disease and extended our findings to studies of human subjects to better understand the pathogenesis of severe asthma and COPD. In the experimental mouse model, we showed that chronic airway disease could develop after infection with a common type of respiratory virus is cleared to trace levels. The postviral lung disease is manifested by persistent MCM and AHR that is also characteristic of chronic airway disease in humans. When the acute disease appears, it depends on an immune axis that is initiated by IFN-dependent expression of the high-affinity IgE receptor (FcεRI) on cDCs. Crosslinking of this receptor causes cDCs to produce the chemokine CCL28 that subsequently recruits IL-13-producing CD4⁺ T cells to the airway. This pathway thereby links the antiviral response that is often Th1 in character to an allergic-type response that is generally Th2 in character. When the chronic lung disease develops, it is unexpectedly independent of an adaptive immune response. Instead, the chronic inflammation is driven by an innate immune axis that relies on T cell receptor iNKT cells that are programmed to activate macrophages to produce IL-13. Studies of mice as well as isolated cells in coculture show that the interaction between iNKT cells and macrophages depends on contact between the invariant Vα14Jα18-TCR on lung iNKT cells and the nonpolymorphic MHC Class I-like protein CD1d on macrophages as well as NKT cell production of IL-13 that binds to the IL-13R on the macrophage. The activated lung macrophages produce IL-13 and overexpress the IL-13R, and this combination of events establishes a positive feedback loop that promotes the persistent expression of IL-13 as well as other IL-13-induced gene products (e.g., chitinase-like proteins and arachidonate 12/15-lipoxygenase) that are characteristic of an alternative pathway for macrophage activation. This innate immune axis is also activated in the lungs of humans with chronic airway disease due to severe asthma or COPD based on detection of increased numbers of iNKT cells and AAMacs in the lung. These findings provide new insight into the pathogenesis of chronic inflammatory disease with the discovery that the transition from respiratory viral infection into chronic lung disease requires persistent activation of a novel NKT cell–macrophage innate immune axis.

Together, our studies identify an adaptive immune response that mediates acute disease and an innate immune response that drives chronic inflammatory lung disease in experimental and clinical settings (as summarized in Fig. 5.4). However, major questions still need to be addressed to better define the basis for chronic inflammatory airway disease. For example, the presence of a persistent innate immune response suggests that there must be ongoing immune stimulation. In that regard, we did not detect any evidence of infectious virus by 2 weeks after inoculation. However, using highly sensitive PCR probes and virus-clean isolation rooms, we were able to detect very low levels of virus-specific RNA in lung tissue until at least 1 year after viral inoculation (E. Agapov and M. J. Holtzman, unpublished observations). Whether viral persistence is necessary for a chronic immune response is still being defined. For example, cDCs are sites of virus uptake, are activated acutely and perhaps chronically after SeV infection, and are capable of activating NKT cells at low

levels of antigen (Cheng *et al.*, 2007; Grayson *et al.*, 2007a,b). Our results (like that of others) indicate that iNKT cells may also react to CD1d-expressing macrophages by a mechanism that does not require but can be enhanced by high-affinity agonists such as α GalCer (Hegde *et al.*, 2007). Additional work must therefore be directed at the cause of chronic NKT cell–macrophage activation after viral infection, and the possibility that viral remnants contained in cDCs, macrophages, or other cell types might drive this process.

In addition to questions over the role of the viral trigger for the development of chronic disease, there are additional uncertainties over the full nature of the immune response that leads to IL-13-dependent disease after viral infection. For example, we unexpectedly found that conventional T cell-deficient mice continued to develop the same level of IL-13-producing macrophages and IL-13-associated disease after viral infection. We interpreted these findings with T cell deficiency to suggest that other types of immune cells can also influence the chronic disease traits that develop after viral infection. This interpretation prompted further study of the innate immune response and the discovery of the NKT cell–macrophage immune axis. However, we also recognize that there may be additional immune cell populations that contribute to IL-13 production after viral infection. For example, a distinct non-B non-T (NBNT) cell lineage with no expression of T cell, B cell, or NK cell markers is also a potent source of IL-13 (Fallon *et al.*, 2006), and preliminary observations suggest that a similar cell population may also be activated after viral infection (Byers *et al.*, 2009). It is also possible that T cell depletion additionally removes an immune cell population that downregulates production of IL-13. For example, a population of CD4⁺ regulatory T cells (Tregs) is induced by IL-13 and is capable of inhibiting effector T cell responses (Skapenko *et al.*, 2005). We can detect a persistent increase in the number of Tregs in the lung after viral infection (Y. You and M. J. Holtzman, unpublished observations), but the functional role of this cell population still needs to be determined. Moreover, we have provided initial evidence that conventional CD4⁺ T cells contribute to IL-13 production after viral infection, but even the full role of this cell population in the development of postviral lung disease is still uncertain. We must eventually define how these additional immune pathways respond to viral infection and furthermore, how they interact with the new NKT cell–macrophage pathway to provide a comprehensive scheme for chronic inflammatory disease after infection. Future work must also define more precisely how to correct these abnormalities in susceptible individuals.

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FIGURE 5.1.

Proposed scheme for how viruses might trigger chronic inflammatory disease. In a susceptible genetic background, two broad issues must be defined: (A) an altered immune program with the development of a chronic immune response involving APCs, memory cells, and effector cells; and (B) end-organ dysfunction with a transition from epithelial precursor cells (such as Clara cells and ciliated cells) to mucous cells. The present review is focused on alterations in the immune program.

FIGURE 5.2.

Cellular and molecular scheme for an adaptive immune axis leading to acute lung disease after viral infection. Viral infection activates two major immune pathways. (A) In one of these pathways, viruses cause AEC and pDC production of type-I IFN. Subsequent IFNAR signaling leads to upregulation of Fc ϵ RI α expression on resident lung cDCs. In turn, Fc ϵ RI activation by viral antigen and antiviral IgE leads to production of CCL28 and recruitment of CCR10-expressing IL-13-producing Th2 cells to the lung. Persistent IL-13 production drives differentiation of AEC precursors towards mucous cells (MCM) and airway smooth muscle cells to become more reactive to contractile agonists (AHR). (B) In another pathway, viral infection also leads to maturation of lung cDCs that use CCL5-CCR5 and CCL19/21-CCR7 interactions to migrate to regional lymph nodes. In the nodes, these cDCs regulate MHC Class I-dependent generation of CD8⁺ cytotoxic T cells as well as MHC Class II-dependent production of CD4⁺ Th cells and consequent B cell production of virus-specific IgE. This IgE is thereby available to participate in Fc ϵ RI signaling in the pathway described in (A). Modified from Grayson *et al.* (2007a).

FIGURE 5.3.

Cellular and molecular scheme for an innate immune axis leading to chronic lung disease after viral infection. Virus may directly or indirectly activate an APC and thereby facilitate CD1d-dependent antigen presentation and consequent activation of invariant CD4⁺ 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 Vα14 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 *Chit3L1*, *Chit1*, *Arg1*, and *Alox15* in humans. Modified from Kim *et al.* (2008).

FIGURE 5.4.

Time course for immune events in the development of acute and chronic lung disease after respiratory viral infection. Viral replication is maximal by postinoculation Days 3–5 in concert with immune-response gene expression and subsequent immune cell infiltration into the site of infection. The development of acute disease is characterized by acute AHR and MCM and is manifested at 3 weeks after viral inoculation; this disease is driven by a combined innate and adaptive immune response that includes type-I IFN production, high-affinity IgE receptor expression and CCL28 production by cDCs, and recruitment of IL-13-producing Th2 cells. The development of chronic disease is fully manifested at 7 weeks after viral inoculation and is regulated by APC-dependent activation of iNKT cells that drive the generation of alternatively activated macrophages (AAMacs). Modified from Holtzman *et al.* (2002).