Toll-like Receptors, Notch Ligands, and Cytokines Drive the Chronicity of Lung Inflammation

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Current dogma supports the concept that the expression of a disease-inducing signature cytokine phenotype is important to the maintenance stage of chronic lung disorders. This cytokine phenotype has been characterized as a polarization toward type 2 cytokines, which are profibrotic and immunoregulatory. The biology of this latter activity could mechanistically explain pathogen-induced exacerbation of chronic lung inflammation, as a skewed cytokine profile in the lung alters dendritic cell function, activates fibroblasts, and facilitates a subsequent "second hit" by an infectious pathogen. In this setting, cytokine biology is also linked to Toll-like receptors (TLRs) in the maintenance of lung immunity, as the activity of this receptor–ligand system by both leukocytes and stromal cells is likely an important component of disease chronicity. The participation of dendritic cells via TLRs in chronic lung disease could facilitate communication circuits established between antigen-presenting cells and lymphocytes. Data suggest that TLR activation via myeloid differentiation factor 88 adaptor protein leads to the induction of a Notch ligand known as Delta-like-4 on dendritic cells that activate the Notch receptor on T cells, promoting a helper T-cell type 1 cytokine response. It is likely that the evolution of host defense signals designed to recognize patterns emitted from a hostile microbial environment may now be superimposed on adaptive immunity and provide the underpinning to support the maintenance of chronic lung disease.

Keywords: virus; dendritic cell; innate immunity

Chronic lung disease is observed in a wide variety of disorders, including infectious diseases, autoimmune disorders of connective tissue, obstructive lung disease, and disorders for which the etiology is unknown (1, 2). The clinical manifestations of these lung disorders are the likely consequences of an initial host response to an etiologic agent(s) followed by tissue injury and progressive lung pathology. Unfortunately, the etiology and mechanisms of the chronicity and exacerbated pathology are not well understood. Thus, the clinical management of many of these chronic pulmonary diseases is difficult and unrewarding, as cytotoxic and immunosuppressive therapies are often used without efficacy (3, 4). This treatment strategy reflects, in part, the limited understanding of the mechanisms that maintain the interstitial lung response.

Although the etiology of many interstitial lung diseases is not known, it is likely that the host response to a persistent agent is key to the chronicity of these diseases. Investigations

have shown that the continuous host response to a persistent challenge often polarizes the cytokine environment toward a type 2 cytokine phenotype (5, 6). This cytokine skewing may be one of the mechanisms associated with the evolution of disease chronicity, as a cytokine bias has been associated with the pathology of certain chronic lung diseases (7–9). Studies have shown that cytokines such as IL-4, IL-13, and transforming growth factor (TGF)- β may dictate pathology associated with chronic diseases by altering immune cell function and dictating fibroblast activation (10, 11). These latter biologic activities are outcomes of the paradigm that labels type 1 cytokines as augmenting the host response to pathogens and suppressing tissue-remodeling activities, whereas type 2 cytokines can dampen the host response and increase stromal cell growth and remodeling activities.

Although it has been appreciated that specific cytokines can indeed facilitate fibroblast activation and matrix deposition, it is now known that type 2 cytokines possess immunoregulatory activities and their continued local expression *in vivo* may place the host at risk for increased lung infections, especially those due to respiratory viruses. The activity of these cytokines may explain why certain chronic lung diseases with a type 2 phenotype are either predisposed to exacerbated viral infections or are associated with the presence of viral products (12–14). In the context of infectious and immune-mediated chronic lung diseases, studies have identified that both type 2 cytokines and viral products are associated with evolving lung pathology (13, 14). Thus, the progression and clinical manifestation of chronic alterations found in these enigmatic diseases may be dictated by the cytokine phenotype and the host's response to a subsequent viral pathogen superimposed on the initial etiologic agent, constituting a "two-hit" mechanism that results in augmented pathology (Figure 1).

ANIMAL MODELS OF CHRONIC LUNG INFLAMMATION POSSESS SPECIFIC CYTOKINE PHENOTYPES

Data derived from a variety of models demonstrate that a number of inflammatory systems are involved in the induction of cytokines, which subsequently play a role in the initiation and maintenance of chronic experimental pulmonary inflammation. However, the mechanistic contribution of various cytokines during the evolution, and more importantly, the maintenance of disease chronicity has been addressed only more recently. For example, *in vivo* studies assessing the development of chronic lung granulomas induced by mycobacterial antigen have demonstrated that IFN- γ , IL-12, and tumor necrosis factor were necessary cytokines for lung lesion progression (15). In contrast, experimental pulmonary inflammation initiated by complex antigens, such as those derived from various allergens and parasites, was showed to be maintained by IL-4 and IL-13 (16, 17). In the context of chronic lung inflammation, these observations served as the basis for the establishment of models that exhibit either a type 1 or type 2 inflammatory response within the lung. The establishment of models with signature cytokine phenotypes has

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Figure 1. Host response during chronic pulmonary disease. Evolution of chronic lung inflammation driven by type 2 cytokines, which induce a susceptible state for lung tissue to become infected with a respiratory virus. DC = dendritic cells.

been accomplished by the presensitization of mice with either *Mycobacterium* species (BCG) or *Schistosoma mansoni* antigen followed by a pulmonary challenge with sized Sephadex beads coated with a known amount of purified protein derivative (PPD) or schistosome egg antigen. In addition, challenging naive mouse lungs with *Schistosoma mansoni* eggs results in a progressive immune response that initially is a type 1 cytokine response, containing mostly small and large mononuclear cells. This immune response then transitions into a type 2 cytokine lesion containing mononuclear cells, eosinophils, and a significant number of fibroblasts with accompanying collagen deposition (Figure 2). Although these models have been used to investigate leukocyte recruitment in the lung, they have not been fully used to address either the mechanism of tissue remodeling in the setting of chronic pulmonary inflammation or alterations in the host response to a subsequent pathogen challenge. The use of defined lung models that are controlled by type 1 and type 2 cytokines will prove important in understanding the cellular and molecular regulation of the host response, which is due to a skewed cytokine phenotype, resulting in matrix-induced pathology and an exacerbated response to a subsequent pathogen challenge to the lung. Furthermore, these antigen-driven immune-mediated models do not result in immune tolerance with a reduced inflammatory signature, as is observed in certain experimental systems where

repeated antigen challenge can tolerize subsequent lung inflammation.

CYTOKINE PHENOTYPE OF HUMAN CHRONIC LUNG DISEASE

A number of chronic diseases can be defined in part by the expression pattern of cytokines, which can be mechanistically linked to the disease pathology. In specific disease states the key cytokine(s) become an identifiable therapeutic target for the development of an efficacious pharmacologic strategy. The best example of targeting a single cytokine for effective treatment of a chronic disease is the treatment of rheumatoid arthritis and Crohn's disease with antibodies to tumor necrosis factor (18). In keeping with this paradigm, the pathology of certain interstitial lung disorders has been reported to exhibit a cytokine phenotype with characteristics of a polarized type 2 cytokine profile (7–10). Clinical investigations have identified that elevated levels of either transcripts or protein for IL-4, IL-10, IL-13, and TGF-β are associated with interstitial pulmonary fibrosis. Although these data do not necessarily establish a causal relationship between an individual cytokine and lung pathology, they are in keeping with known paradigms of cytokine biology. Chronic expression of type 2 cytokines facilitates the chronic

Figure 2. Hematoxylin-and-eosin-stained sections showing the progression of chronic lung response and kinetic assessment of cytokine, collagen, and cellular components 4, 8, and 16 days after lung challenge with *Schistosoma mansoni* egg antigen. Mφ = macrophages.

disease process, whereas elevated levels of IFN- γ have been hypothesized to antagonize the remodeling aspect of specific chronic lung disease and to cross-regulate the production of type 2 cytokines. The differential activities of these polarized cytokines have been put into play in clinical trials where IFN- γ 1b has been used to treat patients with interstitial pulmonary fibrosis (19, 20). However, the end results of this clinical trial were disappointing, as little efficacy was noted (20). Thus, there remains significant room for developing new therapeutics and therapeutic strategies to treat chronic lung disease and the accompanying exacerbations.

CYTOKINE POLARIZATION AND THE EXACERBATION OF PULMONARY VIRAL INFECTIONS

The increased expression of type 2 cytokines clearly alters the biology of diverse systems. Regarding the immune response, an increase in type 2 cytokines provides the mechanism for the expression of additional subclasses of antibody (IgE), the participation of an additional subpopulation of leukocytes (eosinophils), and the involvement of alternately activated (reparative) macrophages (21, 22). This macrophage phenotype is thought to be key to reparative processes via activating fibroblasts and providing an environment that facilitates matrix deposition. In addition, the skewing of the cytokine phenotype away from an IFN- γ -induced intracellular pathogen-clearing response toward a cytokine-directing "reparative response" may leave the host at risk for an exacerbated viral infection. Previous reports have demonstrated that decreased IFN- γ production is associated with more severe rhinovirus infection and a delay in the elimination of the virus (23). This reduced type 1 response to rhinovirus has been reported to increase both asthma severity and viral shedding during rhinovirus infection. This suggests an exacerbated response to respiratory illnesses among patients with a cytokine polarization away from a type 1 response (23). In the context of chronic interstitial disease, data from a number of studies provide evidence that products from DNA viruses (cytomegalovirus, Epstein-Barr virus, and herpesvirus) are associated with lung tissue recovered from a significant number of patients with chronic pulmonary disease (13, 14). Although there is yet only correlative information regarding a type 2 cytokine environment and recoverable viral components from these clinical specimens, it is of interest to speculate that there may be a cause-and-effect relationship between these two findings.

EXPERIMENTAL MODELS WITH AN ALTERED CYTOKINE PHENOTYPE EXHIBIT INCREASED PATHOLOGY DURING VIRAL INFECTION

Mouse herpesvirus strain 68 (MuHV-68) is a murine gamma-herpesvirus tropic for lung tissue. This virus is closely related to human Epstein-Barr virus and Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8), two DNA viruses that have been associated with lung tissue recovered from patients with interstitial pulmonary fibrosis (13, 14). Wild-type mice infected via the respiratory route with MuHV-68 exhibited a lytic infection of alveolar epithelial cells with a subsequent latent infection of B cells and an elevation of CD8⁺ T cells (24). Immunocompetent wild-type mice challenged with MuHV-68 did not exhibit pronounced chronic changes in the lung, which is quite different from mice with a type 2 cytokine bias (25, 26). Intranasal challenge with MuHV-68 in IFN- γ receptor knockout mice induced a type 2 cytokine bias and a striking pathologic picture, characterized by an altered cytokine profile and significant fibrosis of the lungs. Interestingly, histopathology of the lungs of IFN- γ receptor^{-/-} mice challenged with MuHV-68 was found to be

similar to the pattern described for the lungs of patients with interstitial pulmonary fibrosis (26). These changes include myofibroblastic foci, vascular alterations, hyperplasia of type II alveolar epithelial cells, altered expression of surfactant proteins, significant fibrosis, and an imbalance of the cytokine profile with polarization toward a type 2 phenotype. In the C57BL/6 background, IFN- γ receptor^{-/-} mice challenged with MuHV-68 maintained a progressive interstitial fibrosis that did not resolve, which could be the result of reactivation of latent virus causing renewed infection and a continuation of the chronicity of the fibrotic response (26).

VIRUS-DERIVED GENE PRODUCTS TARGET IMMUNE MEDIATORS AND CONTRIBUTE TO LUNG PATHOLOGY

Like other family members of large DNA viruses, MuHV-68 encodes several proteins that have been either demonstrated or postulated to subvert host immune responses. These include K3 protein, a modulator of major histocompatibility (MHC) class I expression on the surface of cells (27); M11, a Bcl-2 homolog (28); ORF4, a complement-regulatory protein (29); M3, a highly secreted chemokine-binding protein (30, 31); and ORF74, a G protein-coupled receptor (vGPCR) with sequence homology to chemokine receptor CXCR2 (32). Previous studies have shown that both the chemokine-binding protein and vGPCR play a prominent role in the chronic aspect of the infectious process (30–32). The chemokine-binding protein appears to control T cell trafficking and to block chemokine-dependent CD8⁺ T cell recruitment during the expansion of latently infected cells. In addition, vGPCR from MuHV-68 serves as a proliferative signal for cells and, in concert with vBcl-2, can enhance the growth and survival of MuHV-68-infected cells. These virus-derived factors could aid in explaining the interesting pathology associated with chronic interstitial lung disease of humans, as it is known that the histologic picture of interstitial pulmonary fibrosis is consistent with islands of myofibroblastic foci with reduced immune cell recruitment (33). The mechanism for this pathologic picture could be the result of the virus targeting chemokines, by regulating their levels at the local tissue level and reducing leukocyte recruitment (M3 activity) and by the activity of vGPCR to increase nonimmune cell proliferation.

VIRAL INFECTIONS DISRUPT DENDRITIC CELL FUNCTIONS AND MAY CONTRIBUTE TO THE CHRONICITY OF LUNG DISEASE

Dendritic cells play an important role in the host immune response by participating in immune surveillance and presenting antigen via either the MHC class I or MHC class II pathway (34, 35). These latter events are coincident with dendritic cell maturation, which increases the expression of the costimulatory molecules CD80 and CD86, MHC classes I and II, and specific chemokine receptors (36–38). These activities are of particular importance for the host's antiviral strategies, as any alterations in their normal function or blocking of dendritic cell maturation could contribute to immunosuppression, thereby allowing a persistent viral infection (39). Interestingly, the tissue environment supported by either type 1 or type 2 cytokines also greatly influences dendritic cell activity (40, 41).

Our data suggest that MuHV-68 preferentially infects immature dendritic cells (Figure 3). We believe this process is key to the developing chronic lung response and subsequent pathology, such as tissue-remodeling problems. These pathologic processes are likely due to the inability of immature dendritic cells to function properly and, as has been reported for human

Figure 3. Murine herpesvirus strain 68 preferentially infects immature dendritic cells (A) and not mature dendritic cells (B). Data demonstrate that immature bone marrow–derived dendritic cells contain significant viral titers, whereas LPS-treated dendritic cells driven to maturity do not have a significant increase in viral titers relative to mock control.

herpesvirus, block dendritic cell maturation (39, 42). Thus, the host response can neither clear antigen effectively nor express the appropriate levels of IL-12, the latter contributing to the skewing of the cytokine environment in the lung (43). Furthermore, a reduction in IL-12 levels in concert with a persisting stimulus has been reported to be a contributing factor to the chronicity of experimental lung inflammation (44). In the setting of a type 2 cytokine–polarized environment, the blocking of dendritic cell function and/or maturation by MuHV-68 could serve as a mechanism for the continued chronicity of lung pathology and the inability of the host to mount an appropriate immune response.

MECHANISMS THAT SUPPORT THE CHRONICITY OF LUNG DISEASE

Alternatively Activated Macrophages and Their Contribution to Disease Chronicity

The scientific community's appreciation of the classical immune activation pathway for macrophages is based on studies performed more than 50 years ago, in which bacterial infections in mice resulted in an augmentation of the antimicrobial activities of macrophages in an antigen-independent manner. These investigations stimulated a flurry of research, which ultimately identified IFN- γ as a cytokine, which provided the stimulus for "classically activated macrophages" (M1). Although many investigations have validated this pathway in multiple immune responses, there are clearly other macrophage populations that exhibit a different phenotype. For example, studies have shown that cancer, asthma, atherosclerosis, and certain infectious processes contain a novel population of "alternatively activated macrophages," or M2 macrophages (45–52). These macrophages, activated in part by IL-4 and/or IL-13, likely provide the underpinnings for the maintenance of chronic disease via their reported role in regulating specific immune events, promoting fibroblast growth, and angiogenic activities. The contribution of alternatively activated macrophages to pulmonary disease chronicity is not well understood; however, they do represent one end of a continuum of functional macrophage activation states that are directed by signals found in the tissue environment. This alternative activation pathway results in the induction of a number of events, including the expression of macrophage mannose receptor and MHC class II expression, an increase in phagocytosis, the expression of various CC chemokines (CCL2, CCL6, CCL17, and CCL22), amplified production of the IL-1 receptor antagonist and the IL-1 decoy receptor, overexpression of TGF- β , and the augmented expression of the novel gene product Ym-1/2. Thus the alternative activated macrophages are clearly not quiescent cells whose activities have been suppressed, but are fully functional immune cells that have been programmed by a cytokine phenotype (IL-4/IL-13) to participate differently in an inflammatory event.

TLRs and the Adaptor Molecule Myeloid Differentiation Factor 88 Regulate Cytokine-dependent Responses and Alter Chronic Lung Inflammation

The immune response is a highly regulated event involving a number of sophisticated and cross-regulated pathways shared by cellular players of the innate and acquired immune system (52–62). Whereas the TLRs are known to be underpinnings of the innate immune response, they are also intimately involved in events that direct the adaptive immune response (49–51). Therefore, the TLR/adaptor molecule system is not only involved in early immune surveillance, but is part of the immune continuum that progresses in lockstep with the development and maintenance of acquired immunity. Interestingly, TLRs have been identified as contributors to the diversity of the adaptive immune response by influencing the type 1/type 2 biasing found in specific immune responses. For example, a pivotal role for TLR molecules in aiding the evolution of a type 1–polarized acquire immune response has been identified via the induction of IL-12 and IL-23, and the expression of both MHC class II:peptide and costimulatory molecules (CD80 and CD86). Just as intriguing are investigations demonstrating that type 2 immune deviation is dependent, in part, on the activation of specific TLRs found on antigen-presenting cells. Subsequent investigations have demonstrated that activation of TLR2 induces a helper T cell type 2 (Th2) immune response and can promote type 2 cytokine–dependent responses. Furthermore, studies have identified the key role that myeloid differentiation factor 88 (MyD88) plays in acquired immune events. MyD88 is a critical adaptor molecule shared by the majority of TLRs and is important for TLR intracellular signaling activities. The interest in this molecule has been accelerated by findings showing that in the absence of MyD88 (MyD88^{−/−} mice) Th1 responses are blunted, whereas Th2-dependent reactions are augmented. Our data demonstrate that the cellularity and cytokine phenotype of pulmonary granulomatous inflammation are significantly altered in MyD88^{−/−} mice (Figure 4), as well as TLR9^{−/−} mice (Figure 5). Whereas a significant amount of data is currently being accumulating on the role of TLRs in the deviation of the acquired immune response, there is little knowledge regarding how TLRs participate in the pathology associated with chronic lung disease.

Notch Ligands Regulate Cytokine-dependent Responses and Participate in Chronic Lung Inflammation

It is clear that the activation of TLRs on antigen-presenting cells can lead to the expression of IL-12 and subsequently activate the expression of IFN- γ from Th1 cells. Interestingly, the lack of IL-12 production via limited TLR activation during the inflammatory/immune response appears to lead to Th2 responses, suggesting that this type of response is a default pathway that occurs in the absence of IL-12. Investigations have demonstrated that MyD88 knockout mice were not able to generate

Figure 4. Hematoxylin-and-eosin (H&E)-stained sections of lung lesions from animals with developing type 2 immune granulomas: (A and B) wild-type (WT) mice and (C and D) myeloid differentiation factor 88 (MyD88)^{-/-} mice. A significant increase in size and cellularity is associated with the MyD88^{-/-} mice. Original magnification: (A and C) $\times 10$; (B and D) $\times 40$.

a Th1 response when challenged with an infectious agent (57–59, 63–66). Also, MyD88-mediated cytokines appear to provide a suppressive signal for a Th2-type response. The caveat in these studies is that not all Th1-inducing stimuli default to Th2 responses in the absence of IL-12, suggesting that other signals may exist on antigen-presenting cells to elicit a Th1 response.

Just like the early biology concerning the TLRs had its origin in developmental biology and then transitioned into inflammation and immunity, the field of Notch and Notch ligands is another system that had its early discovery in developmental biology but now appears to be a key bridge between antigen-presenting cell and T-cell communication circuits. Studies suggest that the Notch ligands Delta-like-1–4 and Jagged, expressed on dendritic cells, can provide novel activation signals for the development of either Th1 and Th2 cells, respectively (67). Interestingly, the expression of Delta-like-4 (DL4) on dendritic cells is induced via TLRs that use the MyD88 pathway. Activation of TLR via MyD88 results in significant DL4 expression. Using antibodies directed against DL4, we set up an experiment to determine the role of this membrane-bound Notch ligand in polarizing cytokine production. Lymphocytes recovered from the lung lymph nodes of animals treated with high-titer antibodies to DL4 with developing type 2 lung granuloma demon-

strated an altered response when challenged *in vivo* with antigen (Figure 6). This study showed that production of both IL-5 and IL-13 from antigen-challenged lymphocytes recovered from animals treated with antibody to DL4 was enhanced over control antibody-treated animals. These investigations underscore the importance of a novel immune-activating system during the development of chronic lung inflammation. The data in these studies identify a critical role for DL4 in the regulation and/or development of Th1- and Th2-mediated responses.

CONCLUSIONS

It is becoming increasingly clear that novel investigative approaches are needed to more fully understand the mechanisms that influence the pathologic changes associated with chronic lung inflammation. The significance of the above-described studies can be traced to the clinic, where efficacious therapeutic options for the treatment of many chronic lung diseases are not readily available. Thus, the generation of mechanistic data from experiments designed to study the partnerships that align specific mediator cascades, resulting in chronic lung pathology, will provide fertile grounds for the exploration of the next generation of therapeutics.

Figure 5. Hematoxylin-and-eosin (H&E)-stained sections of lung lesions from animals with developing type 2 immune responses: (A and B) wild-type (WT) mice and (C and D) Toll-like receptor 9 (TLR9)^{-/-} mice. Increased cellularity and the presence of stromal cell proliferative foci are demonstrated in the TLR9^{-/-} mice. Original magnification: (A and C) $\times 10$; (B and D) $\times 40$.

Figure 6. Increase in lymphocyte-derived IL-5 and IL-13 assessed from T cells recovered from lung lymph nodes of animals with developing type 2 granulomas treated *in vivo* with antibodies to the Notch ligand Delta-like 4 and stimulated *in vitro* with schistosome egg antigen (SEA).

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