VIRAL IMMUNOLOGY
Volume 21, Number 2, 2008

Pp. 115–122
DOI: 10.1089/vim.2007.0110

Regulation of Immunity to Respiratory Syncytial Virus by Dendritic Cells, Toll-Like Receptors, and Notch

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ABSTRACT

The activation and maintenance of pulmonary viral disease is regulated at multiple levels and determined by the early innate response to the pathogenic stimuli. Subsequent activation events that rely directly and indirectly on the virus itself can alter the development and severity of the ensuing immunopathologic responses. In the present review we outline several interconnected mechanisms that rely on the early recognition of viral nucleic acid for the most appropriate anti-viral immune responses, including TLRs and Notch activation in DCs and T cells. Deviation or persistence of the immune response to respiratory viruses may impact significantly on the severity of the responses. While these mechanisms are likely similar in most respiratory viral infections, this review will focus on findings with respiratory syncytial virus (RSV) infections.

INTRODUCTION

THE INDUCTION OF IMMUNE RESPONSES IN THE HOST often determines the nature and the severity of the ensuing illness during infectious diseases. Studies have shown that patients with severe respiratory viral infections have an increased risk for the development of chronic pulmonary diseases (1–5). A number of respiratory viruses have been implicated in the induction of pulmonary diseases, including infections with rhinovirus, influenza, parainfluenza, and adenovirus. Recent studies in children (6) and adults (7) support this contention as it relates to severe asthma exacerbations. Thus, viral infections not only can directly alter the health of an individual, but may also alter the immune environment within infected tissues and thus allow progression of other chronic responses. Several investigators have focused upon respiratory disease using a model of respiratory syncytial virus (RSV) infection, as this virus has epidemiologic links to the development of chronic airway disease. Recent evidence suggests that RSV has a significant role in elderly patient populations as well as in patients with chronic obstructive pulmonary disease. Whereas RSV is rarely fatal in infants, the impact of RSV on the elderly has only recently begun to be recognized and may be associated with nearly as many deaths as influenza (8). The specific mechanism(s) of immune regulation identified in RSV studies may be relevant to other viral infections that must be recognized and cleared properly or a more pathogenic disease progression could result. This review will address the regulation of cellular immune responses during viral infection that result in the activation of appropriate antiviral responses and focus on immune responses in RSV infection models.

DENDRITIC CELLS AND RECOGNITION OF INFECTIOUS AGENTS

In order to monitor pathogenic insults at mucosal surfaces a complex network of innate immune cells are positioned within the tissue with the ability to recognize

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microorganisms. The surveillance function can be accomplished by resident macrophages that have the ability to quickly phagocytize bacteria and fungi for clearance prior to colonization, thereby avoiding inappropriate activation of the acquired immune system. The epithelial cell barriers that have been set up also provide a reasonable means of protecting the host from colonization, and upon infection can initiate innate immune signals for the recruitment of additional phagocytic cell populations. However, in the case of viral infections, it is difficult, if not entirely impossible, for the innate immune response to clear the virus without activation of the acquired immune system. Thus, the resident and recruited dendritic cell (DC) becomes a critical link for recognition and transport of the viral stimuli to the draining lymph node for activation of the antiviral, cell-mediated responses. The determining factor for the outcome of the response depends upon how the DCs are activated and in what manner the cells interact with the T cells. In addition, the nature of the resulting acquired immune response likely also depends upon the subsets of DC that are recruited, activated, and participate in the initiation of the viral responses.

The ability to monitor the pulmonary environment for pathogen infection is regulated at several levels. During viral infections the innate immune response has adapted mechanisms of pattern recognition to allow immediate and effective identification of the invading microorganisms. These include the toll-like receptor (TLR) activation pathways, both MyD88-dependent and independent, as well as cytoplasmic triggers that include RIG-I and PKR pathways (9–11). TLR4 was the first to be identified to have an effect in RSV infection via its interaction with the F protein of RSV (12). While controversial (13–15), a number of clinical studies have identified a TLR4 polymorphism associated with susceptibility to severe RSV infection (16–21). A recent study demonstrated that the altered TLR4 protein can confer altered responses in epithelial cells and peripheral blood mononuclear cells (22). In our own studies comparing the response to RSV infection in bone marrow–derived DCs from wild-type versus TLR4-deficient DCs (C3H/HeJ), an early and significant reduction in chemokine production with no alteration in IL-12 was observed (data not shown). Thus, the role of TLR4 may depend upon the cell type and location of the infection. Other TLR molecules that recognize viral components are found in the endosomal compartment, where viruses uncoat and initially release their nucleic acid prior to replication. Once the nucleic acid begins to replicate in the cytoplasm, the relatively high levels appear to activate cytoplasmic triggers such as RIG-I, which drive strong type I IFN responses (23–25). The expression of TLRs in DC subsets may be defining for their function of monitoring the environment. While myeloid/conventional lineage DCs (cDCs) have a relatively wide range of TLRs, including TLR2, TLR3, TLR4, TLR5, and TLR6, the plasmacytoid DCs (pDCs) primarily express TLR7 and TLR9. Because TLR7 and TLR9 are primary recognition molecules for ssRNA and unmethylated CpG sequences, the pDC likely plays a prominent role in the initial phases of viral infection for monitoring and eliciting the early responses. The notion that pDCs may play an important role in regulation of the immune response is logical given that the pDC is the predominant producer of IFN-α, a major innate cytokine involved in antiviral immunity (26–28). This has been supported in multiple studies that have demonstrated that the removal of pDCs during viral infections leads to decreased viral clearance and increased immunopathology (29–31). Critical experiments on pDC function initially came after the development of antibodies that could specifically deplete pDCs. Subsequently, the initial experiments for pulmonary responses were performed by depleting pDCs during allergic responses, and demonstrated that in the absence of pDCs the allergic response was greatly enhanced (32). The pDC subset was suggested to have a suppressive response on T-cell activation. Recent studies, however, have indicated that pDCs play multiple roles that include antiviral effects by directly limiting viral replication through the production of type I IFN, as well as providing important immune regulatory functions by directing the phenotype of both the CD4 helper T-cell responses and enhancing the CD8 cytotoxic T-cell responses. Thus, the activation of the once-underestimated pDC has been demonstrated to have a critical role in determining the direction of the immune response, as well as the severity of virus-induced immune responses. This latter concept was prominently identified in a seminal study using herpes simplex virus infection that demonstrated the necessity of pDC migration into the lymph node for development of the most effective CTL responses by affecting the nature of cDC activation (31). This latter concept was recently supported in experimental RSV infection by depleting pDCs that led to development of severe pathophysiology and altered immune responses (29). Additional unpublished studies by our laboratory using TLR7$^{-/-}$ mice have indicated that TLR7 and pDCs are the primary source of IFN-α. The lack of signaling through this pathway alters the immune response to RSV infection (data not shown) in a similar manner as that described with MyD88$^{-/-}$ animals (33), leading to increased pathogenesis. Together, these studies begin to describe a system in which the pDC subset participates in the overall antiviral immune response in a coordinated effort with the cDC subset.

Myeloid DCs also play a central role in antiviral immunity and have been subdivided in several ways. Most recently the function of this subset may be best defined

based on the co-stimulatory molecule display and the cytokine production profile. Subsets of cDCs that express high levels of CD40 and CD80, and produce high levels of IL-12 are more efficient for promoting a Th-1 type response, whereas those that express OX40L and lower levels of IL-12 promote a predominantly Th-2 response (24,34–36). The activation of cDCs through specific TLR molecules induces important instructive signals expressed during viral infection, including co-stimulatory molecules (CD40 and CD80) and cytokine responses (IL-12 and type I IFN). Together these signals promote a Th-1-type response. While numerous signals that prompt cDCs to become "Th-2 cell inducers" have been suggested, one airway epithelial cell–derived molecule, TSLP, has aroused the interest of researchers (37,38). TSLP directly activates DCs and promotes Th-2 responses through the expression of OX40, as opposed to other critical co-stimulatory molecules (39). In addition, TSLP promotes the production of CCL17 and CCL22, which preferentially bind to CCR4, a chemokine receptor found at high levels on Th-2 cells (40,41). Thus, the determination of whether cDCs will promote a Th-1- or Th-2-mediated response may be dependent upon the nature of the signal that it receives from the pulmonary environment. In addition, specific signals, such as TSLP versus IFN-α, may determine the nature of the immune response, and depend entirely upon the cell population (epithelial cell versus pDC) that supplies the virus-induced cytokines for maturation of the cDC prior to interaction with the T cell. Activation within the lymph node in the presence of properly activated pDCs have also been shown to be critical for an appropriate immune response via subsequent cDC activation (31).

A complex relationship is beginning to develop between the different DC subsets and the regulation of immune responses within the lung that may depend upon how these different APC populations are initially activated. Why is it that RSV drives such a profound and undesirable response compared to other respiratory viruses? This information may be gleaned from the mechanism of how RSV infects cells compared to the other respiratory viruses. The mode of infection of RSV appears to be through either membrane fusion and subsequent release of ssRNA into the cytoplasmic compartment, or through clathrin-mediated endocytosis that was pH-independent, as opposed to entry into the cell via a receptor-mediated endosomic compartment through specific receptors that are pH-dependent (42,43). The ability of RSV to enter the cell directly into the cytoplasm is likely the reason that RIG-I is the primary activation pathway, whereas TLR-induced type I IFN is not produced until later time points of infection, when pH-dependent endosomic events occur (44). Once RSV begins to expand its RNA its components may depend upon autophagy-associated and other mechanisms to activate a number of innate immune pathways that depend upon endosomic entry for the TLR-induced activation events discussed above (45). While the literature at present is not entirely clear in this area, the delay in TLR-mediated activation prior to transport to the endosome may give RSV an advantage due to a delay in TLR-induced mediator production. In fact, a recent study demonstrated that pDCs depend upon autophagy for IFN-α production during viral infections, such as that with VSV, that enter the cell via a cytoplasmic route, whereas those that enter via the endoplasmic compartment do not depend upon autophagy (46). Autophagy pathways may provide a logical way to activate the acquired immune system to facilitate viral clearance, but could also provide a process for dysregulation during chronic or severe disease (47). A better understanding of these activation events may allow additional avenues of therapeutic control during complex disease phenotypes within the lung immune environment, as well as provide additional information that may allow better vaccine design.

ADAPTIVE IMMUNE RESPONSES TO RESPIRATORY SYNCYTIAL VIRUS

The innate immune response is initiated to quickly protect the host from overwhelming infectious organisms, but can also tailor the adaptive immune response to the invading pathogens. During respiratory viral infections the balance of CD4 and CD8 responses manages the progression of the response. It is now clear that RSV-specific T cells are both protective and pathogenic. In mice depleted of CD4 and CD8 T cells, RSV persists for several weeks but no overt disease symptoms are observed (48). Individually, both CD4 and CD8 contribute to terminating RSV replication, but often at the cost of significant immunopathology. Some groups have investigated the immune response directed toward specific viral proteins. In studies in which mice were vaccinated with vaccinia vectors containing F, G, N, or M2 protein, Th-2-mediated disease only resulted upon challenge in mice primed with the G protein (48–52). Studies demonstrated that while the F protein primed for both CD4 and CD8 responses, the G protein only generated CD4 memory responses. The defective response that resulted in Th-2-mediated disease and eosinophilia could be alleviated by vaccinating mice with a G-protein vaccinia vector that also contained an RSV-specific CD8 epitope. These latter observations may help explain the disastrous vaccination strategy using formalin-fixed RSV that elicits a strong Th-2-mediated disease in vaccinated and infected infants (53). Nearly all vaccination strategies against RSV using killed or fixed virus led to inefficient antiviral immune re-

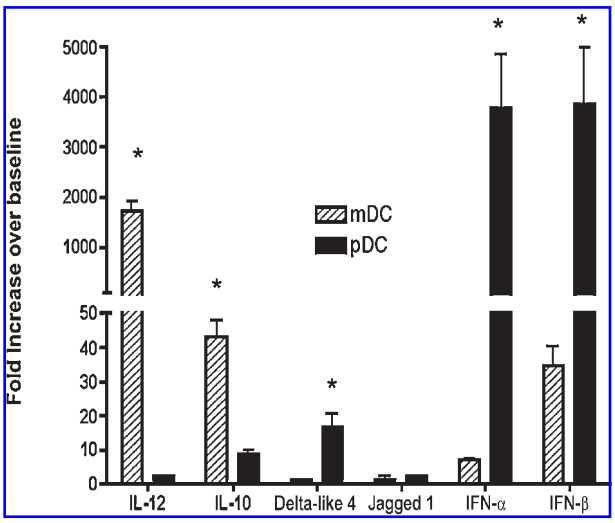

FIG. 1. Peripheral blood mononuclear cells were isolated and $CD14^+$ myeloid cells were purified and incubated for 6 d with GM-CSF and IL-4 to induce mDC differentiation. pDCs were isolated directly from the mononuclear cell population. Cells were infected with RSV (moi = 1.0) and mRNA isolated at 12 h post-challenge.

sponses and increased immunopathology, and have led researchers to begin examining attenuated live vaccine strains (54,55). Thus, the instructive signals used by DCs to activate T cells may depend upon live virus to initiate the most appropriate and least pathogenic responses.

While CD8 T cells play a role in viral clearance, previous studies indicate that RSV may impair CD8 T cells that are recruited to the lung. Using MHC class I tetramer staining, lung CD8 T-cell expansion was shown to occur in response to primary RSV infection (56,57). However, when these RSV-specific CD8 T cells were isolated from the lung, they were impaired in their ability to secrete IFN-γ and upregulate perforin. Interestingly, depletion of $CD8^+$ T cells have led to enhanced Th-2 responses during RSV infection, demonstrating that $CD8^+$ T cells may provide important regulatory signals, such as IFN-γ (58), suggesting that they may not be defective in all cases. In response to RSV, DCs upregulate co-stimulatory molecules, but may also provide specific factors that regulate the acquired responses. While the mechanisms involved in suppressing the functions of CD8 cells by RSV are unknown and still controversial, these mechanisms may allow for recurrent or persistent infections. In other studies, it has also been shown that DCs co-cultured with RSV have a suppressive effect on IFN-γ production by CD4 T cells *in vitro* (59). Thus, a multitude of immunoregulatory responses may stem from inappropriate DC activation.

A number of studies have identified that many of the pathophysiologic changes during RSV infection using experimental models relate to the cytokine phenotype that is generated. In particular, the induction of IL-13 appears to be closely associated with several aspects, including airway hyperreactivity, mucus hypersecretion, and excessive inflammation (60–62). Additional studies in humans have linked specific IL-13 as well as IL-4 haplotypes with a preponderance to develop airways disease (63–65). Thus, the distinct activation signals that drive these responses may be governed genetically and not be controlled by the viral infection alone, leaving distinct subsets of patients at substantial risk. In contrast, the generation of the Th-1 cytotoxic response generally relies on the production of factors such as IL-12 and IFN-α/β from TLR-mediated activation pathways. Pathogen-induced Th-2 responses may preferentially arise in the absence of strong TLR-induced responses that are characterized by low IL-12 levels. Interestingly, studies using cord and peripheral blood from infants that develop severe RSV infections had significantly lower levels of IL-12 production compared to responses in children with mild RSV disease (66,67). This idea is supported by studies that show that $MyD88^{-/-}$ mice, which cannot generate IL-12 in response to viral infection, are incapable of generating Th-1 responses when immunized with complete Freund's adjuvant and ovalbumin (68). RSV infection in $MyD88^{-/-}$ mice generated a predominant Th-2 response with an accompanying increase in pathogenesis that included eosinophilia and mucus hypersecretion (33). While many of the responses observed during severe RSV infections in patients can be attributed to activation by Th-2 cytokines, such as eosinophilia and mucus overproduction, others are associated with acute or Th-1-type responses, such as neutrophilia, fever, and weight loss. It is likely that the most severe and prolonged disease phe-

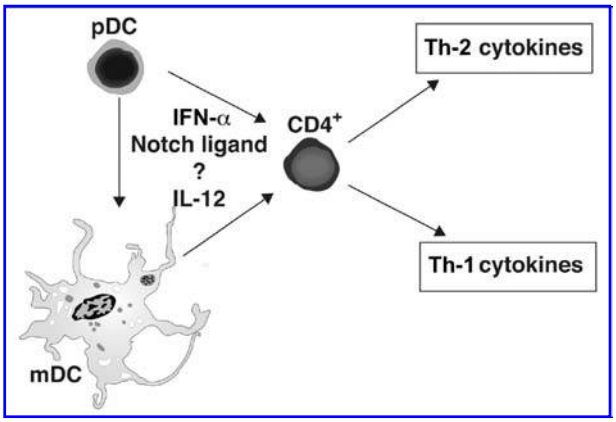

FIG. 2. The activation of the appropriate $CD4^+$ T-cell responses is most appropriately induced by the interaction with both pDC and mDC subsets that each supply important signals for T-cell differentiation.

notypes are a combination of a complex interaction of both cytokine phenotypes, and both must be considered.

An alternative explanation is that specific pathogens stimulate Th-2 responses through DC receptors and signals that are not fully defined, and it is not merely the absence of Th-1-inducing instructive signals. Recent evidence for this latter possibility comes from studies indicating that expression of the Notch ligands Delta-like and Jagged can provide instructional signals for the development of Th-1 and Th-2 cells, respectively (69).

THE ROLE OF NOTCH AND NOTCH LIGANDS FOR REGULATION OF VIRAL RESPONSES

Notch family molecules provide an activation network that has been traditionally associated with development, but has also become well known for regulating complex immune responses (69–71). There are four mammalian Notch receptors (Notch1 though Notch 4) with activation pathways that are not fully understood (70,72,73). A key molecule, MAML, is required to recruit co-activators for transcriptional activation by Notch (74–76). In recent years Notch has been shown to be involved in T-cell lineage maturation in the thymus, allowing double negative pro-T cells to mature into double positive CD4/CD8$^+$ T cells (77–79). In the mature immune system the Notch pathway has been described as a signaling mechanism involved in regulating cell lineage choices for CD4$^+$ T cells. Upregulation of the Delta-like Notch ligands in DCs polarized Th-1 cells, whereas the Notch ligand Jagged induced Th-2 cell polarization (80). Studies have separately demonstrated that Notch activation can be utilized for the generation of Th-1, Th-2, or Treg cell generation, but depends upon additional signals (80–84). Recent studies using genetically altered animals have indicated that Th-2 immune responses are fully dependent upon Notch activation, while Th-1 responses can develop in the absence of Notch signaling (85,86). In recent studies in our lab we have concentrated on Delta-like 4, which is the primary Notch ligand that is upregulated by RSV infection of DCs (87). When specifically blocking Delta-like 4 *in vivo* by passive immunization during RSV infection, a more intense pathogenic response including increases in airway hyperreactivity and mucus hypersecretion was observed. The response was characterized by elevated Th-2 cytokine production that could be reversed *in vitro* by culturing T cells with rdll4 in re-stimulation studies. Altogether, it appears that the role of notch ligand expression by DCs can differentially regulate the outcome of a viral immune response.

More recently we have also characterized whether there was differential expression of notch ligands by subsets of DCs. In particular, we have continued to focus on differences between the expression in cDCs versus pDC subsets after RSV infection, as studies have suggested that different DC subsets may differentially express notch ligands (88). Our recent data indicate that when we examine these two DC subsets from human peripheral blood (Fig. 1), we observe that pDCs but not cDCs express a high level of dll4 after RSV infection. Furthermore, when we examine a number of other innate mediators, we find that the pDCs predictably express much higher levels of IFN-α/β, while the cDCs express significantly more IL-10 and IL-12. Thus, these DC subsets may provide distinct but equally important instructive signals to help differentiate the T-cell-mediated immune responses.

SUMMARY

Determining how the different subsets of DCs work together to promote the most efficient antiviral response with minimal pathogenesis by driving a Th-1 versus Th-2 cytokine profile (Fig. 2) will be an onerous task for researchers. The generation of the most effective antiviral responses requires multiple directional signals. Without these interacting cues the immune response would be altered and potentially lead to long-term sequelae and persistent pathogenic pulmonary disease. While the relevance of DC, TLRs, and Notch in the activation of the mature immune system is a story that is only beginning, it provides an important concept of how the maturing immune system relies on a diverse set of molecules and cell populations to fine-tune the system for the most appropriate and least pathogenic responses. A better understanding of these and other signals not only will aid in detecting potential patient populations that are at risk, but may offer additional avenues for more informed vaccine strategies.

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Received December 21, 2007; accepted February 18, 2008.