The Role of CC Chemokine Receptors 6 and 7 in the Immune Response to Respiratory Syncytial Virus

by

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Abstract

Chemokines and chemokine receptors promote the migration of immune cells during infectious stimuli as well as under homeostatic conditions. In chronic disease, chemokines can also promote influx of cells that contributes to pathological inflammation. Respiratory syncytial virus (RSV) is a negative sense RNA virus that is the primary cause of hospitalization in children under the age of 2. Pathologic immune responses are thought to be responsible for RSV-associated disease. This study investigated the role of two chemokine receptors, CC chemokine receptors 6 and 7 (CCR6 and CCR7), expressed by leukocytes, in the immune response to RSV infection. Animals deficient in CCR6 and infected with RSV had enhanced viral clearance, diminished mucus production, and generated a protective Th1 effector T cell response in comparison to wild-type mice. The tempered pathological response in CCR6-/- animals was due to the impaired recruitment of dendritic cells (DC) in the lung, which promoted Th2 cytokines and mucus production when adoptively transferred into CCR6-/- mice. Conversely, CCR7-/- animals infected with RSV exhibited an exacerbated, dysregulated effector T cell response in the lung as a result of impaired migration of cells to the lymph nodes and thus local priming of the immune response. Restoration of immune cell trafficking to the lymph nodes in bone marrow chimeras reestablished effector T cell responses and abrogated local pathology. These studies demonstrate important roles for chemokine receptor-mediated immune cell trafficking in contributing to tissue pathology as well as modulating responses to RSV infection.

Chapter I

Introduction

Overview

Respiratory viruses are responsible for the highest disease burden in children younger than five (1). Several of the most common respiratory viruses include influenza, rhinovirus, respiratory syncytial virus, parainfluenza and metapneumovirus. A number of features common to respiratory viruses contribute to the difficulty in controlling and treating viral infections. Most respiratory viruses have RNA-based genomes, which allow for mutation and thus multiple infections within and between seasons (2). Viral infections increase the chances of bacterial co-infection by damaging lung epithelial cells (3, 4), and most have evolved mechanisms to avoid detection by the immune system (5, 6). While respiratory viruses represent serious pathogens in their own right, it has been recognized that respiratory viruses are the primary cause of exacerbations of disease, including asthma and chronic obstructive pulmonary disorder (7). A major unresolved issue in the field is whether disease associated with respiratory viral infections is a result of viral pathology or the response of the immune system. The relative contribution of viral replication and immunopathology to pulmonary complications is an important consideration for the development of appropriate preventive treatments.

Respiratory syncytial virus (RSV) is a ubiquitous virus that represents a major clinical problem as the leading cause of severe lower respiratory tract infection in infants

and the elderly (8-10). Contributing to the health-related economic burden is the correlation between early infection with RSV and the later development of asthma and allergy (11, 12). Additionally, RSV has been demonstrated to be a major opportunistic pathogen in individuals that are immunocompromised (13, 14). The pathology associated with RSV infection – excessive inflammatory cell infiltration, hypersecretion of mucus, and airway blockade and damage – has been attributed to the body's immune response. For example, studies that depleted lymphocytes in mouse models of infection found highly diminished pathology, albeit with reduced clearance of virus (15, 16). More tellingly, a formalin-inactivated RSV vaccine developed in the 1960's proved highly detrimental, with individuals exhibiting enhanced eosinophilia and Th2 immune responses, resulting in enhanced disease (17). Contributing to the complexity of the immune response to RSV is the ability of the virus itself to evade immune detection, such as by down-regulating the important innate antiviral cytokines, the Type I interferons (18, 19). More importantly, and less well understood, is the fact that T and B cell-mediated immunologic memory never completely develops against RSV, and so re-infections occur frequently throughout an individual's life. The complicated interplay between viral- and immune-mediated pathology during RSV infection highlights the need for further study that will inform subsequent treatment strategies and development of an effective vaccine.

Chemokines and their receptors on immune cells play a key role in the recruitment of leukocytes into the lung upon pathogen stimulation. In particular, chemokine receptors 6 and 7 (CCR6 and CCR7, respectively) dictate the migration of important cells – dendritic cells (DC) and T cells – that mediate both protection and

pathology associated with RSV infection. CCR6 and CCR7 are upregulated at different stages of DC and T cell maturation to ensure appropriate activation of the immune response: CCR6 mediates immature DC trafficking to sites of pathogen stimulation, while CCR7 permits mature DC trafficking to the lymph node once it has been activated. Alternatively, CCR6 allows effector/memory T cells to migrate to tissue after activation in the lymph nodes, whereas CCR7 permits naïve T cell trafficking through the lymphoid system under homeostatic conditions (Fig 1.1). Elucidating the differential roles of CCR6 and CCR7 on DCs and T cells will contribute to a deeper understanding of the immunological mechanisms responsible for appropriate immunity to RSV, as well as RSV-associated disease.

Respiratory Syncytial Virus

Viral Biology

Respiratory syncytial virus is a single stranded, negative sense RNA virus belonging to the paramyxoviridae family, which also includes parainfluenza, measles and mumps viruses (20). The RSV nonsegmented genome is enclosed by a nucleocapsid within a lipid envelope, which is acquired through fusion with the host cell membrane. Most of the ten proteins encoded by the viral genome are required for the transcriptional activity of the virus, while three surface glycoproteins – the attachment (G), fusion (F) and small hydrophobic (SH) – serve to bind to host cells, and two nonstructural proteins – NS1 and NS2 – act to interfere with host cell anti-viral activity (21). Two strains of RSV have been identified, strain A and B, which alternate in their predominance during any one epidemic of RSV (22, 23). Strain differences and subtypes of each strain result from

variability in the G glycoprotein (24). Because the body produces antibodies to the G protein, likely, antigenic diversity contributes to the inefficient long-term immunity generated against RSV, as well as the difficulty in producing a vaccine.

Host Cell Binding and Replication

Airway epithelial cells are the primary targets of RSV infection, and entry of the virus into these cells occurs via the virus-derived surface attachment (G) and fusion (F) glycoproteins in conjunction with host cell molecules. In vitro studies have revealed glycosaminoglycans (GAGs) to be important host surface molecules accessed by the RSV virion for entry, and in particular, the GAGs heparan sulfate and chondroitin sulphate B (25-27). The G glycoprotein on the surface of the RSV virion was initially thought to be the primary mediator of attachment to host cell GAGs (28), however, it has been demonstrated that viral infectivity is maintained in the absence of the G protein (29). More detailed studies revealed the F protein to also contribute to viral attachment via GAGs (30), in addition to a yet-to-be-identified, GAG-independent pathway of host cell entry (31). Protein-protein interactions between the virus and host likely occur in addition to GAG binding, as the F glycoprotein undergoes conformational change to facilitate host membrane fusion, however, a specific host cellular receptor for viral entry has yet to be identified (32).

Fusion of the RSV nucleocapsid, which harbors the F and G glycoproteins, with host cell plasma membrane allows the RSV virion to be delivered directly into the cell's cytoplasm. Replication of the RSV genome proceeds here via a viral RNA polymerase, which serves to both duplicate the negative-sense genome, as well as transcribe the genome into a positive-sense, complimentary "anti-genome" (33). The complimentary

RNA is translated by host cell machinery into viral proteins, which are packaged into new virions. Cell-to-cell infectivity is facilitated by the F glycoprotein, and this forms the characteristic syncytia – large, multinucleated cells – for which the virus is named.

Host Immune Response

The immune response to RSV infection is initiated in airway epithelial cells, the major site of productive viral replication, and results in the activation of genes that promote an antiviral state. Activation of antiviral signaling pathways occurs through innate pathogen recognition receptors found on host cells, including retinoic acid-inducible gene 1 (RIG-1) and Toll Like Receptors (TLRs). RIG-1 is an innate receptor found in the cytoplasm of cells, and recognizes double-stranded RNA, which is the intermediate product produced by RSV during viral replication. TLRs represent a large family of surface as well as intracellular (endosomal) receptors that recognize a variety of conserved motifs found on pathogens. RIG-1, TLR3 and TLR4 have been found to participate in the immune response to RSV infection (34-38): TLR3 is an endosomal receptor and like RIG-1, binds double-stranded RNA; Lui et al found a temporal relationship between RIG-1 and TLR3 receptor use in a model of airway epithelial cell infection whereby RIG-1 mediated the early response to RSV whereas TLR3 was important at later timepoints (36). TLR4 is a cell surface receptor that recognizes cell wall components of gram-negative bacteria, most notably, lipopolysaccharide. In vivo studies found that mice deficient in TLR4 and infected with RSV exhibited reduced cytotoxicity and impaired viral clearance (34, 37, 38). These studies furthermore showed that the RSV F glycoprotein directly associated with TLR4 (34, 38). The result of RSV recognition through RIG-1, TLR3 and TLR4 is the activation of the transcription factors,

NFκB and IRF3. NFκB promotes expression of proinflammatory genes, such as TNFα and IL-8, which allows for immune cell recruitment, while IRF3 permits Type I interferon (IFN) expression, which is important in antiviral immunity.

Innate Immunity

Epithelial cell infection by RSV results in the production of pro-inflammatory cytokines and chemokines that recruit innate cells to the lung, including Natural Killer (NK) cells, macrophages, and dendritic cells. NK cells are lymphocyte-like cells that recognize virally-infected host cells via interaction with surface MHC Class I molecules and directly promotes their killing. During RSV infection, NK cells are recruited into the lung and function as the primary source of interferon-gamma before T cells arrive, thus mediating early viral clearance (39, 40). Macrophages are innate phagocytic cells that, like epithelial cells, produce pro-inflammatory cytokines upon pathogenic stimuli. In studies where macrophages were depleted during RSV infection, NK cell recruitment and activation was impaired; however, overall viral clearance and the T cell response was unaffected (41). In addition to enhancing NK cell activity, macrophages play a major role in viral uptake itself; mice deficient for several surfactant proteins – innate pathogen opsonins – had enhanced RSV-associated disease that was attributed to the inability of macrophages to take up virus (42-44). Together, macrophages and NK cells participate in the early innate response to RSV by shaping the cytokine environment for appropriate activation of T cells.

Dendritic cells are key innate immune cells not only for their ability to take up antigen, like macrophages, but for their direct role in priming and influencing the T cell

response. Two distinct subsets of DC exist in the lung: conventional DCs, which are myeloid in origin and present antigen to T cells, and plasmacytoid DCs, which are lymphoid in origin and serve both to influence the T cell response as well as produce large amounts of IFN-alpha upon viral stimulation (45). After infection with RSV, both subsets of DC migrate to the lung (46). Conventional DCs directly access the T cell response by presenting RSV-associated peptides to T cells, resulting in their activation (47). Plasmacytoid dendritic cells produce IFN-alpha upon stimulation with RSV in vitro (48), although a direct, protective role for IFN-alpha during RSV infection has not been definitively shown, particularly given a number of studies demonstrating the poor induction of Type I IFN by RSV (discussed further below) (5, 49, 50). In vivo, however, pDCs are associated with a protective response to RSV, as depletion of these cells during infection resulted in reduced viral clearance and enhanced lung pathology (51), while repletion of pDCs enhanced the anti-viral response (52). Thus, both subsets of DC represent important components of the immune response to RSV.

Adaptive Immunity

The ultimate clearance of and pathology associated with RSV infection depends on T cell-mediated immunity. The T cell response against RSV has been extensively studied using mouse models of disease as well as human clinical studies. Murine models have shown T cells to be indispensible for proper viral clearance, yet they also mediate RSV immunopathology (15, 53). These aspects of RSV immunity are mediated by T cell effectors, including CD4+ T helper (Th)1 and Th2 cells, which produce the signature cytokines IFNγ (Th1) and IL-4, IL-5 and IL-13 (Th2). CD8+ cytotoxic T cells produce

IFNγ as well as Fas and perforin effector molecules, although IFNγ has been shown to be the primary mediator by which RSV-specific CD8 T cells clear virus (54). It is generally accepted that a predominant Th2 response correlates with enhanced RSV-associated pathology, while a cytotoxic or Th1 (IFNγ) response is protective (55, 56). This paradigm was recognized in the early vaccine trial in the 1960s, which used a formalin-inactivated virus to inoculate individuals, and resulted in enhanced disease, characterized by eosinophilia and predominant Th2 responses, upon natural infection (57-60). Subsequent work in mouse models attempted to define the immunogenic properties of RSV that were responsible for the vaccine-induced pathological response using vaccinia virus constructs expressing individual RSV proteins. These studies found CD8+ cytotoxic and Th1 responses to be elicited by the RSV F and M2 proteins, while the RSV G protein promoted only CD4+ Th1 and Th2 responses (61-63). Though the vaccinia construct presents a complication in likely contributing to the host immune response, these studies have helped identify immunodominant epitopes of RSV, which will require further study using non-immunogenic components.

In human disease, the paradigm of a protective Th1 and a pathologic Th2 response in association with RSV infection is not clear. Diminished levels of IFNγ, but not IL-4, were found in nasal washes of children experiencing severe RSV-associated diseases compared to those with less severe disease (64). Another study found increased levels of IFNγ in infants with RSV-induced wheezing compared to control subjects (65), and Garofolo et al found no correlation between presence of Th2 cytokines and wheezing (66). In 24 infants ventilated due to RSV infection, bronchoalveolar lavage fluid analysis revealed some with high IFNγ levels and no IL-4, some with high IL-4 levels and no

IFNγ and some infants with low levels of both cytokines (67). Finally, peripheral blood mononuclear cells from infected infants produced equivalent levels of both IL-4 and IFNγ compared to cells from healthy controls (68). The lack of a consistent link between particular immune mediators and disease severity demonstrates that other factors, including genetic predisposition, play a role in individual responses to RSV infection.

Another major concern in RSV research is the inability of individuals to generate long-lasting immunity to RSV. This phenomenon has been attributed to poor B cell as well as T cell responses. RSV-specific antibodies can protect against severe disease (69), yet acute infection generates very low numbers of plasma cells and low levels of serum antibodies, and reinfection results in only small increases in both (70, 71). Similar to the B cell response, it appears that RSV induces poor CD8+ T cell memory and may even actively suppress effector CD8 responses (72). The mechanisms for such weak recall responses are unclear, but evidence suggests that the virus itself contributes to actively interfere with immune responses.

Genetic Predisposition

Given the highly variable human data on immune factors associated with severity of RSV disease, much study has been done on genetic determinants of RSV pathogenesis. Furthermore, it remains controversial regarding the extent to which RSV viral load corresponds to pathology (73), and so genetics clearly play a prominent role in determining the degree of (or lack of sufficient) response to infection. Studies of gene polymorphisms have tended to fall into two categories, depending on whether the pathology associated with RSV is believed to come from initial viral fusion and

replication, or whether it arises from the subsequent immune (T cell) response (74). In the case of the former, surfactant proteins, TLR4, and the fractalkine receptor, whose ligand has structural similarities to the RSV G protein, have been investigated (75-78). In the latter case, studies have focused on polymorphisms in innate cytokines such as IL-6, as well as T cell cytokines such as IL-4 and IFNγ (79-81). Though some polymorphisms have been found in genes in study pools of infants hospitalized with RSV, particularly in surfactants, TLR4 and IL-4, the biological significance of their association with RSV severity remains to be elucidated. Variation in study parameters, such as RSV disease outcome, as well as likely multiple gene associations, indicates that study of genetic association with disease requires more comprehensive focus.

Immune Evasion

Most viruses have mechanisms for subverting immune cell recognition and/or function to promote their own propagation. RSV is no exception, and has the ability to specifically impair host cell induction of Type I IFNs - IFNα and β. These innate cytokines broadly function to limit viral replication in infected cells by inducing apoptosis, promoting MHC Class I upregulation, which increases viral peptide presentation to CD8 T cells, and reducing protein synthesis. Studies have shown the two nonstructural proteins of RSV, NS1 and NS2, to have a key (and seemingly only) function in inhibiting Type I IFN production in epithelial cells, macrophages and dendritic cells (5). Several mechanisms by which the NS1 and NS2 proteins prevent IFNα/β production have been identified, including impairing nuclear translocation of IRF3 and NFκB (82), by decreasing STAT2 protein levels (49, 50) and by directly

associating with RIG-1 to prevent downstream signaling (83). The latter study demonstrated that the NS2 protein inhibited the production of IFNβ in DCs, and it was further shown that deletion of NS1 increased the expression of costimulatory markers and production of cytokines in DCs (84). Thus, RSV has the capability to prevent the maturation of DCs in a number of ways. It is clear that the subversion of DC function has consequences on the T cell response, as infection of DCs with RSV reduces their capacity to activate CD4 T cells (85, 86), which may be a result of impaired synapse assembly between the cells (87). RSV then acts on several cell types to prevent both anti-viral and pro-inflammatory cytokine production to thwart a robust immune response.

Asthma and RSV-Associated Exacerbation

Asthma is a chronic disease associated with recurrent episodes of wheezing caused by bronchiole constriction, airway obstruction, and inflammatory cell infiltration and persistence (88). Disease prevalence has risen over the years, and the so-called "hygiene hypothesis" attributes this to cleaner living conditions, which prevents exposure to pathogens that would otherwise prime the immune system to respond appropriately to pathogenic versus innocuous antigen (89). While there has been a correlation found between early exposure to RSV and subsequent development of asthma, this is not the case for other respiratory viruses, including influenza (90, 91). The role of early viral infection in the later development of asthma is therefore controversial. However, respiratory viruses clearly play a major role in exacerbating disease in established asthmatics (92). Acute exacerbations account for most hospitalizations and are characterized by an increase in asthmatic respiratory symptoms such as wheezing, chest

tightness, and shortness of breath. RSV and rhinovirus have been identified as the most prevalent viruses found in exacerbated disease (93, 94), with rhinovirus contributing primarily to exacerbations later in life (95) and RSV linked to earlier and more severe exacerbations (96).

The study of RSV-associated asthma exacerbations has proven difficult, as many results have been inconclusive due to variations in models of disease. For example, features of exacerbation such as airway hyperreactivity have been found in models where RSV infection is followed by cockroach allergen or ovalbumin challenge (96, 97), whereas others have found exacerbation of allergic responses only when RSV infection occurs during or after sensitization (98). On the other hand, transgenic mice have proven very useful in elucidating cell types and mediators that likely participate in exacerbated disease. Here, studies using chemokine receptor knockout mice have implicated various cells involved in promoting the virus-associated pathologic lung environment, such as CCR6-expressing DCs and CCR1+ effector T cells (99-102). The study of RSV and other respiratory viruses in the exacerbation of asthma is an important area requiring further study, and serves to highlight the need for a more thorough understanding of RSV-associated immunopathology.

Current Treatment and Strategies for Vaccine Development

Most of the treatment strategies for infants with severe RSV are palliative. Adrenaline and bronchodilators act at early stages of infection to relieve wheezing by opening the small airways (103). Corticosteroids are administered to counteract the immune response, however a number of studies have questioned the clinical benefit of

steroid use (104, 105). The only approved antiviral for RSV is ribavirin, which inhibits synthesis of viral structural proteins, thus attenuating replication (106). Initial evaluation of ribavirin was encouraging, however, administration has been restricted to high risk infants due to concern about teratogenic effects (106). The most promising therapy available today is prophylactic, in particular, a humanized mouse IgG1 monoclonal antibody directed against the F glycoprotein of RSV, called Palivizumab. Studies have demonstrated the efficacy and safety of Palivizumab, but it is cost prohibitive and requires multiple hospital visits (107, 108). There is thus a clear need for a preventive vaccine that can be administered early and in a single dose.

There are several challenges to developing a vaccine to RSV. The first is the fact that the target group is infants, which have immature immune systems as well as maternal antibodies that may suppress immune responses to RSV (109). Another issue is the potentially highly immunogenic nature of RSV that was demonstrated in the first attempt at vaccine development: here a formalin-inactivated RSV vaccine candidate resulted in the development of severe bronchiolitis, eosinophilia, Th2 cytokines and non-neutralizing antibodies upon subsequent infection with RSV, features not characteristic of natural infection (60). Thus, current vaccine candidates must be appropriately attenuated, but still elicit immunity. Such approaches have included the development of temperature-sensitive viruses and viruses with deletion of non-essential genes (110, 111). Strategies outside of live virus attenuation include development of recombinant viruses, which combine human RSV proteins with those from other animals (112), and subunit vaccines, which use purified RSV proteins such as the F, G and M proteins and various combinations of each (113). The lack of a current vaccine demonstrates the clear

difficulty in proving the efficacy of many of the aforementioned strategies, however, a few are in clinical trials, indicating that there is promise for early, protective immunity to RSV.

Chemokines and Chemokine Receptors

Chemokines are a large family of small soluble proteins that signal through G-protein–coupled receptors and are functionally associated with immune cell recruitment and activation. Chemokines have also been shown to be important in lymphocyte homeostatic migration, lymphoid organ development, angiogenesis, and polarization of Th1 and Th2 effector responses (114-117). In addition to a wide range of functions, chemokines are redundant and promiscuous in nature: in many cases, several chemokines can bind a single receptor, and most can bind multiple receptors. However, as a family, these mediators have significant structural and sequence homology and thus are classified into groups based on the position of the first two N-terminal cysteine residues in the conserved amino acid sequences. The groups include the CC, CXC, C, and CX3C chemokines, with receptor usage restricted to the same subclass, i.e., CC ligands (CCL) will bind CC receptors (CCR). This classification system was established in an attempt to simplify a field that to date has identified more than 50 chemokines and approximately 20 receptors.

Homeostatic Versus Inflammatory Chemokines

A useful way to classify chemokines is through their purported function under either homeostatic or inflammatory conditions. Chemokines may be expressed

constitutively by certain tissues or in the absence of inflammatory stimuli, reflecting a homeostatic role. For example, high endothelial venules, which are structures lining the endothelium that allow for lymphocyte movement into and out of secondary lymphoid structures, constitutively express CCL19 and CCL21, two chemokines that bind a common receptor, CCR7. CCR7 is expressed on naïve T cells; thus, binding of the receptor to either of its ligands allows for T cell extravasation from the circulation into the T-cell zones of lymphoid tissue (118). This permits T-cell sampling of antigen for potential immune activation. High endothelial venules also express CXCL13, a chemokine that binds CXCR5 found on B cells. This likewise permits B-cell migration into and positioning within B-cell follicles in lymph nodes (119). CCL19/21 and CXCL13 thus highlight an essential homeostatic function in the regulation of lymphocyte migration and positioning within lymphoid tissue, as proper lymph node development is critical to initiating and maintaining acquired immunity.

The more commonly known role for chemokines is in immune cell chemotaxis during recruitment of leukocytes to sites of pathogenic infiltration. Many chemokines are upregulated upon proinflammatory stimulation, such as by cytokine cascades or through ligation of TLRs. These induced chemokines promote the influx of immune cells that then serve to eradicate the pathogen. For example, CXCL8 is the best characterized acute phase chemokine released upon pathogenic stimulation, serving to recruit CXCR1+/CXCR2+ neutrophils, the prototypical innate cell that initiates the immune cascade (120). The adaptive response is also directly accessed by chemokines, as exemplified in mucosal tissue. Here, epithelial cells selectively upregulate CCL2 and CCL20, ligands for CCR2 and CCR6, respectively (121). These receptors permit the

recruitment of immature dendritic cells to inflamed tissue for uptake of antigen and subsequent priming of the T cell response.

CC Chemokine Receptor 6

CC chemokine receptor 6 (CCR6) is unique from most other receptors in the chemokine family in that it binds a single ligand, the chemokine CCL20. CCL20 is expressed by epithelial cells in mucosal tissue, including the gut, lung and skin, and functions as both a homeostatic and inflammatory chemokine (122). A key role for this receptor/ligand pair has been found in several stages of intestinal lymphoid organ development, particularly in the recruitment of B cells to cryptopatches and their subsequent organization into isolated lymphoid follicles of Peyer's patches (123). In the gut and other mucosal tissue, upregulation of CCL20 has been found to contribute to the pathological recruitment of DCs and T cells in autoimmune diseases such as psoriasis and rheumatoid arthritis (124, 125), as well as inflammatory conditions such as inflammatory bowel disease and asthma (126, 127). Thus, the CCL20/CCR6 pathway is important in mediating leukocyte migration in different immune settings.

CCR6 is expressed on B cells, immature dendritic cells and effector/memory T cells. This expression pattern suggests a key role for CCR6 during an immune response. Studies have shown CCR6+ DCs to participate in initiating innate responses to infection, as CCR6-/- animals demonstrated reduced pathogen control in the gut and lung due to the impaired accumulation of DCs (128, 129). Likewise, generation of antibody responses were impaired in mice deficient for CCR6 in a model of enteric rotavirus (130). A specific use of CCR6 by antigen-experienced T cells during an infection has not been definitively shown in vivo, however, as discussed above and further below, auto-antigen

specific T cells infiltrate tissue in a CCR6-dependent manner and promote pathological cytokine responses (131).

CC Chemokine Receptor 7

CC chemokine receptor 7 (CCR7) is a key receptor that participates in homeostatic migration as well as coordinates the adaptive immune response. The migration pattern of naïve lymphocytes involves circulation from the blood stream into the lymphatic system through specialized structures, high endothelial venules, and subsequent positioning into T and B cell zones of secondary lymphoid organs (132, 133). This enables lymphocytes to sample the antigenic environment for potential activation that when antigen-specific, allows lymphocytes to traffic to inflamed tissue, or when not activated, permits lymphocytes to recirculate back to blood. This process is mediated by CCR7 expressed on naïve lymphocytes in response to the two ligands of CCR7, CCL19 and CCL21, which are expressed by stromal cells of high endothelial venules and lymphatic vessels, and by fibroblastic reticular cells within lymph nodes (134). Conversely, dendritic cells selectively upregulate CCR7 upon pathogen stimulation, allowing them to mature and traffic from inflamed tissue into afferent lymph vessels where they enter secondary lymphoid organs and present antigen to naïve lymphocytes (135). Mice deficient in CCR7 also revealed the key role of this chemokine receptor not only in mediating migration events, but also for the appropriate organization of lymphoid tissue. Thus, CCR7-/- animals have altered structural segregation of B and T cell zones within lymphoid organs (136). CCR7 is therefore an important regulator both of immune cell homing as well as lymphoid tissue organization.

The role of CCR7 in mediating lymphocyte and DC migration is crucial also for maintaining peripheral tolerance. Animals deficient in CCR7 display generalized, multi-organ autoimmunity characterized by lymphocytic infiltrates in peripheral organs as well as circulating antibodies specific for tissue antigens (137). Though mechanisms of peripheral tolerance are not clear, multiple lines of evidence suggest that steady state DCs with a semi-mature phenotype, including expression of CCR7, permit the constant migration of these DCs to lymph nodes where tissue-derived antigens are presented for T cell tolerance (138-140). Peripheral tolerance may also be hindered by the functional impairment of T regulatory cells (Tregs) due to their inability to migrate to lymph nodes. Though in vitro suppressive activity of Tregs is intact in CCR7-/- animals, in vivo migration of CCR7-/- Tregs to lymph nodes is impaired, and this correlated with development of inflammatory bowel disease in mice (141). It is clear that CCR7 acting on different cell types plays a major part in maintaining tolerance to tissue-specific antigens.

The immune response to viral and bacterial pathogens has been investigated in CCR7-/- mice. Interestingly, while each of these studies found differential requirements of CCR7 on different subsets of T cell, effective clearance was observed for each pathogen (142-144). For example, CCR7-/- animals infected with *Listeria monocytogenes* had defects in MHC Class I^a-restricted CD8 T cells, but not in MHC Class I^b-restricted CD8 or MHC Class II-restricted CD4 T cells (143). Similarly, in mice infected with influenza, antigen-specific CD8 but not CD4 T cell generation was impaired, which nonetheless provided protection (142). Thus, while priming and full

generation of T cell responses may be impaired in the absence of CCR7, compensating mechanisms are clearly in place such that clearance of pathogens is maintained.

Th1 and Th2 Effector T Cells

Appropriate handling of infectious stimuli requires that T cells initiate particular effector responses tailored to the pathogen. The two canonical subsets of effector CD4+ T cells – Th1 and Th2 – have been extensively studied and characterized, and have discrete pathways of differentiation and function during immune responses (145). The differentiation of naïve T cells into either Th1 or Th2 cells depends upon the local cytokine environment as well as the manner in which DCs have been activated. When DCs are induced to produce IL-12, such as by TLR ligation, naïve T cells will preferentially skew toward a Th1 phenotype and produce IFNγ. Induction of the Th1 phenotype is achieved by signaling through the Signal Transducer and Activator of Transcription (STAT) protein, STAT4, and expression of the lineage-specific transcription factor T-bet (146). In the presence of IL-4, naïve T cells will preferentially differentiate into Th2 cells, producing the signature cytokines IL-4, IL-5 and IL-13. Here, Th2 lineage commitment occurs through STAT6 signaling and expression of the lineage-specific transcription factor, GATA3 (147). Maintenance of Th1 and Th2 phenotypes occurs both by negative regulation of the lineage not being induced and by epigenetic silencing of the appropriate locus (148).

Each Th effector subset mediates immunity to different types of stimuli. The generation of a Th1-dominated response promotes immunity to intracellular pathogens while Th2 responses allow clearance of multicellular organisms such as helminthes (149).

Dysregulated or displaced effector responses promote pathology, such as asthma in the case of Th2 cells and autoimmunity in the case of Th1 cells (149), although as discussed below, the long-standing paradigm of Th1 cells promoting autoimmune states no longer appears to be the case. In addition to having distinct cytokine and functional profiles, Th1 and Th2 subsets also express differential chemokine receptors, indicating that particular chemokine environments may promote the recruitment and/or differentiation of Th1 or Th2 cells. For example, CXCR3 is primarily expressed by Th1 cells, allowing for their migration via CXCL9/10/11, whereas Th2 cells preferentially express CCR4 and respond to CCL17 and CCL22 (150). The multiple ways in which Th1 and Th2 cells differ demonstrates their unique roles under different immune stimuli.

T Regulatory Cells and Th17 Effector T Cells

In addition to the canonical Th1 and Th2 effector T cell subsets described above, two additional subsets of CD4+ T cell have been identified – T regulatory and Th17 cells. Both sets express specific transcription factors that identify these subsets as separate lineages from Th1 and Th2 CD4 T cells. While Th17 cells represent an effector cell like Th1 and Th2 cells, T regulatory cells function in the opposite manner, to suppress ongoing T cell responses. However, recent data has also alluded to more flexible differentiation programs exhibited by Th17 and T regulatory cells, and so both of these cell types appear to differ from Th1 and Th2 cells in several ways.

T regulatory cells (Tregs) are key modulatory cells that function both to restrain an ongoing immune response as well as prevent responses against innocuous or self-derived antigen. These functional distinctions have led to the identification of two types

of T regulatory cells, the so-called “natural” Tregs and “induced” Tregs. The former arises in the thymus in a manner similar to CD4 and CD8 T cells and generates a T cell receptor repertoire specific for self antigens, thus highlighting the role of natural Tregs in maintaining peripheral tolerance (151). The latter arises from naïve T cells in secondary lymph nodes in response to pathogen stimulation and presentation of pathogen-derived antigen, thus identifying induced Tregs as participating in the modulation of immune responses in an antigen-specific manner (152). Despite the seeming clear-cut distinction between the Treg subsets, the phenotypic and functional characterization of Tregs remains identical, making the strict identification of naturally arising Tregs versus induced Tregs difficult.

The master regulator of Treg development and maintenance, and phenotypic identifier of Tregs, is the lineage-specific transcription factor, forkhead box P3 (Foxp3). The importance of Foxp3 – and by extension, Tregs – in the maintenance of peripheral tolerance is emphasized by mice and humans deficient in Foxp3. Here, mice (scurfy mice) develop a fatal autoimmune condition characterized by hyper-responsive CD4 T cells (153), and humans manifest profound immune dysregulation that is often fatal within the first year of life in males, referred to as immunodysregulation, polyendocrinopathy and enteropathy, X-linked (IPEX) syndrome (154). In addition to Foxp3, Tregs highly express the IL-2 receptor, or CD25, which is required for Treg maintenance and propagation (154, 155). Because Foxp3 is intracellular, isolation of Tregs has therefore relied on the population of cells highly expressing CD25 in conjunction with CD4. However, during immune stimulation, activated effector T cells likewise express CD25, though to a lesser degree than Tregs, making the identification

(and isolation) of Tregs difficult. Moreover, induced Tregs have been known to downregulate CD25 upon stimulation in vivo (156). Recently, generation of a transgenic mouse that expresses Foxp3 in conjunction with green fluorescent protein (GFP) has greatly aided in the identification of Tregs, here relying on Foxp3 instead of variability of CD25 expression (157).

Tregs function to suppress T cell responses in a variety of ways. The first is through secretion of the modulatory cytokines IL-10 and TGFβ, which attenuates effector cell function by reducing cytokine secretion from activated T cells (158, 159). The second mechanism is by direct elimination of effector cells though use of perforin/granzyme and Fas ligand (160-162). Another way Tregs restrain effector responses is through IL-2 (the ligand for CD25) blockade or consumption, which then promotes anergy instead of effector cell expansion and cytokine production (163, 164). Lastly, it appears that Tregs can also influence DCs by inducing them to produce immunosuppressive molecules such as indoleamine 2,3-dioxygenase (165), or by interfering with DC maturation (166, 167), which can then prevent DC activation of T cells. Each of these mechanisms implies a close proximity of Tregs to their target cell, though interestingly, modulation of DC function indicates that antigen-specificity, a hallmark of Treg biology, is not necessarily required for Treg function.

Th17 cells are a recently identified subset of CD4+ effector T cell that produces the signature effector cytokine, IL-17. The identification of Th17 cells as a separate T cell lineage arose in autoimmune studies where severity of disease was found to be associated not with IL-12 (and hence, Th1 effector cells), but with IL-23, a cytokine that shares the p40 subunit with IL-12 and heterodimerizes with p19 to function. Thus, both p40- and

p19-deficient mice had significantly reduced severity of disease in different models of autoimmunity, while p23 (the subunit that heterodimerizes with p40 to form functional IL-12)-deficient mice remained susceptible (168, 169). Subsequently, IL-23 was found to induce Th17 cell development, and further studies confirmed the direct role of IL-17 in promoting disease in a number of murine models of autoimmunity including experimental autoimmune encephalitis, collagen-induced arthritis and colitis (169-171).

Th17 cells express the proinflammatory cytokines IL-17A (IL-17), IL-17F, IL-21 and IL-22 and are primarily found and function at the mucosal surface of the gut. Here, Th17 effectors mediate immunity to pathogens, in particular, gram-negative bacteria and fungi (172, 173). This occurs through induction of proinflammatory cytokines in other cell types, as well as through IL-17-mediated recruitment and activation of neutrophils (174). While the various effector cytokines produced by Th17 cells participate in immunity, some discrete functions have been found. For example, while IL-17A and IL-17F share significant homology, IL-17F had only a marginal role in the development of autoimmunity in mouse models of EAE and arthritis (175). IL-22 appears to have anti-inflammatory properties in some settings, and has even been proposed to be a distinct lineage of CD4 T cell involved in epidermal immunity and autoimmunity (psoriasis) (176-178). The effector functions of IL-21 track well with IL-17, however, IL-21 participates in Th17 cell development and thus can act at a pivotal point of differentiation by preventing regulatory cell development in favor of effector cells (179). Th17 cells therefore play a role in different immune settings due to the various roles of Th17 effector cytokines.

The differentiation of naïve T cells into Th17 cells is interesting in that it requires cytokines with opposing functions: Th17 development proceeds from production of the regulatory cytokine, TGFβ, in conjunction with the pro-inflammatory cytokine, IL-6. The action of these cytokines promotes upregulation of the Th17-specific transcription factor STAT3, which induces IL-21 expression (180). IFNγ and IL-4 produced by differentiating Th1 and Th2 cells act in an autocrine way to promote commitment to Th1 and Th2 lineages, and it appears that IL-21 performs this function in Th17 cells (181). Furthermore, IL-21 in conjunction with STAT3 induces the upregulation of the Th17 lineage specific transcription factors, RORα and RORγt (182). Full commitment to the Th17 lineage also involves responsiveness to IL-23 and thus upregulation of the IL-23 receptor on Th17 cells, which appears to depend both on STAT3-mediated IL-6 and IL-21 signaling as well as RORγt (180, 181). IL-23 is produced by antigen presenting cells and is essential for full and sustained differentiation of Th17 cells, as IL-23p19-deficient mice have significantly reduced numbers of Th17 cells (171). Th17 differentiation is clearly a complicated process with integration of a number of signaling pathways and cytokines, which may serve to highlight the importance of regulating Th17 development.

A unique feature of Tregs and Th17 cells in relation to Th1 and Th2 cells is their reciprocal developmental pathways, which appears to allow flexibility in differentiation that was always considered a permanent feature of differentiating effector CD4 T cells. For example, induced Treg and Th17 development both rely on TGFβ, with Th17 cells further requiring IL-6; the outcome of Treg versus Th17 development therefore depends on the relative concentration of IL-6 in the environment (183). Recent studies have shown that both in vitro and under pathological conditions in vivo, Tregs can convert to

IL-17-producing Th17 cells with proinflammatory properties (184, 185). Interestingly, it does not appear that Th17 cells can reciprocally convert to Tregs, however, Th17 cells have been shown to co-produce IFNγ in Th1-skewing conditions whereas Th1 cells did not produce IL-17 in Th17-skewing conditions (186). Though further investigation is needed, it appears that Tregs and Th17 cells have less strict differentiation programs and can alter their phenotypes when required by the immune environment.

The novelty of and relationship between Tregs and Th17 cells has resulted in a boon of studies on the role of these cells in disease, particularly as both subsets are identified by their expression of CCR6. However, the opposing function of Tregs and Th17 cells has made investigations controversial, which is particularly evident in recent studies using murine models of experimental autoimmune encephalitis (EAE). Here one study found CCR6-/- animals to be more susceptible to EAE due to a reduced frequency of local T regulatory cells (187), while another group found CCR6-/- animals to be protected against EAE, which was attributed to a reduction in local Th17 cell accumulation (188). Much more work is needed to clarify how Tregs and Th17 cells are regulated, especially in their use of CCR6.

Bronchus Associated Lymphoid Tissue

A dramatic example of how altered migration patterns of cells can impact on immune outcome is evidenced in CCR7-/- mice, and is also relevant to human disease. CCR7-/- animals manifest ectopic lymphoid tissue in the lungs at an early stage of development. This tissue is referred to as bronchus associated lymphoid tissue (BALT) and has features similar to secondary lymphoid organs, including organized T and B cell

zones, stromal cells and high endothelial venules (189). The exact mechanism for why BALT develops in CCR7-/- lungs is not clear, however it appears that impaired migration of Tregs to the lymph nodes is at least partly responsible for BALT formation (189).

BALT is an example of ectopic or tertiary lymphoid organs (TLO) that are not normally present in immunocompetent humans, but instead develop in peripheral tissue in response to chronic inflammation, particularly autoimmune disease, or infection. The overall composition of TLOs resembles secondary lymphoid organs, however, TLOs develop in response to environmental stimuli and thus range in overall organization depending on anatomical site and associated disease (190). As discussed above, the basic features constituting TLOs are similar to secondary lymphoid organs, i.e., aggregates of lymphocytes organized into discrete T and B cell areas that harbor antigen presenting cells (APCs), high endothelial venules and stromal cells. Similarly, signals that mediate secondary lymphoid organ development are also involved in TLO development, most notably, lymphotoxin α and β (191, 192). However, many features of TLO development remain unclear or unlike that of secondary lymphoid organs, such as the seeming lack of a requirement for lymphoid tissue inducer and organizer cells, which are necessary for secondary lymph node organogenesis (190). Morphologically, TLOs are not encapsulated, do not have afferent lymph vessels and potentially lack the complicated system of “corridors” that regulates the flow of APCs, lymphocytes and chemokines to distinct areas of secondary lymphoid organs (193). It is thus an area of active research to fully define the developmental and functional features of TLOs, particularly as tissue that is highly infiltrated, i.e. has highly organized ectopic lymphoid tissue, correlates with enhanced severity of disease (194).

Summary

The studies presented in this thesis investigated the role of two important chemokine receptors in the trafficking of leukocytes in response to RSV infection. The overall hypothesis was that immune cell migration would be impaired in CCR6- and CCR7-deficient mice, impacting on the subsequent immune response to RSV infection. This hypothesis is based on the paradigm of immune cell trafficking mediated by CCR6 and CCR7 on DCs and T cells shown in Fig 1.1. The results of these studies elucidated a pathological role of CCR6 on DCs in promoting a Th2-based response to RSV, while T cell responses were enhanced and dysregulated in the absence of CCR7, which was partly due to local development of lymphoid tissue, when animals were infected with RSV. It is clear that chemokine-receptor-mediated immune cell migration can both enhance (and promote pathology) and regulate the adaptive response to RSV infection.

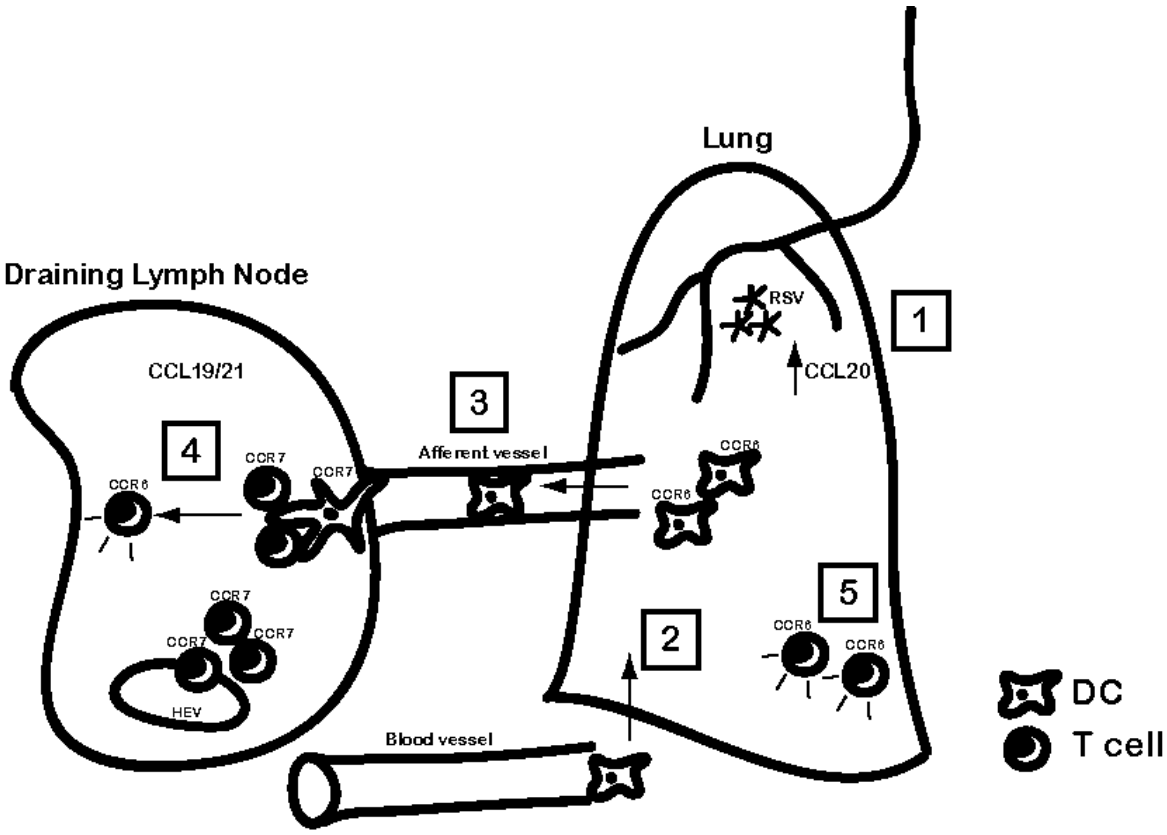

Figure 1.1 CCR6- and CCR7-mediated DC and T cell trafficking upon RSV infection.

Airway RSV infection upregulates CCL20 production by lung epithelial cells **[1]** and promotes the influx of circulating DCs expressing CCR6 **[2]**. Uptake of RSV by lung DC activates the DC, which upregulates CCR7 and migrates into the lung draining lymph node in response to CCL19/CCL21 **[3]**. The fully mature, CCR7-expressing DC presents antigen to naïve, CCR7+ T cells in the lymph nodes, and antigen-specific T cells proliferate and differentiate into effector T cells expressing CCR6 **[4]**. Effector T cells then migrate back to the lung environment to produce cytokines and eradicate the infection **[5]**.

Chapter II

CCL20/CCR6 Blockade Enhances Immunity to Respiratory Syncytial Virus by Impairing Recruitment of Dendritic Cells

Abstract

Chemokines are important mediators of the immune response to pathogens, but can also promote chronic inflammatory states. Chemokine receptor 6 (CCR6) is found on immature dendritic cells and effector/memory T cells, and binds a single ligand, CCL20, with high affinity. Here we investigated the role of CCL20 and CCR6 in a pulmonary viral infection caused by respiratory syncytial virus (RSV), a ubiquitous virus that can cause severe pulmonary complications. Neutralization of CCL20 during RSV infection significantly reduced lung pathology and favored a Th1 effector response. CCR6-deficient animals recapitulated this phenotype, and additionally showed enhanced viral clearance when compared to WT mice. No differences were observed in migration of T cells to the lungs of CCR6-/- animals, however, a significant reduction was observed in numbers of conventional DCs (cDCs), but not plasmacytoid DCs, in CCR6-/- mice. A pathogenic phenotype could be reconstituted in CCR6-/- mice by supplying cDCs into the airway, indicating that mere number of cDCs dictates the adverse response. Our data suggest that blockade of the CCL20/CCR6 pathway provides an environment whereby the attenuated recruitment of cDCs alters the balance of innate immune cells and mediates the efficient antiviral response to RSV.

Introduction

Respiratory syncytial virus (RSV) is a pervasive virus that is the most common cause of hospitalization in children under the age of 2 (195). RSV can also adversely affect the elderly and immunocompromised individuals, causing severe lower respiratory tract infection (196). Although both Th1 and Th2 effector responses may be generated, Th2 immunity is responsible for RSV-associated pathology, including airway damage and mucus hypersecretion (197). RSV represents a recurrent problem throughout life because immunologic memory never fully develops (198). Furthermore, studies have demonstrated a correlation between early exposure to RSV and the later development of asthma (11, 199). No vaccine currently exists, and early attempts to develop a vaccine proved detrimental, as individuals inoculated with a formalin-inactivated form of virus demonstrated enhanced pulmonary eosinophilia and Th2 responses (57). Clearly, further investigation is needed to clarify the fine balance between immune protection and pathology during RSV infection.

Chemokines are key mediators of leukocyte recruitment during pathogenic insult, and also play a prominent role in homeostasis (200). Most chemokines are promiscuous in that they can bind multiple receptors. CC chemokine receptor 6 (CCR6) is unique in the latter regard in that it binds a single chemokine, CCL20 (201). CCL20 is a homeostatic chemokine, with a prominent role in organizing lymphoid tissue in the gut (123), but is also upregulated upon pro-inflammatory stimulation (122). This dual function of CCL20 is evident in the cells expressing its corresponding receptor, CCR6, and contributes to a role for these cells in various immune settings.

CCR6 is found on immature dendritic cells (DC), B cells, effector/memory T cells and T regulatory cells (202-205). Studies have identified CCR6 as contributing to the pathology of inflammatory conditions such as asthma (100), autoimmune disorders such as rheumatoid arthritis (206) and graft versus host disease (207). However, studies examining the effect of CCR6 deficiency on pathogen clearance showed defective DC recruitment, resulting in reduced T cell-mediated control of infection (128, 208). Thus, CCR6 contributes to both immune pathogenesis and appropriate immunity to pathogens.

Herein, we investigated the impact of CCR6 deficiency on appropriate immunity to RSV, and the contribution of DCs and T cells to the response. We used a blocking antibody to CCL20 and CCR6-deficient mice to show that RSV-induced pathology was attenuated in these animals, and a predominant Th1 effector response was generated. CCR6-/- mice had a significant decrease in conventional DCs (cDCs) in the lungs, however, plasmacytoid DCs (pDCs), which have been demonstrated to limit viral replication and modulate immunopathology following RSV infection (52, 209), were recruited equally. Several studies have elucidated the differential roles played by cDCs and pDCs in the lung, and suggest a critical balance between these DC subsets in order for appropriate responses to occur to both innocuous and pathogenic stimuli (209, 210). Our data support this concept, suggesting that appropriate immunity to RSV involves an altered balance of DC subsets, where reduced recruitment of cDCs promotes a beneficial Th1-based environment.

Results

Treatment with anti-CCL20 alters the immune response to RSV infection Previous studies demonstrated a pathological role for CCR6 in mouse models of disease (100, 187, 211). To determine whether CCR6/CCL20 played a role during RSV-induced disease, we infected mice intratracheally with $5x10^4$ PFU RSV and assayed for production of CCL20 in BALF. We found increased levels of CCL20 at days 1 and 2 post-RSV challenge (Fig 2.1A), indicating that CCL20 is important in mediating leukocyte recruitment early upon infection with RSV.

To determine whether CCL20 had a pathogenic role, Balb/c mice were treated with a neutralizing antibody to CCL20 prior to and during infection with RSV (Fig 2.1B). Histological examination of lungs revealed a decrease in mucus production in mice treated with anti-CCL20 (Fig 2.1C). Consistent with this, anti-CCL20-treated mice had significantly reduced expression of the mucus-associated genes, *Muc5ac* and *Gob5* (Fig 2.1D). RSV-specific T cell responses were next assessed by restimulating lymph node cultures with RSV. Mice treated with anti-CCL20 exhibited reduced expression of the Th2 cytokines, IL-4 and IL-13, but showed no difference from control-treated mice in the Th1 cytokine, IFNγ (Fig 2.1E). Together, these studies indicate that CCR6-CCL20 plays a pathological role during RSV infection, and influences the nature of the T cell response.

Differential expression of CCR6 on leukocytes during RSV infection To investigate the contribution of leukocytes during RSV infection, the expression pattern of CCR6 on subsets of DCs and T cells was examined in the lung by flow cytometry. A significant increase was seen in numbers of CCR6+ MHC II+ CD11b+ CD11c+ conventional dendritic cells (cDCs) on day 2 following RSV infection (Fig 2.2A). An insignificant

increase was seen in CCR6+ CD11c+ B220+ plasmacytoid dendritic cells (pDCs) (Fig 2.2B). When T cell subsets were assessed, a significant increase was found in numbers of CCR6+ CD69+ (activated) CD4+ T cells at day 6 post-infection (Fig 2.2C), but not CCR6+ CD69+ CD8+ T cells (data not shown). These data indicate that CCR6-expressing cDCs and CD4 T cells are increased in the lung after RSV infection, and thus may play a role in the anti-viral immune response.

CCR6-/- mice display reduced pathology but control RSV more efficiently To assess the mechanism and specific cells involved in the altered pathogenic phenotype seen in mice treated with anti-CCL20, mice deficient in CCR6 were used in our RSV model. Like anti-CCL20-treated mice, CCR6-/- mice demonstrated reduced mucus production in the lungs, as shown histologically (Fig 2.3A), and by significantly lower expression of *Gob5* (Fig 2.3B). CCR6-/- mice also exhibited significantly lower airway resistance compared to WT mice (Fig 2.3C).

To determine whether the decreased pathology observed in CCR6-/- animals affected their ability to control infection with RSV, whole lungs were isolated at day 3 to determine viral titer. CCR6-/- mice had significantly fewer plaques than WT mice, suggesting that CCR6-/- animals were better able to control viral replication (Fig 2.3D). To insure that both strains were infected equally, transcript levels of the RSV G protein were measured by RT-PCR, with no differences found at either day 1 or day 2 post-infection (Fig 2.3E). Because CCR6-/- animals had more efficient handling of RSV, we examined early recruitment of NK cells, which have been shown to participate in RSV clearance prior to T cell involvement (40). Paradoxically, a 50% reduction in NK cells

was seen in CCR6-/- mice at day 2, suggesting that other innate cells are involved in the early anti-viral response to RSV in these animals (data not shown). Thus, CCR6-/- mice appear to have a decreased pathological response to RSV coincident with more efficient viral clearance.

Altered T lymphocyte response in CCR6-/- mice To examine the T cell phenotype in CCR6-/- animals after RSV infection, migration of CD69+ CD4 T cells into the lungs was assessed by flow cytometry. No differences were seen between WT and CCR6-/- mice in numbers of activated CD4+ T cells at days 6 or 8 (Fig 2.4A). We also assayed for this subset of T cells in the lymph nodes and likewise found no differences between the groups (data not shown).

RSV-specific effector T cell responses were next assessed by lymph node restimulation with RSV. CCR6-/- mice produced significantly less IL-4 and IL-13, but exhibited no difference from WT mice in IFNγ production (Fig 2.4B, left panel). In support of this data, lymph node cells stimulated with anti-CD3/anti-CD28 also demonstrated Th2 skewing by WT animals (Fig 2.4B, right panel). Recent work has identified CCR6 to be a primary receptor on Th17 cells (205). In our model, we found RSV-specific induction of IL-17, but no difference in this induction, or in the recruitment of Th17 cells, by CCR6-/- mice (data not shown). This is not surprising, as studies have shown that Th17 development is inhibited by Type I IFN (212, 213); furthermore, Hashimoto et al found augmented IL-17 in mice deficient for STAT1, the transcription factor responsible for IFN-α/β expression, after infection with RSV (214). Thus, IL-17

does not appear to play a role in influencing the phenotype of CCR6-/- animals after RSV infection.

Effector cytokine expression was then examined in the lungs of WT and CCR6-/- mice by RT-PCR. Similar to the response generated in the lymph node, CCR6-/- mice expressed significantly lower levels of IL-4 and IL-13 (Fig 2.4C). However, reduced expression of IFNγ was also seen in CCR6-/- mice (Fig 2.4C). Because we saw similar numbers of CD69+ CD4 T cells in the lungs of WT and CCR6-/- animals, we examined the intracellular cytokine profile of lung CD4 T cells. Consistent with the expression data, CCR6-/- animals displayed reduced numbers of IL-4+ and IFNγ+ CD4 T cells in the lung (Fig 2.4D). Intracellular cytokine staining in the lymph node likewise reflected our Bioplex data, with CCR6-/- animals showing reduced numbers of IL-4+ CD4 T cells but similar numbers of IFNγ+ CD4 T cells compared to WT animals (data not shown). Together, these data indicate that after infection with RSV, CCR6-/- mice exhibit an altered Th cytokine phenotype in lungs and lymph nodes, with a less pathogenic profile of cytokines in the lung.

To further examine the cytokine milieu, BALF was collected early after RSV infection and chemokines associated with a Th1 immune environment were assessed (117). Significantly higher production of CXCL10 and CCL5 was found in the BALF of CCR6-/- mice; CXCL9, which binds the same receptor as CXCL10, was also elevated, but was not significantly different in CCR6-/- mice (Fig 2.4E). Thus, the early milieu in CCR6-/- animals may influence the subsequent response by providing a more favorable environment for activation of appropriate subsets of effector cells.

Conventional DCs promote Th2 pathology upon RSV infection Because CCR6-/- animals cleared virus more efficiently and showed an overall decrease in Th effector cytokine production in the lung, we hypothesized that innate cells, such as macrophages and dendritic cells, were influencing the early antiviral response to RSV. Macrophage recruitment was first assessed by flow cytometry, with no difference found between the strains in macrophage numbers (data not shown). Interestingly a recent study demonstrated that macrophage depletion had no effect on T cell responses to RSV infection (41). Dendritic cells, on the other hand, both participate in activating RSV-specific T cells (cDCs) (47), as well as promote direct anti-viral immunity through production of IFN-α (pDCs) upon RSV stimulation (48). When cDC and pDC subsets were assessed, CCR6-/- mice had significantly fewer cDCs recruited into the lungs at day 2 post-infection (Fig 2.5A). However, no difference was found in numbers of pDCs (Fig 2.5B). Consistent with the latter data, no difference was found in the production of IFN-α in whole lung and BALF of WT and CCR6-/- mice (data not shown). To further confirm that the defective trafficking of cDCs in CCR6-/- animals was due to the absence of CCR6, we assayed for CCR6+ cDCs in animals treated with anti-CCL20 and infected with RSV. We found significantly reduced numbers of CCR6+ cDCs in mice that were administered anti-CCL20 (Fig 2.5C). Together with our T cell data, these studies suggest that the absence of early recruitment of cDCs alters the immune response to RSV in CCR6-/- animals, potentially by abrogating the Th2 response while maintaining effective IFNγ production.

To test whether cDC administration to CCR6-/- animals could recapitulate the lung pathology and Th2-biased responses seen in WT mice, $5x10^5$ WT BMDCs were

transferred intratracheally (215-217) into CCR6-/- animals immediately prior to intranasal administration of RSV. Upon histological analysis, CCR6-/- mice receiving either WT or CCR6-/- cDCs had increased mucus production compared to CCR6-/- mice not receiving cDCs (Fig 2.5D). Measurement of *Muc5ac* and *Gob5* gene expression supports the histology (Fig 2.5E). When RSV-specific cytokine responses were assessed, CCR6-/- mice receiving either WT or CCR6-/- cDCs produced elevated levels of IL-4 and IL-13, but showed no difference in IFNγ compared to CCR6-/- mice not receiving cDCs (Fig 2.5F). While the Th2 cytokine responses were not statistically significant, the trends along with the pathology suggest that merely supplying additional cDCs, regardless of CCR6 expression, partially reconstitutes the response. This supports a role for cDCs in promoting the Th2-biased pathology after RSV infection in WT mice, and that CCR6 mediates the recruitment (and possibly positioning) of this subset of DCs into the lung.

CCR6-/- cDCs have competent antigen presenting function The above data demonstrates that CCR6-/- cDCs are capable of priming a Th2 response when transferred into the lungs of CCR6-/- animals. To further confirm the functional capacity of CCR6-/- cDCs *in vitro*, cDC ability to restimulate a primed RSV T cell response was examined. Bone marrow-derived cDCs were stimulated with RSV and co-cultured with CD4+ T cells isolated from lymph nodes of WT and CCR6-/- mice at day 8 post-RSV infection. In agreement with our lymph node restimulation data, CCR6-/- T cells were skewed toward a Th1 phenotype, irrespective of origin of DC in the culture (Fig 2.6A). Thus, CCR6-/- cDCs are capable of antigen presentation to the same extent as WT cDCs.

Next, cDC contribution to a primary T cell response was examined using DO11.10 mice. Conventional DCs were pulsed with OVA peptide and co-cultured with CD4+ T cells isolated from the spleens of DO11.10 mice. Upon analysis of cytokine production, CCR6-/- cDCs were equally able to elicit Th1 and Th2 cytokine production from OVA-specific T cells (Fig 2.6B). Together, these studies suggest that cDCs from CCR6-/- animals are capable of priming both Th1 and Th2 responses, but upon RSV infection, CCR6-/- cDCs are skewed by their *in vivo* environment, allowing them to preferably prime a Th1 response.

Discussion

This study investigated the role of CCR6 and its ligand, CCL20, in a model of pulmonary viral infection induced by respiratory syncytial virus. Previous studies demonstrated this receptor to be essential for pathogen clearance in the gut, such as in models using *S. typhimurium* (128) and murine enteric rotavirus (130), as well as in the lung, using *A. fumigatus* (129). However, CCR6 has also been implicated in the pathology of diseases such as asthma, inflammatory bowel disease and psoriasis (99, 124, 218). In our studies, mice treated with an antibody to CCL20 and infected with RSV exhibited significantly decreased mucus production in the lung and generated a T cell response favoring IFNγ-production. Further investigation using CCR6-/- mice showed that these animals likewise had attenuated RSV-induced pathology and a predominant Th1 effector response when compared to WT mice. Examination of leukocyte trafficking to the lungs of CCR6-/- animals revealed no differences in T cell accumulation, but significant decreases in early cDC recruitment. Effector T cell cytokines were reduced in

CCR6-/- animals, however, viral clearance was enhanced. Together, these data suggest that the early pulmonary environment, characterized by a reduction in cDC recruitment, is key in shaping the subsequent immune response to RSV by CCR6-/- mice.

Several recent studies have demonstrated the importance of pulmonary DCs in mediating the immune response to RSV. Different subsets of cDCs, including CD103+ and CD103- populations, migrate to the lymph nodes and activate RSV-specific CD4 and CD8 T cells (47), while pDCs mobilize directly to mucosal tissue upon RSV infection (47, 219). Interestingly, RSV has been shown to interfere with cDC and pDC function, resulting in reduced cytokine production and impaired T cell activation (87, 220). In the latter study, T cell activation was assessed using proliferation assays, while our studies examined RSV-specific effector cytokine production. It is likely that these pathways are differently regulated and therefore account for the differences seen in T cell activation. The DC studies suggest that subversion of DC function may impact on the inadequate long-term immunity to RSV infection. Thus, DCs clearly play a part in shaping the anti-viral response.

To further investigate the role of DCs during RSV infection, we infected mice that were deficient in the mucosa-specific chemokine receptor, CCR6, which mediates cDC migration to the lung upon inflammatory stimuli (121). When stimulated with RSV, cDCs become activated and upregulate the costimulatory molecules CD40, CD80 and CD86, and when pulsed with OVA and RSV, prime OVA-specific Th1 and Th2 responses (48). In our studies, CCR6-/- cDCs were defective in their ability to traffic to the lung, but were not deficient in their capacity to prime either Th1 or Th2 responses using an *in vitro* co-culture assay with OVA-specific T cells. *In vivo*, however, CCR6-/-

mice generated a predominant Th1 response, and reconstitution of these mice with either WT or CCR6-/- cDCs reverted the phenotype to the mixed Th1/Th2 response, and enhanced lung pathology, seen in WT mice. These latter data indicate that the alteration observed in the CCR6-/- mice is related to the number of cDCs migrating to the lung early in the response.

Alternatively, we found no defect in accumulation of pDCs in the lungs of CCR6-/- mice after RSV infection, and this difference in migratory behavior between cDCs and pDCs highlights the distinct role these DC subsets play in the lung. For example, cDCs activated in the lung have been shown to preferentially induce Th2 effector responses (221), and only under particular stimulatory conditions will induce a Th1 response, such as upon exposure to Type I IFN (222). Plasmacytoid DCs, on the other hand, produce IFN-α upon stimulation with RSV (48), and this Type I environment likely influences the activational state of cDCs. The influence of pDCs during RSV infection has been verified by both depletion (209) and repletion (52) protocols, and demonstrates a beneficial role for pDCs on viral clearance and lung pathology. Together with the current data, it is our view that the balance between pDCs and cDCs is critical for RSV clearance and the nature of the subsequent T cell response.

In support of cDCs promoting a pathogenic response are numerous studies demonstrating that they alone induce a skew toward Th2-associated disease. An original study that isolated cDC subsets indicated that the response to allergen was skewed toward a Th2 response exclusively by a subset of lung DC (223). Subsequently, cDCs have been classified into DC1 and DC2 subsets based upon their ability to activate T cells into Th1 and Th2 effectors, respectively (224, 225). While the present study has not characterized

these subsets, it appears that CCR6 influences the recruitment of cDCs in general and it may be the mucosal environment that dictates the ability of cDCs to promote a pathogenic Th2 response. The nature of the response and environment can be influenced by specific mediators, including epithelial cell-derived thymic stromal lymphopoietin, which promotes the upregulation of OX40 on cDCs and skews T cells toward a Th2 phenotype (226). Other mediators, such as CXCL9, CXCL10 and CCL5, have been associated with promoting a Th1-skewed environment (117), and the present study shows elevated production of these chemokines in BALF of CCR6-/- mice. Thus, the early cytokine environment along with the absence of a Th2-promoting cDC subset may favor generation of a predominant Th1 response.

Overall, using this infection model, WT mice generated IL-13 and IFNγ Th effector responses to RSV, but in the absence of the CCL20/CCR6-associated immune pathway, mice instead initiated an IFNγ-dominated antiviral response. Conventional DC recruitment was impaired in CCR6-/- mice, yet the recruitment and function of pDCs remained intact. When cDCs, whether WT or CCR6-/-, were provided into the lungs, the more pathogenic Th2 response was partially reestablished. This suggests a model whereby a cytokine milieu established by the altered balance of cDC and pDC in CCR6-/- mice favors the generation of a Th1-based, efficient antiviral response without the concurrent Th2-based pathology promoted by cDCs.

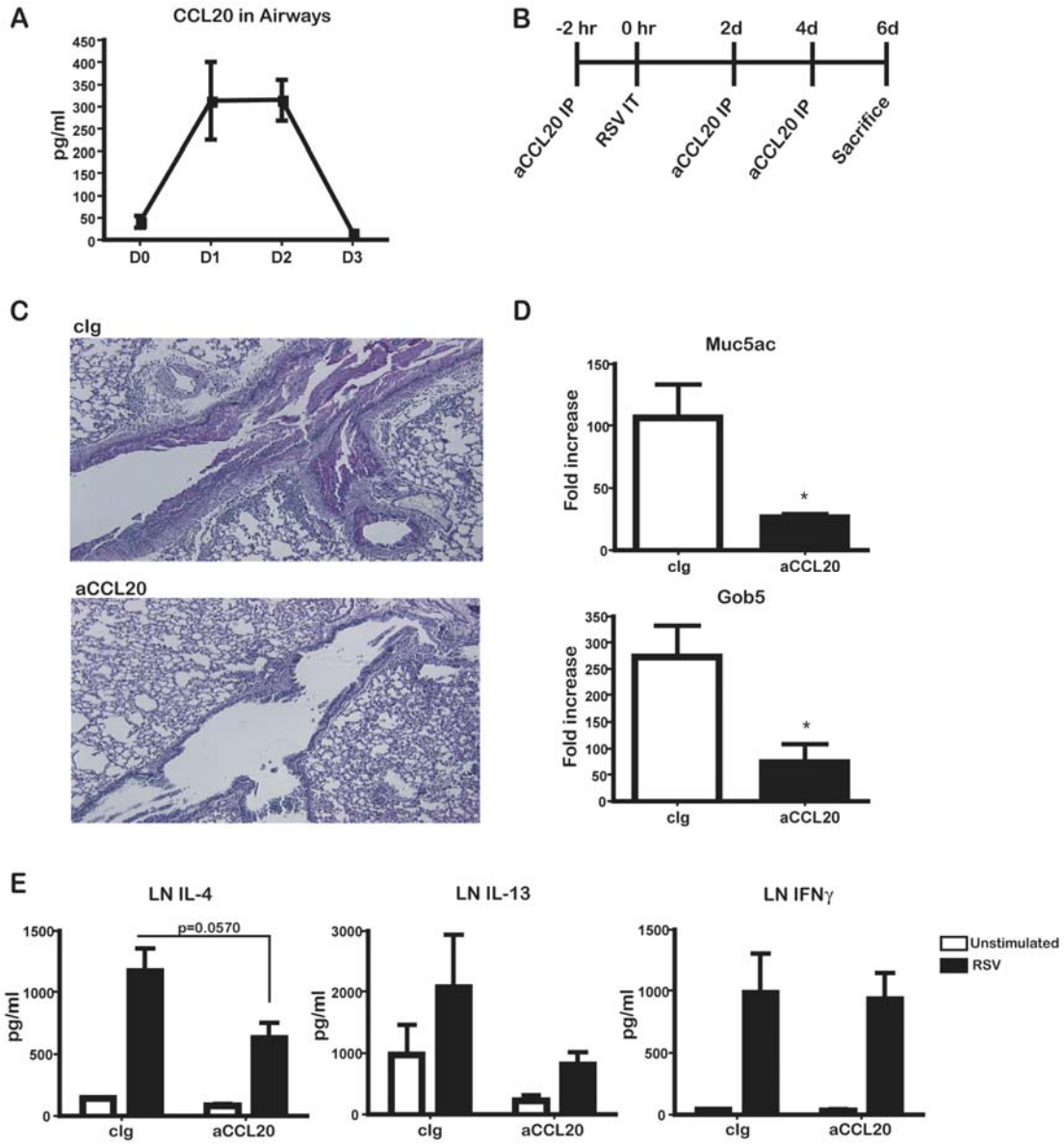

Figure 2.1. Anti-CCL20-treated mice have reduced lung pathology and lower Th2 cytokines

A. Protein levels of CCL20 were determined by ELISA in BALF of BALB/c mice after RSV infection. Data show mean ± SE from five mice/timepoint. **B.** Schematic of anti-CCL20 antibody administration to BALB/c mice. **C.** Histology of mucus production in antibody-treated mice. Data are representative of samples from 4 mice/group, magnified 100x. **D.** Expression of *Muc5ac* and *Gob5* was assessed in lung samples by RT-PCR. Data is expressed as fold increase over uninfected mice and show mean ± SE from five mice/group. **p*<0.05. **E.** RSV-specific T cell cytokines were determined by Bioplex in lymph node cultures restimulated with RSV. Data show mean ± SE from five mice/group. **p*<0.05.

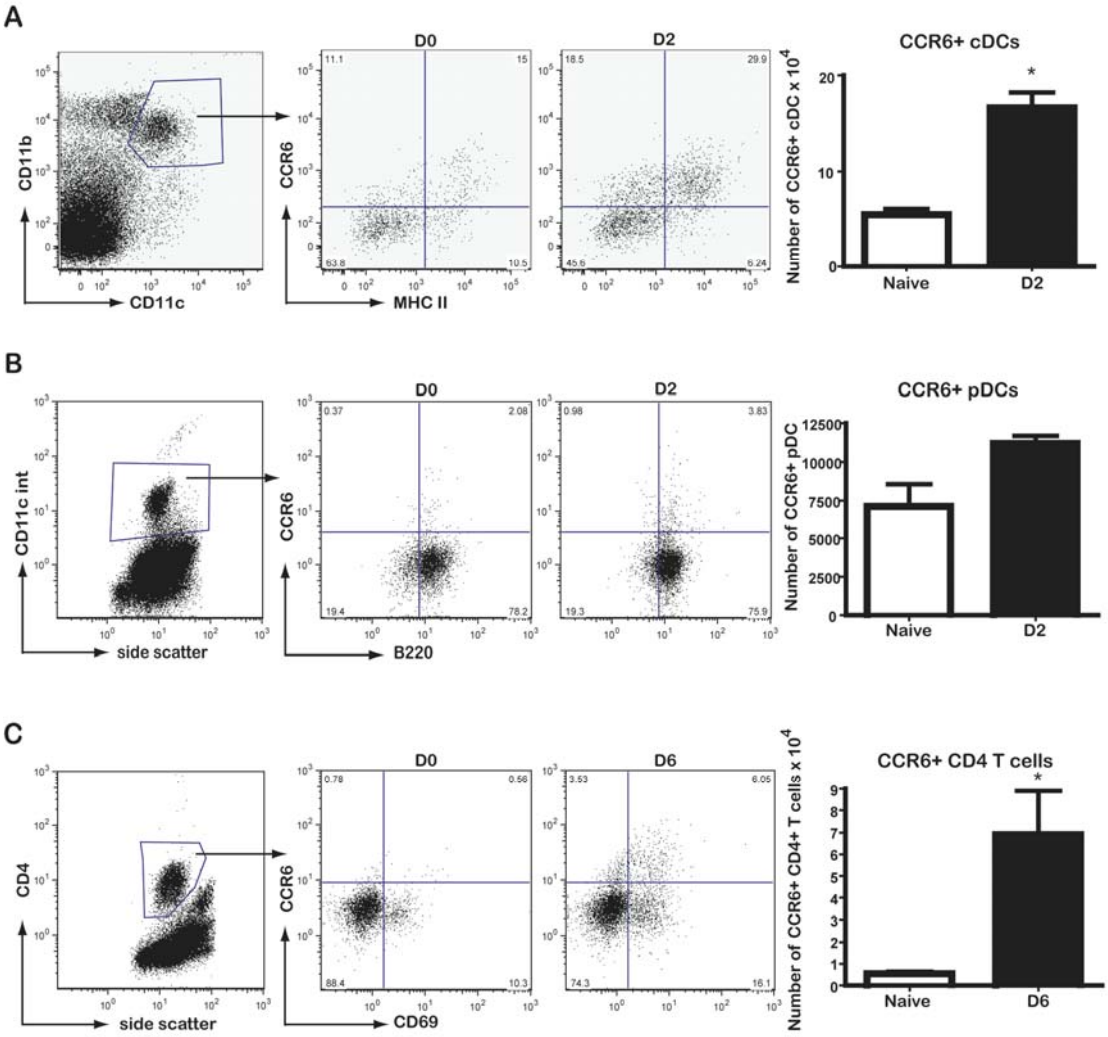

Figure 2.2. CCR6+ cDC and CCR6+ CD4 T cells are increased in lung after RSV infection

A. $CCR6^+$ cDC were assessed in lung tissue by flow cytometry at day 6 post-RSV infection. Autofluorescence was gated out and cells were gated on the $CD11b^+CD11c^+$ population and analyzed for MHC class II and CCR6 expression. Graph shows total numbers of cDC in each sample and represents the mean ± SE from five mice/group. *p<0.05. **B.** $CCR6^+$ pDC were assessed in lung tissue by flow cytometry. Samples were gated on the lymphocyte cell population and the $CD11c^{int}$ population was analyzed for CCR6 and B220 expression. Graph shows total numbers of pDC in each sample and represents the mean ± SE from five mice/group. **C.** $CCR6^+CD69^+$ $CD4^+$ T cells were assessed in lung tissue by flow cytometry. Samples were gated on the lymphocyte population and the $CD4^+$ population was analyzed for CCR6 and CD69 expression. Graph shows total numbers of T cells in each sample and represents the mean ± SE from five mice/group. *p<0.05.

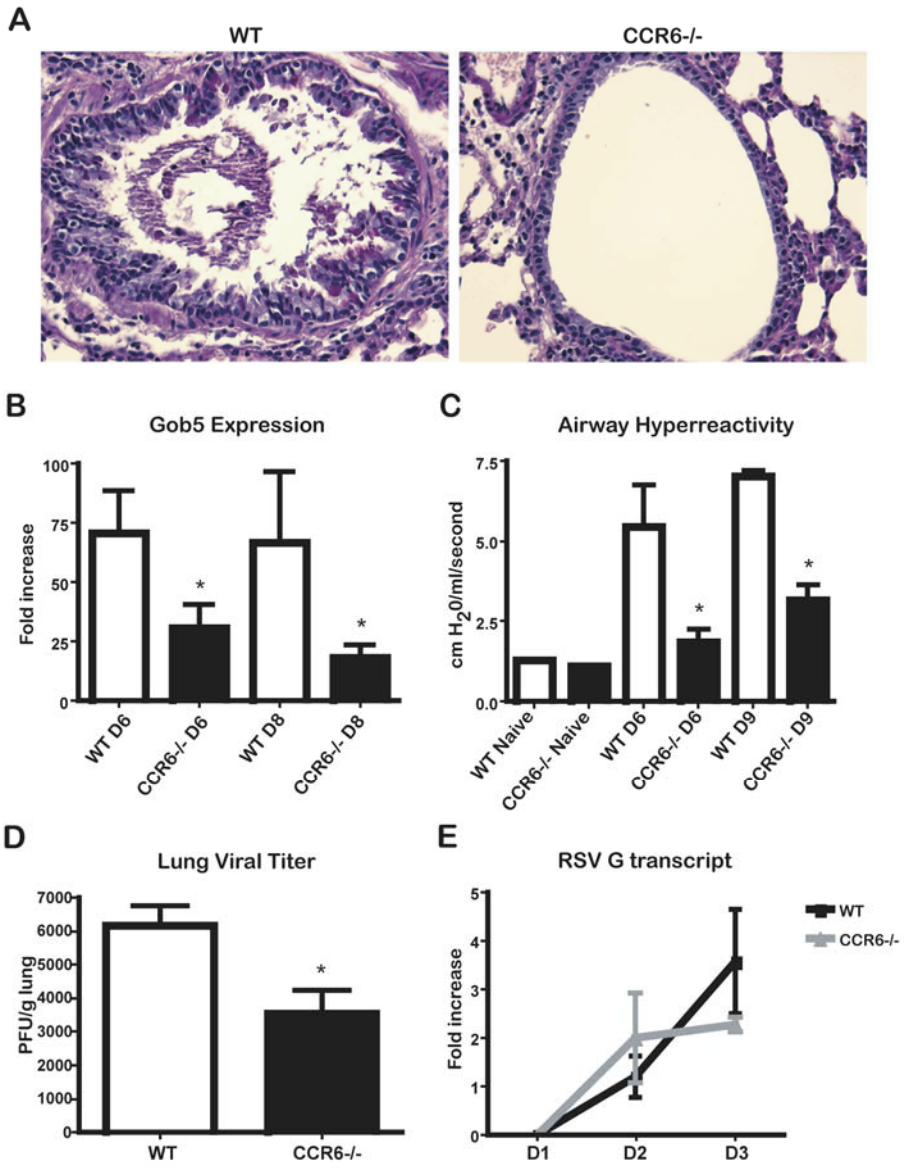

Figure 2.3. CCR6-/- mice have reduced lung pathology and more efficient viral clearance

A. Mucus production in the lung was assessed at day 8 post-infection in CCR6-/- mice by histology. Data are representative of samples from 4 mice/group, magnified 200x. **B.** *Gob5* gene expression was assessed in lung samples by RT-PCR. Data is expressed as fold increase over uninfected mice and represent the mean ± SE from four mice/group/experiment; data are pooled from 2 experiments. **p*<0.05. **C.** Airway hyperreactivity was determined using whole body plethysmography. Data represent the mean ± SE from four mice/group. **p*<0.05. **D.** RSV viral titer was determined by plaque assay at day 3 post-infection. Data represent the mean ± SE from five mice/group. **p*<0.05. **E.** RSV protein G transcript levels were measured in lung samples by RT-PCR. Data is expressed as fold increase over WT challenged mice on day 1, and represent the mean ± SE from four mice/group.

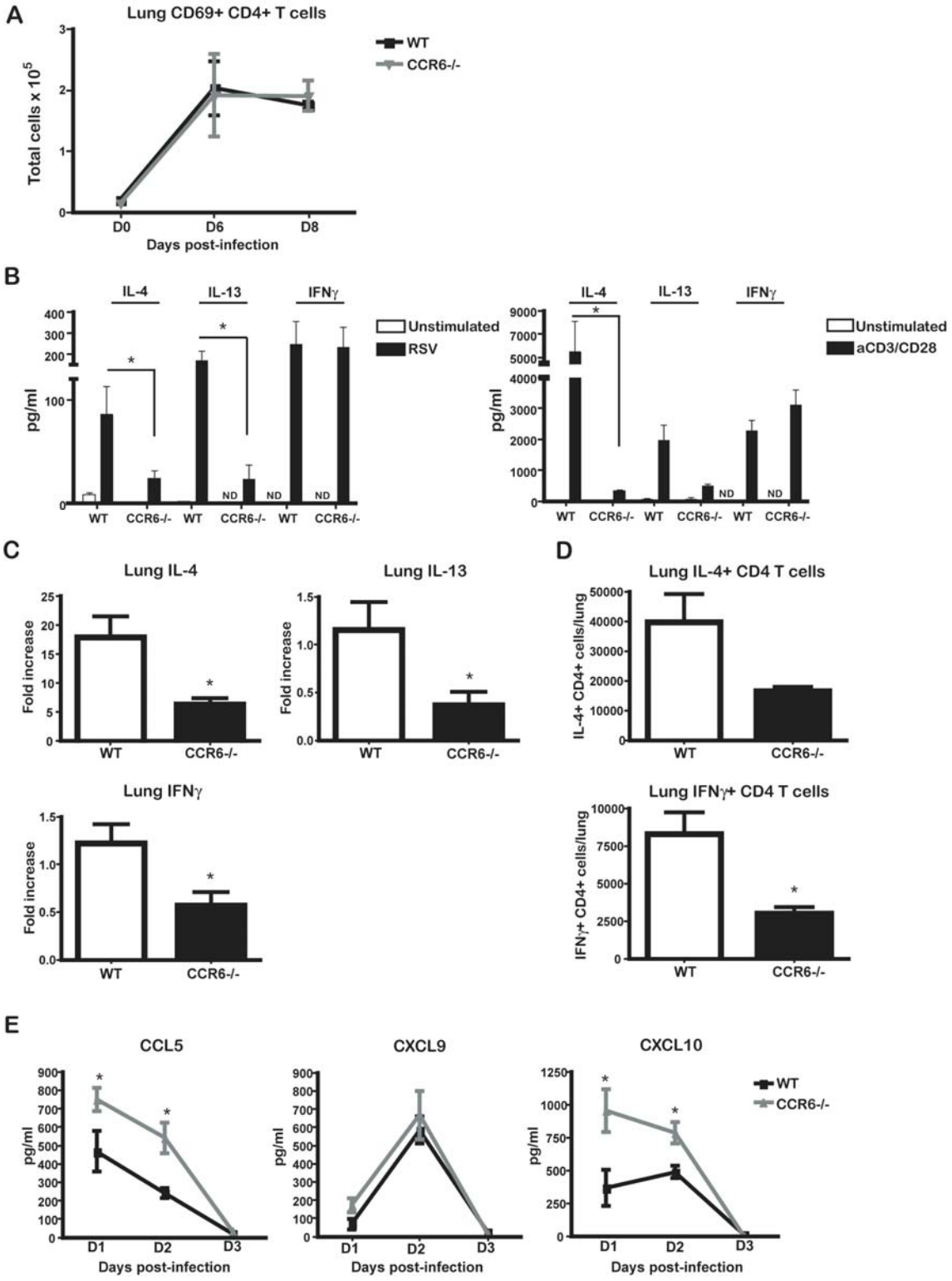
A
Lung CD69+ CD4+ T cells
Total cells x 10^5
Days post-infection
D0
D6
D8
WT
CCR6-/-
B
IL-4
IL-13
IFNγ
pg/ml
Unstimulated
RSV
aCD3/CD28
ND
WT
CCR6-/-
C
Lung IL-4
Lung IL-13
Lung IFNγ
Fold increase
D
Lung IL-4+ CD4 T cells
IL-4+ CD4+ cells/lung
Lung IFNγ+ CD4 T cells
IFNγ+ CD4+ cells/lung
E
CCL5
CXCL9
CXCL10
D1
D2
D3
Days post-infection

Figure 2.4. CCR6-/- mice have an altered T cell response following RSV infection
A. Recruitment of CD69+CD4+ T cells to the lungs was determined by flow cytometry. Data represent the mean ± SE from four mice/group. **B.** RSV-specific T cell cytokines were assessed by Bioplex in lymph node cultures at day 6 post-RSV infection. **Left panel:** lymph node cultures restimulated with RSV. Data represent the mean ± SE from four mice/group. *p<0.05. **Right panel:** lymph node cultures restimulated with anti-CD3/anti-CD28. Data represent the mean ± SE from five mice/group. *p<0.05. **C.** Cytokine gene expression was determined by RT-PCR in lung samples at day 6 post-RSV infection. IL-13 and IFN-γ are expressed as fold increase over WT challenged samples, and IL-4 expressed as fold increase over unchallenged samples. IL-4 and IL-13 data represent the mean ± SE from four mice/group; IFN-γ data represent the mean ± SE from four mice/group/experiment, data are pooled from 3 experiments. *p<0.05. **D.** Intracellular cytokine staining was assessed by flow cytometry in lung samples at day 6 post-RSV infection. Total IL-4+CD4+ and IFN-γ+CD4+ cells are. Data represent the mean ± SE from five mice/group. *p<0.05. **E.** Th1-associated chemokines were assessed by Bioplex in BALF. Data represent the mean ± SE from four mice/group. *p<0.05.

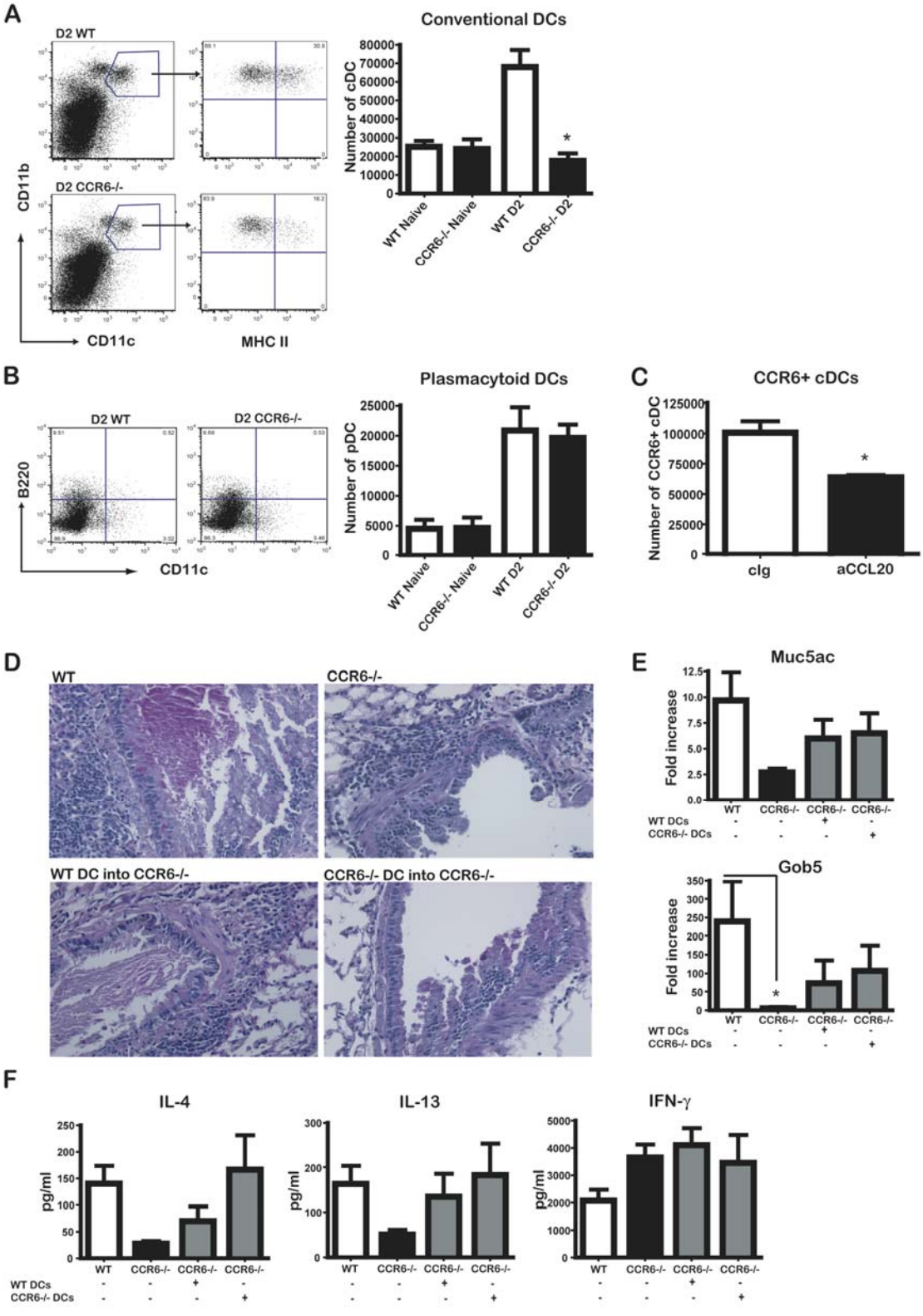
A
D2 WT
D2 CCR6-/-
CD11b
CD11c
MHC II
Conventional DCs
Number of cDC
WT Naive
CCR6-/- Naive
WT D2
CCR6-/- D2
B
D2 WT
D2 CCR6-/-
B220
CD11c
Plasmacytoid DCs
Number of pDC
C
CCR6+ cDCs
Number of CCR6+ cDC
cIg
aCCL20
D
WT
CCR6-/-
WT DC into CCR6-/-
CCR6-/- DC into CCR6-/-
E
Muc5ac
Fold increase
Gob5
WT DCs
CCR6-/- DCs
F
IL-4
IL-13
IFN-γ
pg/ml

Figure 2.5. CCR6-/- mice have reduced cDC recruitment, and reconstitution of animals with cDC promotes Th2 pathology

A. Lung cDC recruitment was assessed by flow cytometry in WT and CCR6-/- mice. Lung cells were gated on CD11b and CD11c expression and analyzed for MHC class II expression. Graph shows mean number of cDC ± SE from four mice/group. *$p<0.05$. **B.** Lung pDC recruitment was assessed by flow cytometry in the same mice as A, gating on $CD11c^{+}B220^{+}$ cells. Data represent the mean ± SE from four mice/group. **C.** CCR6+ cDC recruitment was assessed by flow cytometry in RSV-infected animals treated with anti-CCL20. Data represent the mean ± SE from five mice/group. *$p< 0.05$. **D.** Mucus production was assessed by histology in WT and CCR6-/- animals reconstituted with cDC. Shown are representative samples from 5 mice/group, magnified 400x. **E.** Mucus-associated gene expression was determined in DC transfer groups by RT-PCR, and represented as fold increase over unchallenged samples. X-axis indicates recipient animals; cDC transferred are indicated below the x-axis. Data represent the mean ± SE from 5 mice/group. *$p<0.05$. **F.** RSV-specific T cell cytokines were measured by Bioplex in lymph node cultures of DC transfer groups. Data represent the mean ± SE from five mice/group/experiment, data are pooled from 3 experiments.

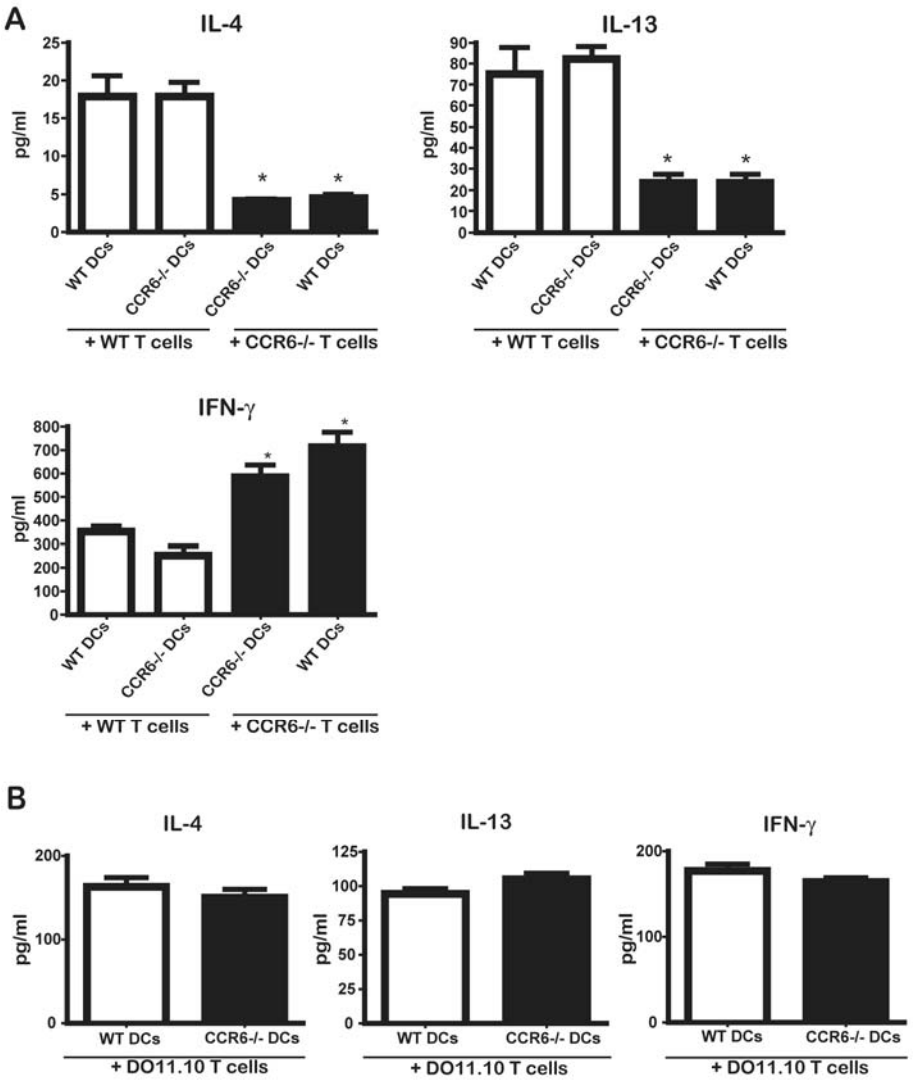

Figure 2.6. CCR6-/- cDC can prime both Th1 and Th2 T cell responses
A. T cells from RSV-challenged mice were co-cultured with RSV-primed CCR6-/- and WT DC to assess the T cell response as determined by IL-4, IL-13 and IFN-γ secretion. Data represent the mean ± SE from triplicates/group. **p*<0.05 **B.** OVA-specific, primary T cell responses as determined by IL-4, IL-13 and IFN-γ secretion were measured after co-culture with OVA-primed CCR6-/- and WT DC. Data represent the mean ± SE from three replicates/group. **p*<0.05.

Chapter III

Inefficient Lymph Node Sensitization during Respiratory Viral Infection Promotes IL-17 Production and Lung Pathology

Abstract

The development of ectopic lymph nodes often corresponds to chronic inflammatory diseases and may contribute to local pathology or enhance local immunity. The current studies investigated the role of ectopic pulmonary lymph nodes upon respiratory syncytial virus (RSV) infection using CC chemokine receptor 7 (CCR7)-deficient mice, which develop tertiary lymph nodes in the lung. CCR7-/- mice exhibited impaired secondary lymph node formation and had enhanced effector T cell responses as well as mucus production in the lung after RSV infection. Strikingly, IL-17 production from CD4 T cells in CCR7-/- mice was most notable. Wildtype animals reconstituted with CCR7-/- bone marrow recapitulated the pathogenic lung phenotype in CCR7-/- mice, while CCR7-/- animals reconstituted with WT bone marrow had normal lymph node development, diminished IL-17 production and reduced lung pathology. Mixed bone marrow chimeras revealed a defect only in CCR7-/- T cells, suggesting that impaired trafficking promotes local effector T cell generation. Lymphotoxin-alpha-deficient mice supported the above observations with increased mucus production and amplified cytokine responses in the lung in the absence of secondary lymph nodes. Thus, immune

cell trafficking to secondary lymph nodes is necessary for appropriate cytokine responses to RSV as well as modulation of the local environment.

Introduction

The classical immune response to pathogens involves the activation and subsequent migration of dendritic cells into lymph nodes where antigen is presented to T cells. Secondary lymphoid organs are specialized structures that are programmed to form during development and are characterized by distinct B cell follicles surrounded by a T cell area harboring DCs and high endothelial venules (227). Segregation of the adaptive immune response in the lymph nodes permits the recognition and proliferation of antigen-specific T cells, which then allows for an efficient effector response in infected tissue.

CC chemokine receptor 7 (CCR7) is an important chemotactic receptor that plays a major role in homeostasis as well as during an immune response. CCR7 responds to two chemokines, CCL19 and CCL21, which are expressed in secondary lymphoid organs and high endothelial venules (228). This expression pattern allows CCR7-expressing, naïve T and B cells to organize into lymphoid tissue as well as migrate throughout the lymphoid system for potential antigen recognition (136). During an immune response, CCR7 is upregulated on DCs, allowing them to migrate to the lymph nodes where they interact with T cells to initiate an adaptive immune response (229). Studies examining the immune response to pathogens in CCR7-deficient animals found effective, but delayed, T cell responses, which was attributed to the impaired trafficking of lymphocytes to draining lymph nodes (142-144, 230). Interestingly, though secondary lymphoid development is defective, CCR7-/- mice have organized tertiary (or ectopic) lymphoid

tissue in the lung, referred to as bronchus associated lymphoid tissue (BALT) (189). In the current studies, we used CCR7-/- animals to determine the contribution of tertiary lymphoid tissue in the lung in mediating immunity to respiratory pathogens.

The priming of the T cell response was investigated in CCR7-/- animals infected with respiratory syncytial virus (RSV), a significant respiratory pathogen in infants and immunocompromised individuals due to altered cytokine responses (231, 232). Our studies revealed a defect in RSV-induced immune responses that correlated with the inability of CCR7-/- animals to develop secondary lymph nodes. While viral clearance was not defective in CCR7-/- mice, enhanced mucus production and increased effector CD4 T cells and inflammatory Th2- and Th17-associated cytokines were observed in the lung. Use of bone marrow chimeras established that the defect was associated with normal secondary lymph node formation even when CCR7-/- T cells were combined with normal immune cells in mixed chimeras. Finally, the total absence of any secondary lymph node development in lymphotoxin-α-/- mice recapitulated the pathologic results observed in CCR7-/- mice. These studies suggest significant differences in the role of local versus secondary lymph node priming during respiratory viral infection and the development of subsequent pulmonary pathology.

Results

Lymph node phenotype and lung pathology are altered in CCR7-/- mice after RSV infection. CCR7 is important in allowing activated DCs and T cells to come together in the lymph node to initiate adaptive immune responses. To assess the T cell response in CCR7-/- mice, animals were infected intratracheally with ~$5x10^4$ PFU of RSV and lung

draining lymph nodes harvested. CCR7-/- mice had significantly reduced overall lymph node development (Fig 3.1A). Leukocyte recruitment was then assessed by flow cytometry, and though statistically significant differences were not found in DC numbers (Fig 3.1B), CCR7-/- animals had significantly fewer CD4+ T cells and T-regulatory cells in the lymph nodes (Fig 3.1C). Despite the diminished accumulation of CD4 T cells, CCR7-/- mice had higher proportions of IL-4+, IFNγ+ and IL-17+ effector T cells (Fig 3.1D). Likewise, restimulation of lymph node cells with RSV showed significantly higher antigen-specific production of Th cytokines by CCR7-/- animals (Fig 3.1E). These data indicate that though CCR7-/- animals have impaired lymph node formation, an enhanced effector T cell response to RSV is generated.

We next examined the lung phenotype of CCR7-/- mice in response to RSV infection. A significant increase in numbers of DCs as well as activated (CD69+) T cells was found in CCR7-/- mice (Fig 3.2A). When effector Th subsets were examined by intracellular cytokine staining, CCR7-/- animals had significantly higher numbers of IL-4-, IFN-γ- and IL-17-producing T cells (Fig 3.2B), as well as T-regulatory cells (Fig 3.2C). Whole lung RNA likewise revealed significant increases in proinflammatory cytokine gene expression (IL-6) as well as enhanced expression of IL-4, IL-17 and IL-21 in CCR7-/- mice, consistent with the intracellular staining (Fig 3.2D). We also noted the upregulation of Foxp3, the transcription factor associated with T-regulatory cell development, in CCR7-/- animals (Fig 3.2D). One of the most detrimental aspects of RSV infection is the hypersecretion of mucus. To assess the degree of mucus production in the lungs of CCR7-/- animals, histological sections stained with periodic acidic schiff were examined, and it was found that CCR7-/- mice had more extensive mucus

production in the airways than their WT counterparts (Fig 3.2E). CCR7-/- mice likewise had elevated expression of the mucus-associated genes, *Muc5ac* and *Gob5* (Fig 3.2F). Despite the high degree of mucus in the airways of CCR7-/- mice, analysis of RSV transcripts in the lung revealed no major defect in control of viral replication when compared to WT animals (Fig 3.2G). Taken together, CCR7-/- animals have an enhanced local pathological response to RSV infection, characterized by increased inflammatory cell recruitment, higher Th2 and Th17 effector cytokine production and abundant mucus secretion.

Bone marrow chimeras recapitulate CCR7-/- lung T cell response. To determine if the pathogenic local environment found in CCR7-/- animals could be recapitulated in WT animals, bone marrow chimeras were generated. WT mice were lethally irradiated and reconstituted with bone marrow from CCR7-/- animals, and after 8 weeks, chimeras were infected with RSV. Chimeras receiving CCR7-/- bone marrow displayed impaired lymph node development to a greater degree than CCR7-/- animals themselves (Fig 3.3A). Like CCR7-/- mice, CCR7-/- chimeras had increased numbers of CD69+ T cells (Fig 3.3B), and particularly IL-17+ CD4 T cells (Fig 3.3C), in the lungs after RSV infection compared to WT chimeras. When lung RNA was assessed, CCR7-/- chimeras had elevated levels of Th2- and Th17-associated cytokines, as well as Foxp3 and Muc5ac (Fig 3.3D). Thus, CCR7-/- chimeras have a similar inflammatory milieu in the lung as CCR7-/- animals after RSV infection, suggesting that impaired trafficking to the lymph node generates an inappropriate local T cell response.

To determine if CCR7-/- animals reconstituted with WT bone marrow could re-establish a proper response to RSV, chimeras were again generated. After RSV infection, lymph node development was rescued in the CCR7-/- mice receiving WT bone marrow (Fig 3.4A), and restimulation of lymph node cells with RSV resulted in a significantly diminished effector cytokine response when compared to CCR7-/- animals reconstituted with CCR7-/- bone marrow (Fig 3.4B). Furthermore, CCR7-/- animals reconstituted with WT bone marrow displayed T cell responses in the lung much like WT animals themselves (Fig 3.4C-E). These data indicate that proper priming of the immune response to RSV infection, mediated by immune cell trafficking to the lymph node, promotes a less pathological immune response in the lung.

To determine whether the defect in T cell effector phenotype in CCR7-/- mice was due to the pathologic lung environment or was intrinsic to the site of activation itself, mixed bone marrow chimeras were generated. To accomplish this analysis, C57BL/6 CD45.1 animals were reconstituted with CD45.2 donor bone marrow cells from C57BL/6 GFP (WT) and CCR7-/- mice (Fig 3.5A). Using this model, we were able to distinguish WT from CCR7-/- cells, as well as take advantage of the WT environment to determine the phenotype of CCR7-/- effector T cells upon RSV infection. Analysis of donor cell populations in the lung and lymph nodes by staining for CD45.2 showed no difference between WT and CCR7-/- in cellular composition in the lung, but significantly more cells originating from WT donors in the lymph nodes after RSV infection (Fig 3.5B). When intracellular cytokines were measured, significantly more IFNγ+ and IL-17+ cells were found to originate from CCR7-/- donor cells in the lung (Fig 3.5C). In the lymph node, significantly more CD4 T cells came from WT donor cells, however effector T cell

cytokines were higher in cells from CCR7-/- donors proportional to total donor CD4 T cells (Fig 3.5D). Thus, the inability of T cells to properly migrate to the secondary lymph nodes appears to contribute significantly to the activation and differentiation of CD4 T cells in CCR7-/- mice after RSV infection.

Lymphotoxin-α-/- mice have enhanced lung pathology following infection with RSV

To confirm the importance of lymph node priming in animals, we acquired lymphotoxin-α (LT-α)-/- mice, which are unable to form secondary lymph nodes due to deletion of lymphotoxin-α (231). Upon infection with RSV, LT-α-/- animals demonstrated significantly more mucus production in the lungs compared to WT animals as indicated by lung histology (Fig 3.6A). Additionally, LT-α-/- mice had enhanced CD69+ effector T cell recruitment (Fig 3.6B) and significantly greater numbers of Th17 and T regulatory cells in the lung, consistent with the milieu of cells seen in CCR7-/- animals after RSV infection (Fig 3.6C). Gene expression analysis revealed LT-α-/- mice to have higher levels of Th2- and Th17-related cytokine expression in the lungs (Fig 3.6D) when compared to WT animals. Therefore, in mice that cannot form secondary lymph nodes and whose only option is to mount an immune response locally, we found a similar pathologic response to RSV infection as that observed in CCR7-/- mice.

Discussion

The current studies investigated the role of immune activation by ectopic lymph node priming in the lung in response to respiratory syncytial virus (RSV). These studies were initiated to better understand the consequences of local immune priming during

pulmonary viral infections. Indeed, the structure of ectopic lymphoid tissue lends itself to immune cell activation because unlike secondary lymphoid organs, tertiary structures do not have afferent lymph vessels and are not encapsulated (193). Thus, lymphocytes and DCs organized into tertiary lymphoid tissue have direct access to stimulating signals and antigens in the local environment. Two recent studies examined the role of bronchus associated lymphoid tissue (BALT) in murine models of influenza and gamma herpes virus infection and found both a protective and detrimental role for BALT, respectively (233, 234). These studies focused on viral clearance as the endpoint for determining the role of BALT. Mechanisms that enhance viral clearance make sense from an evolutionary standpoint, however, an immune response initiated locally, including effector cytokine production, may also promote local pathology.

In human disease, lymphoid neogenesis has been documented in organ-specific autoimmune disorders, such as Graves' disease, rheumatoid arthritis and Sjogren's syndrome (235-237), as well as in chronic inflammatory conditions (238, 239). The stimuli that induces ectopic lymphoid formation is unclear, however studies have shown that T and B cell responses against tissue antigens contribute to the autoimmune inflammatory process; this is particularly the case in diseased organs manifesting germinal center formation, where isolated B cells had undergone somatic hypermutation (240, 241). The necessity of CCR7 for migration of lymphocytes and dendritic cells has been established (136), and without CCR7, tertiary lymph nodes (BALT) form in the lung (189). The organization of BALT appears to depend on dendritic cells, as their depletion led to an alteration in local sensitization (242). Our studies found no significant differences in DC migration to lymph nodes, however there were increases in local DC

numbers in both the CCR7-/- and lymphotoxin-α-/- mice that likely contributed to BALT formation. Development of BALT and other ectopic lymphoid tissue is likely a strategy to ensure the proper clearance of pathogens in inflammatory settings, however, while our studies demonstrate effective RSV viral clearance in CCR7-/- animals, local pathology is significantly enhanced. This could have serious consequences in chronic inflammatory diseases such as chronic obstructive pulmonary disorder, where lymphoid neogenesis has been documented – and infection with RSV correlates with disease severity – by inducing further pathology (243, 244).

The current study further suggests not only that the presence of pre-formed tertiary tissue (BALT) promotes pathological local immune responses, but also that altered trafficking of T cells in general may permit alternative effector cell generation and local pathology. Lymphotoxin-α is an important cytokine produced by lymphoid tissue inducer cells during development and is responsible for key aspects of secondary lymphoid organ development, including proper lymphoid architecture and recirculation of lymphocytes (190). After development, the role of lymphotoxin-α in maintaining secondary lymphoid development during immune responses is mediated by activated lymphocytes (245). Thus, without the cues given by lymphotoxin-α during the immune response to RSV, lymphotoxin-α-/- T cells may be unable to locate to secondary lymphoid organs. Lymphotoxin-α-/- mice do not have baseline ectopic lymphoid tissue in the lungs like CCR7-/- animals, however an alternative immune response developed similar to that observed in CCR7-/- mice after infection with RSV. This indicates that BALT-like tissue was induced to form as a result of the impaired trafficking and thus facilitated the pathologic local immune response in lymphotoxin-α-/- mice.

A notable feature in CCR7-/- animals was the presence of significant numbers of both Th17 and T regulatory cells in the lung. It seems counterintuitive that high numbers of T regulatory cells could co-exist with high numbers of effector T cell populations, however it has been shown that pathologic signals, such as IL-6, can prevent T regulatory function, and this may be irrespective of their accumulation (246). Additionally, a number of recent studies indicate that environmental signals can skew the development of T regulatory cells toward Th17 cells, and thus the overall pathologic lung environment may promote an effector T cell phenotype (184, 247). Interestingly, our mixed chimera data demonstrated a fundamental defect in CCR7-/- T cells irrespective of environment, indicating it was strictly the inability of T cells to traffic properly that supported the development of IL-17 producing effector T cells in the lung. A recent study supports this concept, as local sensitization of antigen into the airway appears to specifically drive IL-17-associated responses (248). Thus, T cells unable to traffic and activated locally within the lung upon viral infection may be predisposed to an altered cytokine generation.

However, a role for T regulatory cells in our model cannot be ruled out. This is particularly the case in the lymph node, where significantly increased production of all Th cytokines was observed in CCR7-/- animals. T regulatory cells have been shown to be responsible for BALT development in CCR7-/- animals (189), and were also demonstrated to be important during RSV infection, as T regulatory cell depletion enhanced mucus production, severity of illness, and CD8 T cell responses in mice (249). Recent studies have further identified mechanisms by which T regulatory cells specifically suppress Th17 and Th2 responses (250, 251). In our model, we were unable to see a reduction in Th effector cytokine production when WT T regulatory cells were

adoptively transferred to CCR7-/- animals (data not shown). This may be a result of the environment as discussed above, in that transferred T regulatory cells encountering pathogenic signals in CCR7-/- mice may be either unable to function as suppressors or are actively converting to effector cells. Such a possibility could have detrimental effects with regard to ectopic lymphoid generation, and studies are currently underway to investigate the potential conversion of T regulatory cells to effectors in a pathologic setting.

The current studies identify a key role for lymph node priming of the T cell response not simply for efficient control of viral replication, but for the overall modulation of local pathology. Animals that did not develop lymph nodes after RSV infection had enhanced effector T cell responses in the lung in addition to significant mucus production. When lymph node development was reestablished, lung pathology was concurrently abolished. Furthermore, an inherent defect in CCR7-/- T cell effector differentiation upon RSV infection was observed, indicating that the inability to properly migrate contributes to a dysregulated local T cell response. Thus, tertiary lymphoid development in response to inflammation may not affect pathogen clearance, but may promote pathological immune responses and thus have serious consequences for an already inflamed tissue state.

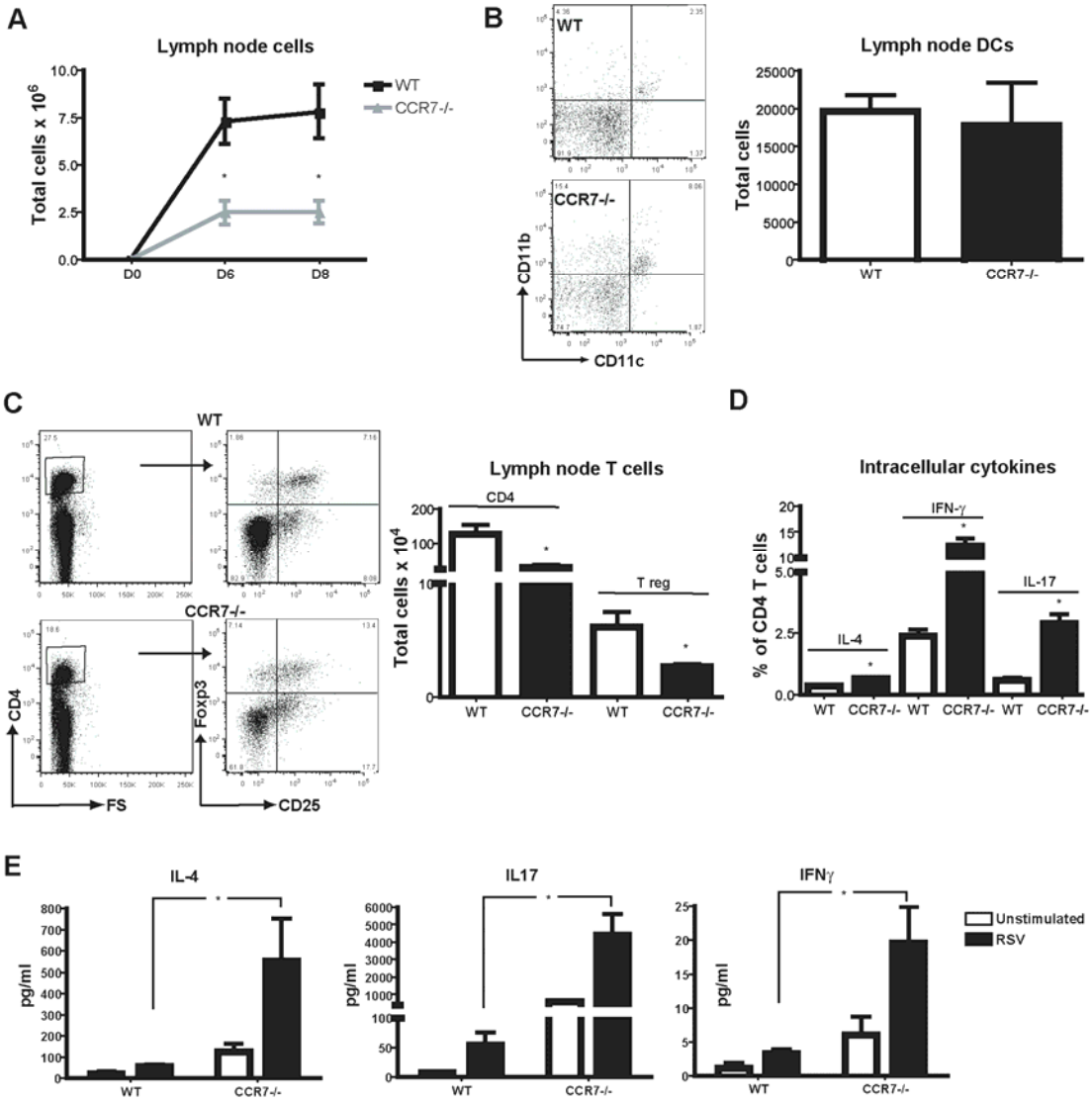

Figure 3.1 Trafficking of T cells is deficient, but effector cytokine production is enhanced in lymph nodes of CCR7-/- mice

A. Total lymph node cells were counted at days 6 and 8 post-RSV infection in CCR7-/- animals. Data represent the mean ± SE from four mice/group/timepoint. **P* < 0.05. **B.** Lymph node DCs were enumerated by flow cytometry. Cells were gated on MHC Class II hi+ cells and CD11b+CD11c+ double positive cells are shown in plots. Data represent the mean ± SE from four mice/group. **P* < 0.05. **C.** Lymph node T cells and T regulatory cells were assessed by flow cytometry. Data represent the mean ± SE from four mice/group. **P* < 0.05. **D.** Intracellular cytokine staining was performed to assess effector T cell cytokine production. Total IL-4+, IFNγ+ and IL-17+ CD4+ T cells are shown. Data represent the mean ± SE from five mice/group. **P* < 0.05. **E.** RSV-specific effector T cell responses were determined by Bioplex in lymph node cultures restimulated with RSV. Data represent the mean ± SE from five mice/group. **P* < 0.05.

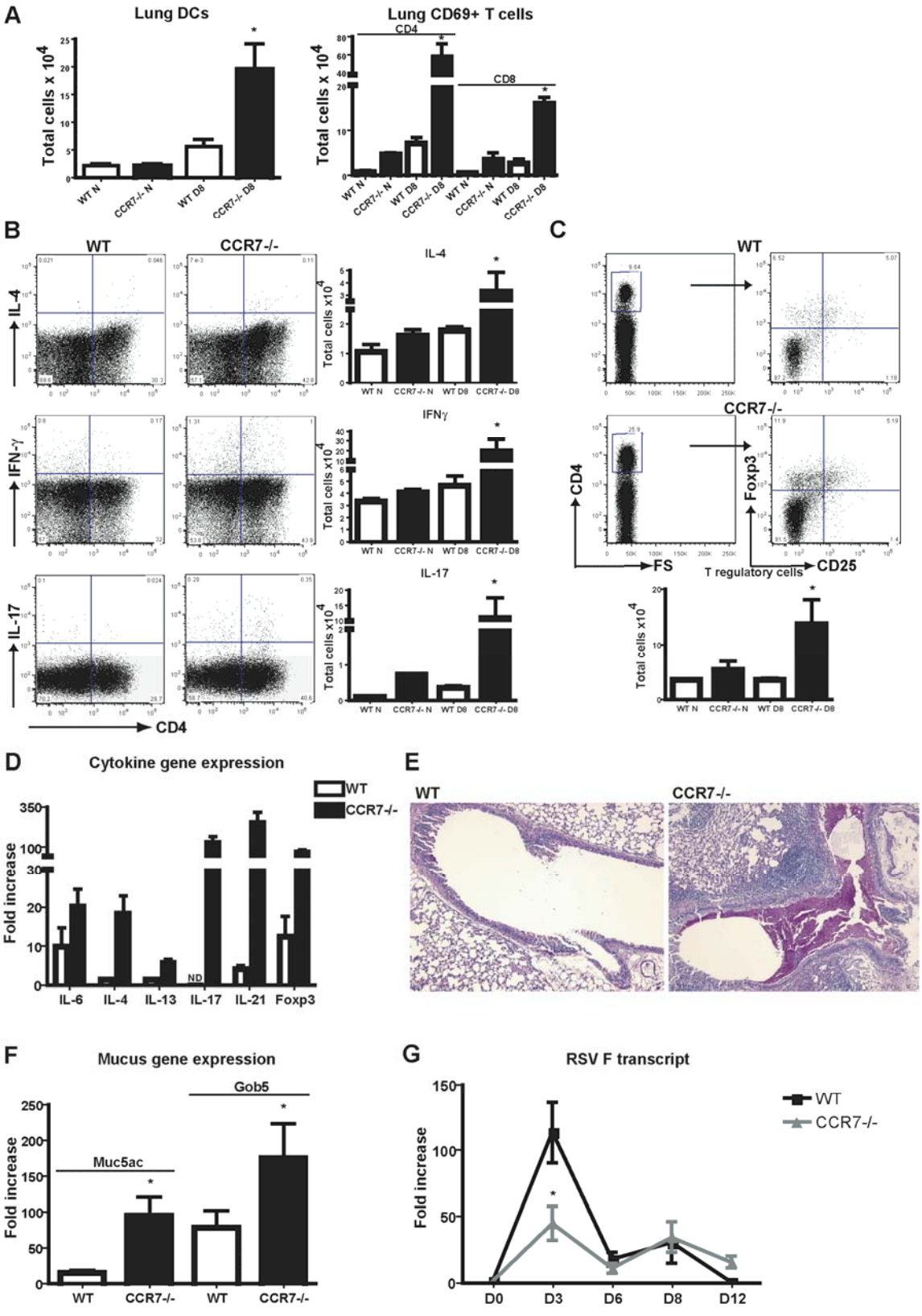
A
Lung DCs
Total cells x 10^4
WT N
CCR7-/- N
WT D8
CCR7-/- D8
Lung CD69+ T cells
CD4
CD8
B
WT
CCR7-/-
IL-4
IFN-γ
IL-17
CD4
Total cells x10^4
C
WT
CCR7-/-
CD4
FS
Foxp3
CD25
T regulatory cells
D
Cytokine gene expression
WT
CCR7-/-
Fold increase
IL-6
IL-4
IL-13
IL-17
IL-21
Foxp3
ND
E
WT
CCR7-/-
F
Mucus gene expression
Muc5ac
Gob5
Fold increase
G
RSV F transcript
Fold increase
D0
D3
D6
D8
D12

Figure 3.2. CCR7-/- mice have enhanced mucus production and increased leukocytes recruited to the lung after RSV infection

A. Lung DCs and activated (CD69+) T cells were assessed by flow cytometry after RSV infection in CCR7-/- animals. Data represent the mean ± SE from four mice/group. **P* < 0.05. **B.** Intracellular cytokine staining was performed to assess effector T cell cytokine production in the lung. Total IL-4+, IFNγ+ and IL-17+ CD4+ T cells are shown. Data represent the mean ± SE from five mice/group. **P* < 0.05. **C.** T regulatory cell recruitment was determined in the lung by flow cytometry. Graphs show total CD25+ Foxp3+ double positive cells from the CD4+ gate. Data represent the mean ± SE from five mice/group. **P* < 0.05. **D.** Expression of cytokine genes was assessed in lung samples by RT-PCR. Data is expressed as fold increase over uninfected mice and represents the mean ± SE from five mice/group. **P* < 0.05. **E.** Mucus production in CCR7-/- mice was assessed at day 12 post-RSV infection by histology. Shown are representative samples from 4 mice/group, magnified 100x. **F.** Expression of *Muc5ac* and *Gob5* was assessed in lung samples by RT-PCR. Data is expressed as fold increase over uninfected mice and represents the mean ± SE from five mice/group. **P* < 0.05. **G.** RSV protein F transcript levels were measured in lung samples by RT-PCR. Data is expressed as fold increase over unchallenged samples, and represent the mean ± SE from four mice/group. **P* < 0.05.

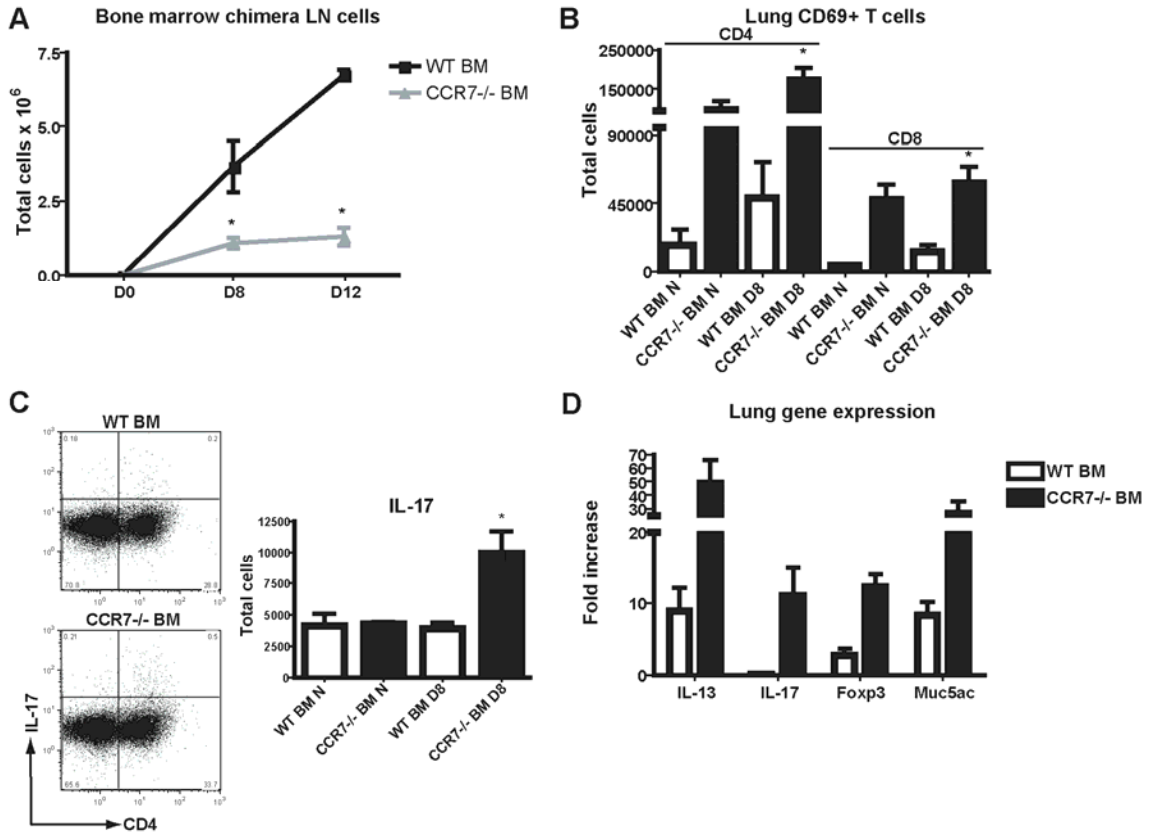

Figure 3.3. WT mice reconstituted with CCR7-/- bone marrow display similar lung pathology as CCR7-/- mice

A. Total lymph node cells were counted at days 8 and 12 post-RSV infection in WT mice reconstituted with CCR7-/- bone marrow. Data represent the mean ± SE from five mice/group/timepoint. **P* < 0.05. **B.** Activated T cell recruitment to the lung was determined by flow cytometry at day 8 post-RSV infection. Data represent the mean ± SE from five mice/group. **P* < 0.05. **C.** Intracellular cytokine staining was performed to assess IL-17+ CD4+ T cell cytokine production. Data represent the mean ± SE from five mice/group. **P* < 0.05. **D.** Expression of cytokine genes was assessed in lung samples by RT-PCR. Data is expressed as fold increase over uninfected mice and represents the mean ± SE from five mice/group. **P* < 0.05.

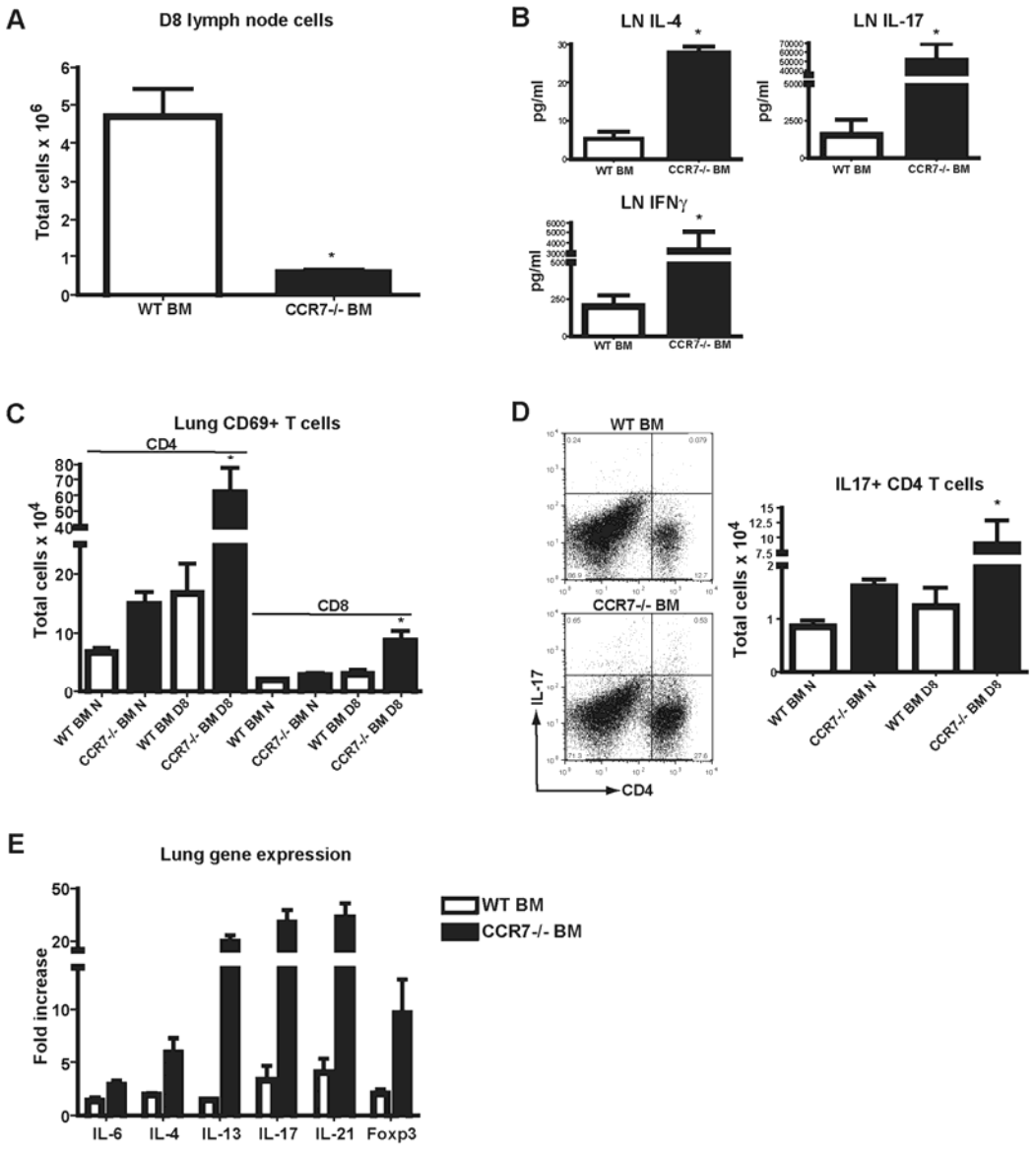

Figure 3.4. CCR7-/- mice reconstituted with WT bone marrow have normal lymph node development and reduced lung pathology

A. Total lymph node cells were counted at day 8 post-RSV infection in CCR7-/- mice reconstituted with WT bone marrow. Data represent the mean ± SE from five mice/group. **P* < 0.05. **B.** The RSV-specific T cell response was determined by Bioplex in lymph nodes restimulated with RSV. Data represent the mean ± SE from five mice/group. **P* < 0.05. **C.** Activated T cell recruitment to the lung was determined by flow cytometry at day 8 post-RSV infection. Data represent the mean ± SE from five mice/group. **P* < 0.05. **D.** Intracellular cytokine staining was performed to assess IL-17+ CD4+ T cell cytokine production. Data represent the mean ± SE from five mice/group. **P* < 0.05. **E.** Expression of cytokine genes was assessed in lung samples by RT-PCR. Data is expressed as fold increase over uninfected mice and represents the mean ± SE from five mice/group. **P* < 0.05.

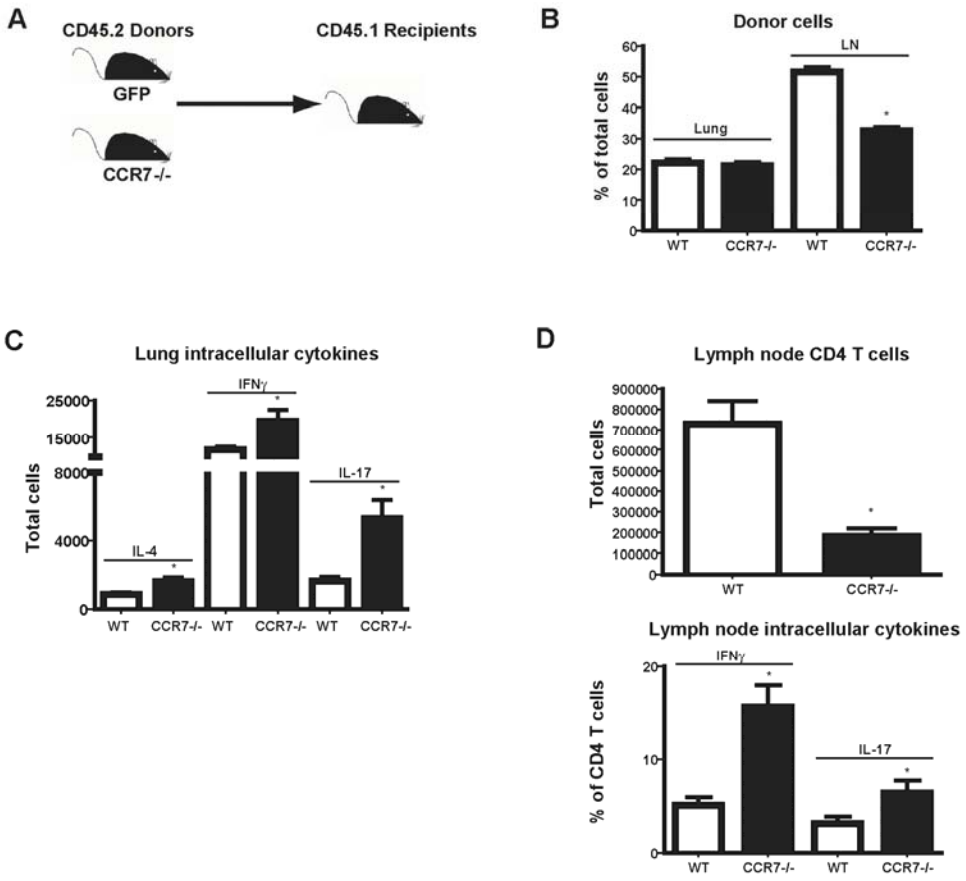

Figure 3.5. Mixed chimeras reveal defect in CCR7-/- effector T cell generation
A. Schematic of mixed bone marrow chimera generation. **B.** Percentage of total lung and lymph node cells that originated from WT or CCR7-/- donors was determined by flow cytometry after infection with RSV. Data represent the mean ± SE from seven mixed chimera mice. **P* < 0.05. **C.** Intracellular cytokine+ cells were determined by flow cytometry and calculated based on the total number of cells from each donor group in the lung. Data represent the mean ± SE from seven mixed chimera mice. **P* < 0.05. **D.** Total lymph node CD4+ T cells were determined by flow cytometry and calculated based on the total number of cells from each donor group in the lymph node. Percentage of lymph node intracellular cytokine+ cells was determined based on the total number of CD4+ T cells from the respective donor.

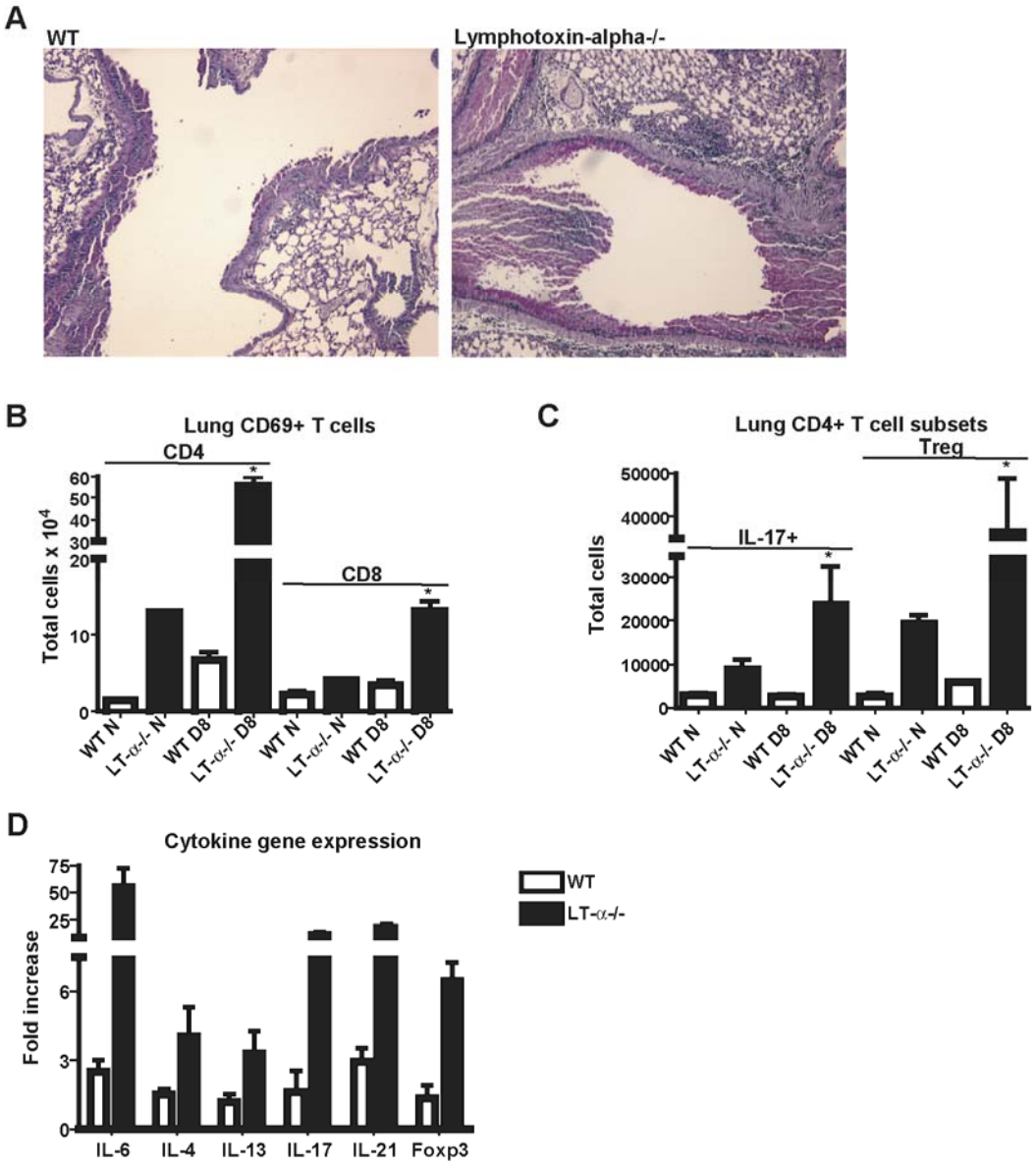

Figure 3.6. Lymphotoxin-alpha-/- mice exhibit lung pathology similar to CCR7-/- animals

A. Mucus production in lymphotoxin-alpha-/- mice was assessed by histology at day 8 post-RSV infection. Shown are representative samples from four mice/group, magnified 100x. **B.** Activated T cell recruitment to the lung was determined by flow cytometry at day 8 post-RSV infection. Data represent the mean ± SE from four mice/group. **P* < 0.05. **C.** Th17 and T regulatory cell recruitment to the lung was determined by flow cytometry at day 8 post-RSV infection. Total CD4+ IL-17+ and CD4+ CD25+ Foxp3+ cells are represented on the graph. Data represent the mean ± SE from four mice/group. **P* < 0.05. **D.** Expression of cytokine genes was assessed in lung samples by RT-PCR. Data is expressed as fold increase over uninfected mice and represents the mean ± SE from four mice/group. **P* < 0.05.

Chapter IV

The Role of CCR7 on Dendritic Cells and T Cells in the Immune Response to Respiratory Syncytial Virus

Abstract

The chemokine receptor-mediated migration of immune cells is critically important in dictating the immune response to pathogenic stimuli. CC chemokine receptor 7 is expressed on T cells and dendritic cells and permits the interaction of these cells in the lymph nodes for activation of adaptive immunity. Herein we investigated the role of CCR7 on DCs and T cells in immunity to respiratory syncytial virus, a ubiquitous virus that can cause severe pathology due to an inappropriate immune response. Although CCR7-/- DCs were unable to migrate to lymph nodes and activate T cells in transfer studies, in vivo DCs from CCR7-/- animals trafficked to the lymph nodes and promoted a dysregulated effector T cell response. Transfer of CCR7-sufficient T regulatory cells was ineffective at modulating the effector response in CCR7-/- animals, and investigation of CCR7-/- T cells themselves, through transfer into recombinant activating gene 1 (RAG1)-/- animals, revealed little involvement of T cells from either WT or CCR7-/- animals in the response to RSV. Thus, while DCs do not appear to be the pathological cell in our model, further investigation is needed to identify the mechanism of T cell effector dysregulation in CCR7-/- animals.

Introduction

Chemokines and their receptors promote proper cell migration that mediates immunity to infection, but can also play a role in promoting inflammation. CC chemokine receptor 7 and its ligands, CCL19 and CCL21, are an important homeostatic chemokine/chemokine receptor pair responsible for lymphocyte circulation through and organization into secondary lymphoid organs (136). In the absence of CCR7, animals develop tertiary lymphoid tissue in the lung, or bronchus associated lymphoid tissue (BALT) (189). While the mechanism of early BALT development in CCR7-/- mice is not fully clear, improper accumulation of T cells and dendritic cells in the lung, together with local expression of lymphoid tissue inducer molecules, such as lymphotoxin-α and β, likely contribute to the formation of pulmonary lymphoid aggregates (190). Previous work in our laboratory found that CCR7-/- animals infected with RSV had an enhanced, local effector T cell response in conjunction with increased mucus production. Herein we investigated the individual roles of DCs and T cells in the dysregulated response to RSV demonstrated in CCR7-/- animals, which could have important implications for the control of viral infections in tissue harboring tertiary lymphoid organs.

RSV is the top cause of hospitalization in infants under the age of two, promoting inflammatory cytokine release and mucus hypersecretion, and is also implicated in the exacerbation of established disease including asthma and chronic obstructive pulmonary disorder (96, 244). Immunopathology is a major feature of RSV-associated disease, and in particular, Th2 effector T cell cytokines drive inappropriate immune responses (56). Current treatment strategies aim to control immune-associated symptoms, and more preventative measures, such as a vaccine, have not been developed. Attempts have been

foiled by the complicated nature of protective immunity to RSV in individuals, and thus it is a priority in RSV research to understand the regulation of immune cells in response to infection.

To understand the dysregulated local effector T cell cytokine production in CCR7-/- animals infected with RSV, we performed transfer studies to elucidate particular roles of DCs and T cells in mediating the viral-induced pathology. We found that transfer of CCR7-sufficient DCs enhanced the RSV-specific effector cytokine response in lymph nodes, while CCR7-sufficent transfer of T regulatory cells failed to modulate effector cytokines in both the lymph node and lung of CCR7-/- animals. To specifically examine CCR7-/- T cell function, we transferred CCR7-/- T cells into recombinant activating gene 1 (RAG1)-/- animals and infected them with RSV. Although increased numbers of activated T cells were found in the lungs of RAG1-/- animals with CCR7-/- T cells, no effector-positive T cells were evident nor was there any notable lymph node development. Further studies are thus needed to clarify the mechanism of T cell dysregulation in CCR7-/- mice after RSV infection.

Results

CCR7 is required for DC migration and T cell activation CCR7 is the primary receptor by which DCs migrate to secondary lymph nodes upon pathogen activation. To determine if CCR7 is necessary for trafficking upon RSV infection, bone marrow-derived DC from WT and CCR7-/- mice were labeled with CFSE, pulsed with RSV and transferred intratracheally into WT mice. After 24 and 48 hours, CFSE+ cDCs were enumerated in lymph nodes. WT but not CCR7-/- cDCs migrated to lymph nodes at both

timepoints (Fig 4.1A). To assess T cell activation by DCs, we transferred OVA-specific CD4 T cells into WT mice intravenously, then administered either WT or CCR7-/- DCs that were pulsed with OVA and RSV intratracheally. After rechallenge with whole OVA, lymph nodes were harvested and restimulated with OVA to assess antigen-specific T cell responses. We found activation of T cells when animals were given WT but not CCR7-/- DCs (Fig 4.1B). This experiment was repeated in CCR7-/- animals to test whether the inflammatory environment played a role in the activation of T cells. Again, only WT DCs were able to prime OVA-specific T cells transferred into CCR7-/- animals, with the possible exception of IL-17 (Fig 4.1C). Collectively, this data indicates that CCR7 is necessary for DC migration to lymph nodes upon infectious stimuli for proper activation of T cells.

WT DC transfer into CCR7-/- animals enhances cytokine response In vivo, CCR7-/- animals infected with RSV displayed an enhanced RSV-specific cytokine response in lymph nodes, indicating that DCs and T cells are interacting. To examine DC trafficking in vivo, CCR7-/- animals were infected with RSV and recruitment of DC subsets were assessed by flow cytometry in lungs and lymph nodes. DCs were significantly enhanced in the lungs of CCR7-/- animals in comparison to WT mice, but in the lymph node, CCR7-/- DC accumulation was not different from WT mice (Fig 4.2A). On the other hand, plasmacytoid DC numbers in CCR7-/- mice in both tissues were comparable to WT mice (Fig 4.2A). The requirements for CCR7 on DCs in the transfer studies discussed in Figure 4.1 thus differ from that observed in CCR7-/- animals infected with RSV. However, T cell responses in CCR7-/- mice were dysregulated, so we transferred WT

DCs into CCR7-/- mice to determine if CCR7-sufficient DCs could restore proper interactions between CCR7-/- T cells and DCs in lymph nodes. When RSV-specific cytokines were measured, CCR7-/- animals given WT DCs displayed enhanced T cell responses compared to CCR7-/- mice without DC transfer (Fig 4.2B). Therefore, together with the flow cytometry data, CCR7-/- DCs do not appear to be the major cause of the pathologic phenotype observed in CCR7-/- animals after RSV infection.

T regulatory cells do not modify T cell responses in CCR7-/- mice T regulatory cells (Tregs) modify effector T cell responses to prevent excessive cytokine release and tissue damage. CCR7-/- Tregs were found to be functionally defective in vivo (189). To determine whether CCR7-sufficient Tregs could modify the effector response in CCR7-/- animals, 2 x 10^5 Tregs were transferred intravenously into CCR7-/- mice one day after infection with RSV. Tregs were obtained from transgenic mice expressing GFP in conjunction with Foxp3, thus allowing for easy identification and tracking of Tregs. Flow cytometric analysis revealed equal numbers of GFP+ Tregs migrating to lymph nodes in WT and CCR7-/- animals, but more Tregs were observed in the spleen of WT compared to CCR7-/- mice (Fig 4.3A). No Tregs were found in lungs of either strain. When lymph node cells were restimulated with RSV, no difference was observed in production of effector cytokines in either WT or CCR7-/- mice receiving Tregs (Fig 4.3B). Mucus and cytokine gene expression levels were then assessed in the lungs. Here, WT animals given Tregs had reduced expression of mucus-associated genes and IL-13, but enhanced levels of IL-17 and IL-21 compared to WT mice (Fig 4.3C left graph). Conversely, no major differences in gene expression levels were observed in CCR7-/- mice receiving Tregs

compared to CCR7-/- mice themselves (Fig 4.3C right graph). Tregs thus appear to have an affect at the local level in WT animals, but not in CCR7-/- animals, while the lymph node cytokine response was not modified in either WT or CCR7-/- mice receiving Tregs.

RAG1-deficient mice with and without T cells control RSV infection To further examine the role of T cells in the phenotype of CCR7-/- mice infected with RSV, CCR7-/- T cells were transferred into recombinant activating gene 1 (RAG1)-/- animals, which do not have endogenous T or B cells. After 21 days, RAG1-/- mice were infected with RSV. Surprisingly, lymph node development was virtually undetectable in RAG1-/- animals at day 8 post-infection. Assessment of T cell phenotype in the lungs showed that RAG1-/- animals reconstituted with CCR7-/- T cells had increased numbers of CD69+ CD4 T cells compared to mice receiving WT T cells (Fig 4.4A), but no T cells positive for effector cytokines were observed in mice reconstituted with either WT or CCR7-/- T cells (Fig 4.4B). Further examination of lung pathology by gene expression analysis revealed no major differences between RAG1-/- mice receiving T cells and animals without T cells in mucus-associated genes, but slightly better handling of viral replication when RAG1-/- mice received either WT or CCR7-/- T cells (Fig 4.4C). Thus, irrespective of T cell transfer, RAG1-/- animals appear to handle RSV infection efficiently and without significant pathology.

Discussion

Effector T cell generation is the key event that dictates an appropriate versus pathologic immune response to respiratory syncytial virus (RSV). Early attempts at

vaccine development revealed that Th2 effector T cell responses were highly detrimental, as individuals inoculated with a formalin-inactivated virus manifested severe symptoms upon natural RSV infection, characterized by high levels of Th2 cytokines and eosinophilia (60). An appropriate T cell response is the CC chemokine receptor 7 (CCR7)-mediated migration of naïve T cells and activated dendritic cells to lymph nodes. In the absence of CCR7, mice initiated an increased, dysregulated effector T cell response to RSV. Herein we investigated the role of CCR7 on subsets of DCs and T cells to discern the mechanism of RSV-induced, altered cytokine generation in CCR7-/- mice. Our studies ruled out a role for DCs as the major cell promoting the pathology observed in CCR7-/- mice, however we were unable to definitively show a defective mechanism associated with CCR7-/- T cells.

Secondary lymph nodes are highly organized, dynamic structures that reflect their key role in allowing the most efficient interactions between DCs and T cells to occur, an interaction dependent on the CCR7/CCL19-21 signaling pathway (136). A curious observation of the current studies is the fact that both DCs and Tregs require close proximity to naïve/differentiating T cells for proper activation (DCs) and regulation (Treg-mediated) to occur, and yet DCs appear to migrate appropriately and activate T cells in the lymph node while Treg migration is impaired and the effector response is dysregulated. Perhaps a compensatory mechanism is available to DCs that allows activation of T cells in the absence of CCR7 that does not exist for Tregs. From an evolutionary standpoint, it may be more imperative to generate an immune response that is dysregulated rather than allow no immune reaction at all.

It is likely that multiple factors contribute to the altered T cell response in CCR7-/- animals. For example, the compensatory mechanism of CCR7-/- DC interaction with T cells may permit inappropriate activation and cytokine production by T cells, in conjunction with an inability of Tregs to properly modulate the response. Also, there may be an intrinsic defect in CCR7-/- T cells. CCR7 participates in the intrathymic migration of developing T cells, both in the early differentiation stage of double negative T cell progenitors (252) and in the progression of immature double positive to single positive thymocytes, which corresponds to migration from the thymic cortex to medulla (253). In CCR7-/- animals, single positive T cells nonetheless matured normally and were exported from thymus; however, mature CCR7-/- T cells showed a potent capacity to promote an autoimmune phenotype, and this was directly attributed to their inability to accumulate in the medulla of the thymus during development (253). Improperly tolerized CCR7-/- T cells may thus be predisposed or primed for activation during immune stimuli, such as infection with RSV.

RAG1-/- animals were intended to be an ideal model for assessing CCR7-/- T cell differentiation and function upon RSV infection without the added complications of CCR7-deficiency on DCs or in the baseline inflammation observed in CCR7-/- animals. This model would also offer another opportunity to elucidate Treg function through transfer of CCR7-sufficient Tregs with Treg-depleted CCR7-/- T cells into RAG1-/- mice. However, RAG1-/- animals showed no evidence of a T cell response in our model, and in fact appeared to control viral infection irrespective of T cell presence. It is not clear whether the kinetics of RSV infection are altered in RAG1-/- animals or whether a particularly potent innate response compensated in these mice in the absence of

lymphocytes. Interestingly, a recent study assessed NK cell development in RAG1-/- mice and found that NK cell precursors derived from the liver in these animals instead of the bone marrow (254). NK cells control the early response to RSV infection, and perhaps the altered pattern of development in RAG1-/- mice allowed a more efficient anti-viral response by NK cells. Further experiments could assess the T cell response in RAG1-/- mice at earlier timepoints after RSV infection, or a stronger immune stimulus could be used, such as influenza, to begin to understand how T cells and Tregs respond to respiratory infection in the absence of CCR7.

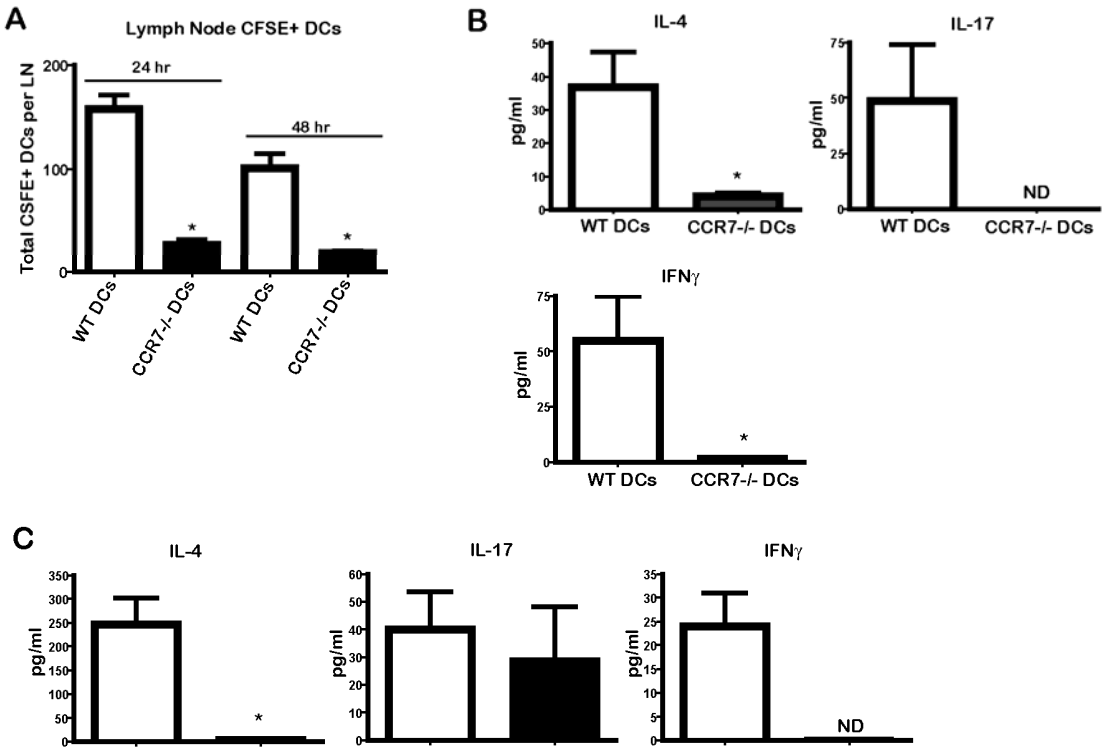

Figure 4.1 CCR7-/- DCs are defective in their capacity to migrate to lymph nodes and activate T cells

A. Ability of DCs deficient in CCR7 to traffic to the lymph nodes was assessed by flow cytometry. Bone marrow-derived, RSV-pulsed DCs were labeled with CFSE and transferred into the airways of WT mice. Lymph nodes were harvested and analyzed for CFSE+ cells at 1 and 2 days post-transfer. Data represent the mean ± SE from five mice/group. **P* < 0.05. **B.** Activation of T cells by CCR7-/- DCs was assessed using OVA-pulsed DCs transferred into WT animals that had received OVA-specific T cells. Graphs show lymph node cells restimulated with OVA to determine the antigen-specific T cell response. Data represent the mean ± SE from five mice/group. **P* < 0.05. **C.** T cell activation was assessed as in **B**, except the transfer was done into CCR7-/- animals to assess a role for the inflammatory environment of CCR7-/- mice. Data represent the mean ± SE from five mice/group. **P* < 0.05.

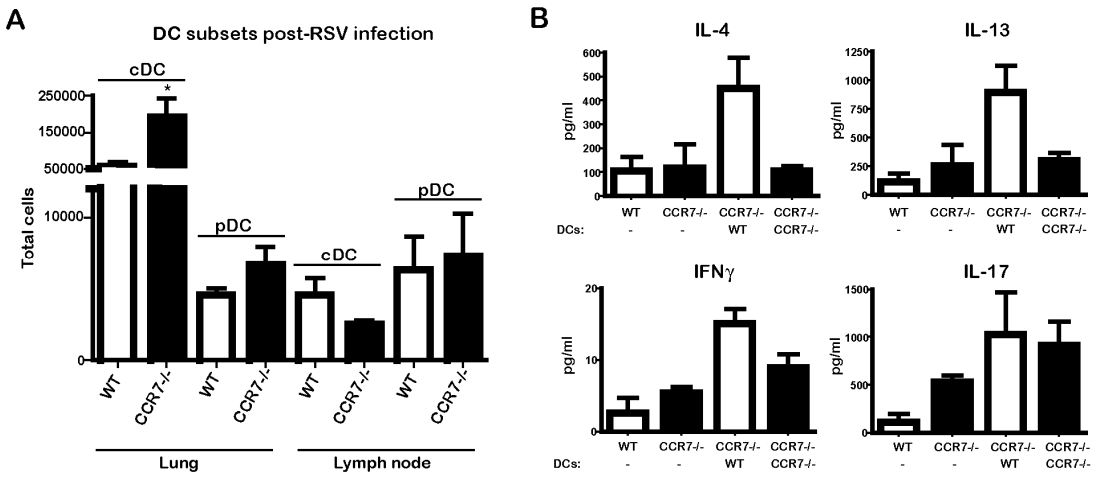

Figure 4.2 In vivo transfer of CCR7-sufficient DCs enhances effector cytokine production in CCR7-/- animals

A. CCR7-/- animals were infected with RSV and subsets of DCs recruited to lungs and lymph nodes were examined by flow cytometry. Shown are the total numbers of CD11b+CD11c+MHCII hi+ conventional (c)DCs and B220+CD11c+ plasmacytoid (p)DCs. Data represent the mean ± SE from four mice/group. **P* < 0.05. **B.** CCR7-sufficient DCs were transferred into CCR7-/- mice and RSV-specific cytokine response assessed by lymph node restimulation with RSV. X-axis shows strain of animal infected with RSV, with DC transfer indicated below. Data represent the mean ± SE from five mice/group.

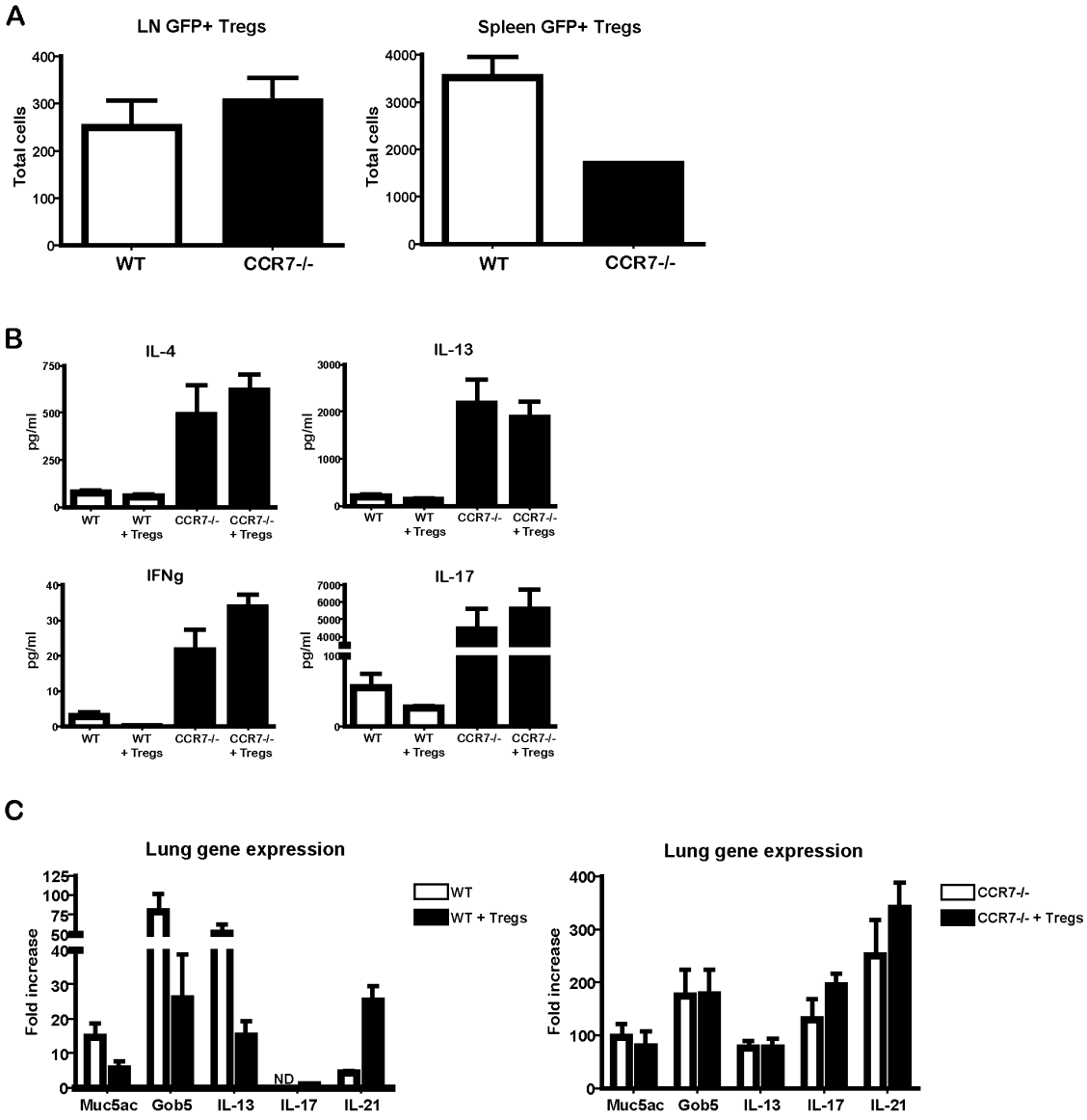

Figure 4.3. Transfer of CCR7-sufficient Tregs modulates mucus-associated gene expression in lungs of WT but not CCR7-/- mice

A. GFP+CCR7+ Tregs transferred into WT and CCR7-/- mice one day after RSV infection were tracked by flow cytometry. Shown are total Tregs identified in the lymph node (left) and spleen (right). Data represent the mean ± SE from four mice/group. **B.** RSV-specific cytokine responses were determined in Treg transfer groups by restimulating lymph node cells with RSV. X-axis indicates groups receiving Tregs. Data represent the mean ± SE from four mice/group. **C.** Gene expression was assessed by RT-PCR in WT and CCR7-/- mice receiving Tregs. Left graph shows gene expression analysis in WT mice and right graph shows gene expression analysis in CCR7-/- mice. Data represent the mean ± SE from four mice/group.

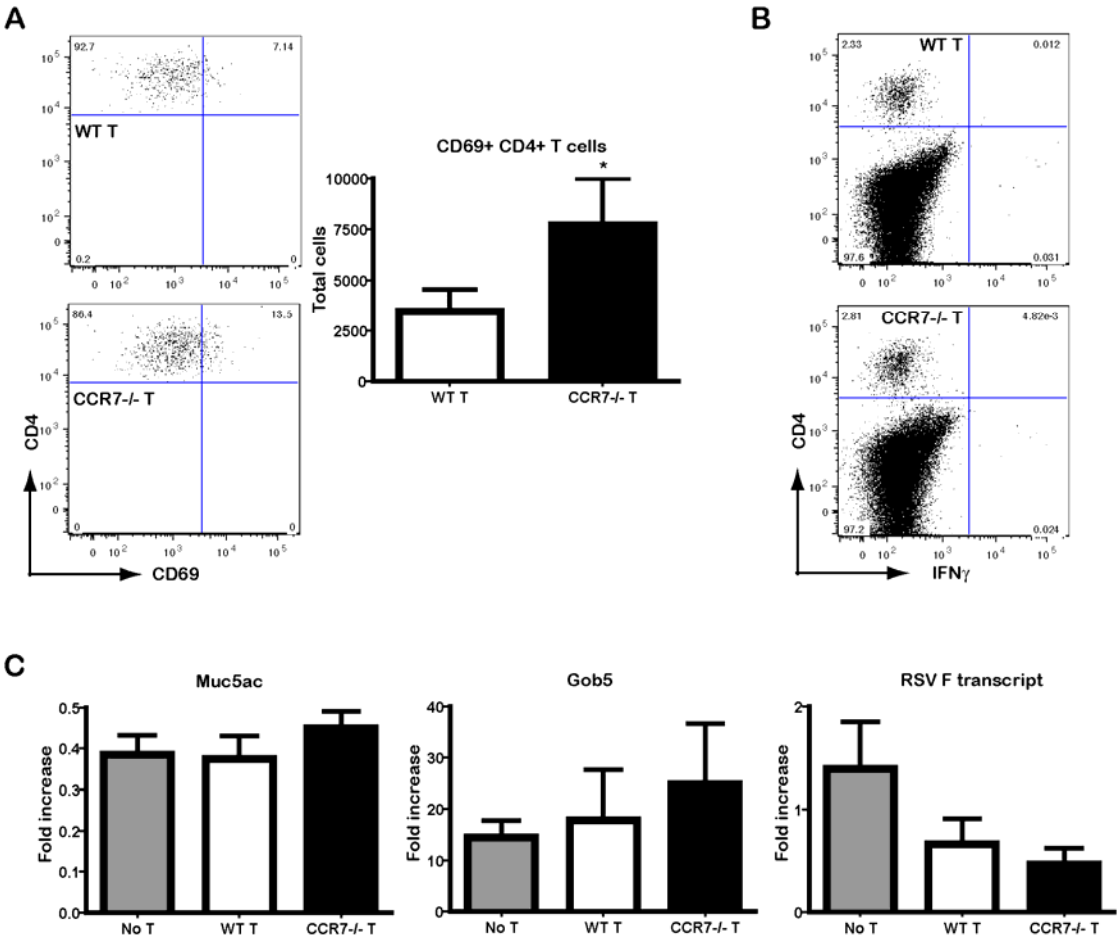

Figure 4.4. RAG1-/- animals with WT or CCR7-/- T cells infected with RSV have no discernable T cell response at day 8 post-infection

A. Activated (CD69+) T cells were assessed by flow cytometry in lungs of RAG1-/- animals with WT (top plot) and CCR7-/- (bottom plot) T cells and infected with RSV. Total cells are enumerated in the graph. Data represent the mean ± SE from five mice/group. **P* < 0.05. **B.** Effector T cell cytokines were examined by intracellular staining in the lungs of RAG1-/- mice with WT (top plot) and CCR7-/- (bottom plot) T cells and infected with RSV. Plots show IFNγ analysis and is representative of IL-17 and IL-4 staining. **C.** Mucus gene expression and RSV transcript levels were measured by RT-PCR in lungs of RAG1-/- mice with WT and CCR7-/- T cells and RAG-/- mice without T cells. Data represent the mean ± SE from five mice/group.

Chapter V

Discussion

The current studies investigated the role of two important chemokine receptors that function under homeostatic conditions as well as during inflammatory responses. Despite the highly redundant nature of the chemokine-chemokine receptor family, which underscores the necessity of proper immune cell trafficking, herein we have identified discrete functions for CCR6 and CCR7 on different cell populations in the immune response to respiratory syncytial virus. Not only does this help to elucidate protective and/or detrimental processes in RSV, but these studies are also broadly applicable to the migration of T cells and DCs in other immune settings, such as pulmonary viral infections, chronic inflammation and autoimmunity.

The differential use of chemokine receptors to enact particular immune outcomes is evidenced in the altered immune response to RSV observed in CCR6-/- mice. This arose due to differences in migration of dendritic cell subsets. The literature is ample on the different roles played by conventional (c) DCs and plasmacytoid (p) DCs during immune activation as well as under non-stimulatory conditions (209, 210). For example, pDCs appear to be less efficient at presenting antigen to T cells, a function primarily mediated by cDCs (255). It is likely that such functional differences reflect a differential use of chemokine receptors by cDCs and pDCs. Thus, we saw impaired recruitment of conventional, but not plasmacytoid, DCs to the lungs of CCR6-/- animals after infection

with RSV. While we suggest that the uninterrupted recruitment of pDCs in CCR6-/- animals promotes a protective phenotype, it is not at all clear the particular role pDCs play during RSV infection. Based on previous studies in our laboratory and others, it appears that pDCs can limit viral replication (51, 52). Clearly IFNα produced by pDCs can achieve this, and though RSV has been documented to severely limit Type I IFN production in a number of cell types, it is not always the case with pDCs (256, 257). Moreover, pDCs do not appear to use the RIG-I pathway as do cDCs in inducing Type I IFN, but instead use TLR7, which may affect the mechanism by which RSV modulates Type I IFN production (258, 259). It may be that pDCs can directly skew the T cell response by upregulating particular costimulatory molecules: both Th1 and Th2 effector responses have been generated through pDC activation and upregulation of CD40L and OX40L, respectively (260, 261). A role for pDCs in influencing the T cell response is also supported by studies indicating that pDCs appear to migrate both directly to inflamed tissue and to lymph nodes via high endothelial venules, but not from tissue to lymph nodes like cDCs (262, 263). Thus, the transport, presentation of antigen and activation of T cells may be the realm of cDCs whereas pDCs may act as a skewing factor, both through local production of IFNα, and through interaction with cDC and T cells in the lymph nodes. Future studies could further define the function of pDCs through transfer and tracking studies and co-culture with T cells with and without cDCs, as well as benefit from CCR6-/- animals as an in vivo model for exploring pDC/cDC ratios and their relation to immune outcome.

A model for cDC participation in the immune response to RSV is the CCR6-mediated recruitment of cDCs from the periphery. An interesting conundrum regarding

CCR6 is the lack of expression of this receptor on monocytes – the primary DC precursor migrating to tissue in response to inflammation. However, peripheral blood-derived monocytes have been found to traffic to tissue in a CCR6-dependent manner, and this study suggested that monocyte-derived DCs were migrating in response to other chemokines (such as CCL2/MCP1), but once in tissue were differentiating and localizing to CCL20 signals (264). In our studies, a notable feature of DCs present in lungs of animals infected with RSV was high expression of MHC Class II, whereas CCR6-/- DCs often had very little expression of this maturation mark. Thus, CCR6 may participate not only in localizing DCs in tissue but also in helping them to mature fully into DCs from monocyte precursors. Chemokines have been documented to affect cellular maturation, as for example, in CCR5 and CCL5-deficient animals, where DCs were unable to upregulate CCR7 upon infectious stimuli and therefore could not traffic to lymph nodes (265). Acquisition of CCR6 by migrating DCs may also play a role in their skewing abilities, as reconstitution of CCR6-/- animals with CCR6 sufficient WT DCs re-established the Th2 responses that were virtually absent in CCR6-/- mice. This could be through CCR6-induced upregulation of particular profiles of costimulatory molecules on DCs that might promote particular effector responses, such as OX40L-expressing DCs and preferential Th2 skewing (266). On the other hand, a cytokine milieu, including IFNα production by pDCs, in the absence of CCR6+ cDCs may simply promote Th1 skewing by cDCs present in CCR6-/- mice.

Despite a difference in CCR6-mediated migratory behavior between pDCs and cDCs, both cell types express CCR7 and migrate to its ligands when activated (267). Interestingly, in CCR7-deficient animals, neither pDC recruitment to the lung nor to the

lymph nodes was significantly altered from WT animals after RSV infection. And while cDC numbers were significantly enhanced in the lungs of CCR7-/- animals after RSV infection, cDC migration to lymph nodes also was not significantly altered. These results again support a differential use of chemokine receptors by pDC and cDC, but also suggest that under particular inflammatory conditions, such as that found in CCR7-/- animals after RSV infection, alternate chemokine receptor usage may be employed. Studies examining a range of chemokine receptor requirements in mediating dendritic cell migration found that in addition to CCR7, cDCs upregulated CXCR4 and migrated to CXCL12 upon maturing stimulus (267). CXCL12 is a lymphoid chemokine that participates in T and B cell localization within secondary lymphoid organs, and so this pathway may be co-opted by dendritic cells in the absence of functional CCR7.

The major defect observed in the CCR7-deficient animals infected with RSV centered not on DCs but instead on T cells. This makes sense considering the primacy of CCR7 in the homeostatic migration of naïve and central memory lymphocytes and the organization of secondary lymphoid tissue, however the fact that T cell activation was altered upon RSV infection simply due to trafficking defects, as evidenced in mixed chimeras, was an unexpected result. It may be that constant exposure to the RSV-induced inflammatory milieu by CCR7-/- T cells trapped in the lung permitted effector cytokine generation in a bystander way. Such an effect has been observed in studies modeling viral infections and using Type I interferons to induce phenotypic T cell activation independent of T cell receptor stimulation (268, 269). It may also be that presence of enhanced numbers of CCR7-/- T cells in the lungs induced local APC-T cell interactions that resulted in RSV-specific effector cytokine generation. It certainly appears that

memory T cells residing in tissue respond to pathogen insult in a similar manner, albeit with less reliance on APC costimulation and no period of proliferation (270). Similarly, the impaired migration pattern of CCR7-/- T cells may both permit local T cell activation and even alter the requirements of these T cells for activation. We attempted to determine whether RSV-specific responses were generated in the lung in WT chimeras reconstituted with CCR7-/- bone marrow by coculturing lung CD4 T cells from previously sensitized animals with RSV-pulsed DCs. However, cytokine detection was minimal, if any. Future studies could make use of an RSV-specific tetramer that would identify virus-specific T cells, thus confirming in mixed chimeras whether local T cell production of effector cytokines by CCR7-/- cells is RSV-specific or indeed a bystander effect of RSV-induced pulmonary inflammation. Antigen-specificity could have consequences for tertiary lymph node formation, as discussed below.

The use of CCR7-/- animals as a model for exploring local T cell activation versus secondary lymph node priming of the immune response was a useful tool in elucidating the functional consequences of impaired trafficking. Though no chemokine-receptor deficient human disorders have been identified, the development of bronchus-associated lymphoid tissue (BALT) in CCR7-/- animals is relevant to human disease. Ectopic or tertiary lymphoid organs (TLO) have been noted to develop in chronically inflamed and autoimmune-targeted organs, including thymus, pancreas and lung (271-273). Much work has been done to characterize the composition and origin of TLO development, and though the initiating stimulus remains unknown (and it is theorized that constant antigen stimulation is responsible for TLO development), evidence suggests that functional germinal center reactions take place in TLOs (274). This indicates a potential

role for TLOs in responding to exogenous antigen stimulation, such as upon infectious stimuli. Recent studies investigated the role of TLOs (BALT) during pulmonary infection, and while one group reported a protective, even advantageous role for BALT in clearing influenza virus (233), a second study demonstrated a deleterious role for BALT by promoting inflammation and harboring latent gamma-herpes virus (234). Our study supports the latter in that we observed increased inflammation and mucus production in the lung, and additionally noted a sustained, activated local T cell response, particularly in T cell derived-IL-17. This implies that handling of infectious stimuli by local TLOs could have detrimental effects on local tissue architecture and integrity via local enhancement of proinflammatory cytokines.

The literature describing and investigating the nature of TLOs use secondary lymph node organ (SLO) development as the model for how TLOs might develop and function. This is a useful approach, and has elucidated several features of TLOs that differ from SLOs, including the apparent lack of afferent lymphatics in TLOs (193); consequently, it is unknown how dendritic cells transport antigen to T cells to initiate local responses. This is consistent with a paucity of data confirming T cell priming in TLOs, although Moyron-Quiroz et al showed in situ T cell proliferation in induced BALT structures after influenza infection, suggesting that interaction of DCs and T cells occurs (233). CCR7 knockout animals may be a useful model to further elucidate the composition and handling of immune stimuli via BALT. Our laboratory has access to RSV that expresses GFP, and this could be used to identify RSV+ DCs in the lung that could be sorted out and cocultured with RSV-sensitized lung T cells to determine whether proliferation and effector generation occurs. Use of fluorescent microscopy

could also localize the RSV-carrying DCs within the lung – potentially to BALT structures. Furthermore, T cell subsets (naïve, memory) and the overall immune response could be characterized upon rechallenge of CCR7-/- animals with RSV. In models of pulmonary viral infection, memory responses occurred both rapidly via local effector cytokine production in affected tissue, as well as in secondary lymph nodes where memory cells became activated, proliferated and trafficked to inflamed tissue for renewed effector functions (275). The concurrent presence of TLOs – acting similarly to SLOs – in infected tissue could therefore influence the intensity of secondary responses; tissue harboring TLOs might be disposed to further pathologic insult upon repeated infection.

In addition to elucidating the importance of lymph node versus local immune priming, requirements for CCR7 on DCs and T cells during RSV infection were investigated in CCR7-/- animals. Unexpectedly, RSV-specific T cell responses occurred in the lymph nodes of CCR7-/- mice, however it is unclear whether this response was actually generated in the lymph nodes: DC trafficking to the lymph nodes was intact, but T cell numbers were significantly reduced in lymph nodes of CCR7-/- mice. The migration of naïve T cells into lymph nodes via high endothelial venules is a process requiring several molecules expressed on the T cell in addition to CCR7, such as selectins and integrins. These molecules are required in each of the steps documented to occur during T cell extravasation from the blood: rolling, activation, adhesion and transmigration (276). While CCR7 signaling appears to activate integrins, allowing for firm adhesion and transmigration, CD62L signaling may perform this function in the absence of CCR7 (277-279); a proportion of naïve CCR7-/- T cells may therefore enter high endothelial venules strictly through integrin-mediated pathways. While these T cells

may be primed by CCR7-/- DCs in the lymph nodes, it is likely that the observed RSV-specific responses are also a result of passive migration of effector T cells from the lung to the lymph nodes. Further elucidation of this issue would examine T cell proliferation in the lymph nodes, which would indicate whether T cell priming was occurring.

Despite the significantly diminished T cell numbers in the lymph nodes of CCR7-/- animals, the effector cytokine response was elevated and clearly dysregulated. Studies have demonstrated that within the lymph nodes, naïve T cells are highly motile, and this is required for T cell localization to T cell zones and optimal interaction with antigen-bearing DCs (134, 280). Naïve T cell motility in lymph nodes was found to depend primarily on the CCR7-CCL19/21 pathway instead of on integrins (281). The disrupted organization and cellular interaction in the lymph node likely contributes to the enhanced cytokine response, particularly with respect to Tregs. Not only were Treg numbers reduced in lymph nodes of CCR7-/- mice, but appropriate signals guiding Tregs in proximity to effector cells were lost, thus hindering their ability to function. In this respect, it is unclear why transfer of CCR7-sufficient Tregs had no effect on the RSV-specific overproduction of cytokines by CCR7-/- lymph node T cells. Splenic Tregs from naïve mice were transferred into CCR7-/- mice one day after RSV infection, and perhaps the lack of antigen specificity prevented their ability to fully function. Indeed, the vast majority of transferred Tregs were recovered from spleens of infected animals instead of lymph nodes, suggesting that few were responding to infection. Further experiments could isolate Tregs from animals previously sensitized to RSV to determine whether antigen-specificity is a key determinant of Treg function in our model.

Alternatively, the lung phenotype in WT mice reconstituted with Tregs showed reduced expression of mucus-associated genes, including IL-13, which helps to validate the functionality of the transferred Tregs. Because WT animals have endogenous functioning Tregs, the full range of function of the transferred Tregs, i.e., no reduction of RSV-specific cytokines in the lymph nodes was observed, may not be evident in these mice. It would be useful to transfer GFP+ Tregs into Treg-depleted, diphtheria-toxin-Foxp3 mice, and perform a comprehensive analysis of how the Tregs are modifying the response to RSV, including assessment of T cell proliferation and antigen-specific cytokine production in the lymph node, as well as DC and lung phenotype. This would clearly help in determining whether the Tregs transferred into CCR7-/- animals are simply not functioning, or perhaps whether increased numbers of Tregs are needed to modulate the response. As discussed further below, Tregs encountering the inflammatory milieu of CCR7-/- mice may also be altering their phenotype through upregulation of molecules associated with effector T cells, such as RORγt, thus altering their ability to suppress.

In the lungs of intact CCR7-/- animals, it is puzzling that large numbers of endogenous Tregs coexisted with Th1, Th2 and Th17 effector cytokines, suggesting that local Tregs were not controlling effector responses. This may reflect the complicated task forced by Foxp3 to perform in the local environment of CCR7-/- mice. The lymph node compartment offers a segregated, non-inflammatory milieu where naïve T cells integrate signals received by the antigen-presenting DC and differentiate accordingly, both into effector and Treg subsets. However, when immune activation is occurring locally, as in CCR7-/- animals, naïve T cells are integrating an array of pro-inflammatory signals from

the lung environment that may alter the regulatory pathways mediated by Foxp3 in induced Tregs. Interestingly, two recent studies found direct interaction of Foxp3 with the Th2-associated transcription factor, IRF4, and the Th17-associated transcription factor, STAT3, that was necessary for modulating Th2 and Th17 responses (250, 251). These studies used gene-specific ablation in Tregs and so assessed the steady-state consequences of natural Tregs deficient in either IRF4 or STAT3. This mechanism of Treg function may have different consequences for induced Tregs developing in a local inflammatory environment: interaction between Foxp3 and STAT3 in CCR7-/- induced Tregs may result in Th17 development instead of Treg suppression of Th17-specific cytokines. Indeed, other studies have documented the co-expression of Foxp3 with T-bet, GATA3 and RORγt in differentiating cells (184, 282, 283), as well as phenotype switching from suppressive Tregs to inflammatory Th17 cells (185). These studies suggest an initial plasticity between regulatory and effector cell development that requires environmental cues to ultimately inform functional outcome. Thus, while Foxp3 may be expressed in CCR7-/- "Tregs", it may not correlate with traditional suppressive function in the lung environment of these animals.

The enhanced production of effector cytokines in the lungs of CCR7-/- animals was proportional to WT animals with respect to IFNγ and IL-4, such that a predominant Th1 response was initiated. However, this was not the case with IL-17. CCR7-/- animals produced levels of this cytokine comparable to levels of IFNγ, whereas WT animals produced levels of IL-17 comparable to that of IL-4. This suggests that in a dysregulated system, IL-17 might be preferentially induced, perhaps in a manner similar to that seen in autoimmune disorders where IL-17 has been shown to be a key pathological cytokine.

Likely this has to do with the developmental relationship between Th17 and Tregs. In our studies, both IL-17 and Foxp3 expression as well as Tregs and Th17 cells themselves were significantly enhanced in CCR7-/- mice, even prior to RSV infection (which was not the case with Th1 and Th2 cytokines or cells). Th17 and Treg cells may have coevolved as an early immune mechanism to prevent microbial invasion at mucosal surfaces but maintain tolerance to commensal organisms. The various functions evidenced by Th17 effector cytokines support this concept – such as recruitment of neutrophils, the prototypical innate responder cells to pathogens – as does the fact that innate cells, such as NK cells and lymphoid tissue inducer cells, as well as adaptive cells with more primitive antigen recognition abilities, such as gamma/delta T cells, produce IL-17 and IL-22 (284, 285). Thus, the conserved mechanism of a Th17/Treg balance meant for mucosal homeostasis goes awry in inflammatory settings, such as that seen in autoimmunity and local immune priming via ectopic lymphoid tissue, allowing unrestrained production of Th17 effector cytokines.

While multiple lines of evidence show the pathology associated with many autoimmune disorders to arise from Th17 cytokines (170, 286), a pathologic role for IL-17 in our model is unclear, as is the role of IL-17 in viral infections in general. Several studies have shown that the innate antiviral cytokines, IFNα and β, inhibit Th17 development (287, 288). Similarly, Hashimoto et al infected mice with RSV and demonstrated augmented IL-17 levels in mice deficient in STAT1, the transcription factor governing Type I IFN production (214). In this model, animals exhibited enhanced mucus production and airway hyperreactivity, and though a direct role of IL-17 in the observed pathology was not established, a separate group found that IL-17 was able to

activate mucin gene expression (289). In other models of viral infection, IL-17 was found to block cytotoxic T cell activity, thus enhancing survival of virally infected cells (290), and to promote neutrophil recruitment, which contributed to acute lung injury during influenza infection (291). Together with the literature, our studies in which both Balb/c and C57BL/6 animals exhibited very little IL-17 production in response to RSV suggest that Th17 responses are detrimental for viral infection. It therefore seems plausible that IL-17 in CCR7-/- animals may be responsible for enhanced mucus production and/or inflammatory cell recruitment. Attempts to block IL-17 in CCR7-/- animals have proven difficult given the high levels of IL-17 present in naïve mice and after RSV infection. Furthermore, IL-13 is also enhanced in CCR7-/- mice and this cytokine has traditionally been associated with mucus production (292). Thus, it may be useful to block these cytokines in another model, such as STAT1-/- animals, to better clarify the contributions of IL-13 and IL-17 to RSV-associated pathology.

Together, these studies assessing the roles of CCR6 and CCR7 during RSV infection elucidate important requirements for these receptors on DCs and T cells, and also show that trafficking of cells can affect the local cytokine milieu, and this ultimately dictates immune outcome. In the absence of CCR6+ cDCs, the environment of the lung promoted innate handling of the virus; thus, while a competent, IFNγ-dominant RSV-specific T cell response was generated in the lymph nodes of CCR6-/- mice, this effector response was not needed. This was evident in the significantly diminished numbers of IFNγ+ T cells in the lungs of CCR6-/- compared to WT mice, despite the equivalent numbers of recruited T cells. Similarly, the altered ability of DCs and T cells to migrate properly in CCR7-/- mice facilitated development of local lymphoid-like tissue that

promoted IL-17 and mucus production in the lung upon viral infection. When trafficking patterns were reestablished in CCR7-/- chimeras, local T cell responses were likewise attenuated. These studies have important implications for chronic inflammatory and autoimmune disorders, in that altered migration of cells (due to persistent antigen stimulation, for example) can generate inflammatory cytokine release that may further enhance and promote local pathology.

These studies additionally suggest a key role for innate cells, particularly an appropriate balance between cDC and pDC, in mediating an appropriate immune response to RSV. Similar to CCR6-/- mice, RAG-/- animals reconstituted with T cells and infected with RSV did not show evidence of a T cell response: lymph nodes were absent, and no CD4+ effector T cell cytokines were present in the lung. These animals perhaps testify to the value of the innate response in both allowing for RSV clearance and diminishing pathology, however, it would be interesting to rechallenge CCR6-/- animals with RSV to determine whether memory responses are generated and how secondary viral challenge is handled. This could shed light on the relative necessity/requirements of acute T cell responses to subsequent development of recall responses, which could then aid in vaccine strategies.

Materials and Methods

Mice. Balb/c, C57BL/6, DO11.10, OTII, RAG1-/- and lymphotoxin-α-/- mice were purchased from Jackson Laboratories. CCR6-/- mice on a Balb/c background were originally provided by Dr. Sergio Lira (New York, NY) and a breeding colony subsequently established at the University of Michigan. CCR7-/- mice were originally provided by Dr. Martin Lipp (Berlin, Germany) and a colony established at the University of Michigan. All animal work was performed in accordance with the University of Michigan Committee on Use and Care of Animals policy.

Respiratory Syncytial Virus. Our laboratory utilizes the antigenic subgroup A strain of RSV, referred to as Line 19. This isolate was obtained from a sick infant at the University of Michigan (293) and has been demonstrated in animal models to mimic human infection by stimulating mucus production, promoting airway hyperreactivity and increasing IL-13 production (294). All mice were infected with $5x10^4$ PFU of RSV/mouse via intratracheal injection.

Bone marrow chimeras. WT or CCR7-/- animals were lethally irradiated with 1000 Rads from a Cesium source, and within 24h were intravenously injected with 5×10^6 bone marrow cells from WT or CCR7-/- mice. Mixed chimeras were generated by irradiating congenic CD45.1 mice and reconstituting them with equal numbers of GFP-expressing WT and non-GFP CCR7-/- bone marrow cells. After 8 weeks, chimeras were infected with RSV.

Antibody administration. 50μg of rat anti-mouse CCL20 Ab (R&D), as well as control rat IgG1 (R&D), was administered to Balb/c mice either intratracheally at the time of

RSV infection, and one day after, until day 2, or intraperitoneally 2 hours prior to infection, and every other day thereafter until day 6, when mice were sacrificed.

Histology. Left lobes from infected mice were removed, perfused with 10% formalin and placed in fresh formalin for an additional 24-48 hours. Routine histologic techniques were used to paraffin-embed this tissue, and 5μm sections of whole lung were stained with periodic acid schiff to detect mucus production.

Real-time Taqman PCR. The smallest lobe was removed and homogenized in 1 ml of Trizol reagent (Invitrogen). RNA was isolated as described (Invitrogen), and 5μg was reverse-transcribed to assess gene expression. Detection of cytokine mRNA in lung samples was determined using pre-developed primer/probe sets (PE Biosystems, Foster City, CA) and analyzed using an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA). GAPDH was analyzed as an internal control and gene expression was normalized to GAPDH. Fold changes in gene expression levels were calculated by comparison of the gene expression in unchallenged mice, which were assigned an arbitrary value of 1. Additional analysis was performed by assigning WT challenged mice a value of 1 and comparing this with CCR6-/- or CCR7-/- challenged samples.

Flow cytometry. Lungs were harvested from infected mice, minced with surgical scissors, and digested with 1mg/ml of collagenase (Roche) for 45min. Samples were dispersed with an 18-gauge needle to obtain single cell suspensions. Lymph nodes were also removed and single cell suspension obtained by passing nodes through a 40μm mesh filter. Cells were resuspended in PBS containing 1% BSA and 0.5% sodium azide (flow buffer). Cells were Fc-blocked (ebioscience) for 10 minutes, then stained for 20 minutes

with fluorophore-conjugated antibodies purchased from BD Pharmingen or eBioscience. Cells were washed 2x and fixed in 4% formalin overnight. For intracellular cytokine staining, cells were first stimulated for 6hr with 50ng/ml of PMA and 10mM of ionomycin, with inclusion of protein transport inhibitor. Routine intracellular staining was performed using the reagents and protocol from BD Biosciences. Analysis was conducted using FlowJo software.

Lymph node restimulation. Mediastinal and cervical lymph nodes were harvested and single cell suspensions obtained by passing nodes though a 40μm nylon mesh filter. Samples were counted and plated in duplicate at $1x10^6$ cells per well. Half the wells for each sample were restimulated with either $4x10^4$ PFU RSV or 1μg/ml of anti-CD3 and anti-CD28 and the other left unstimulated. Cells were incubated at 37°C for 24 hours and supernatants collected for analysis on the BioRad Bioplex 200 system according to the manufacturer's protocol. Kits (Biorad) containing antibody beads to Th cytokines (IL-17, IFNγ, IL-4, IL-5, IL-13) were used to assay for antigen-specific cytokine production in each of the samples.

Airway Response. Airway hyperreactivity was determined using whole body plethysmography with ventilation of anesthetized mice. Briefly, mice were anesthetized with sodium pentobarbital and intubated via cannulation of the trachea with an 18-gauge metal tube. Mice were ventilated with a Harvard pump ventilator (tidal volume, 0.4 ml; frequency, 120 breaths/minute; positive end-expiratory pressure, 2.5 to 3.0 cm H_2O; Harvard Apparatus, Holliston, MA) and challenged intravenously with 0.2 mg of methacholine. Resistance was calculated by dividing the change in pressure (Ptp) by the

change in flow (F) (Ptp/F; units = cm H_2O/ml/second). The peak airway resistance was recorded as a measure of airway hyperreactivity.

Viral plaque assay. Whole lungs were harvested and ground with sand using a mortar and pestle. Samples were spun 2x and supernatants serially diluted onto an ~90% confluent monolayer of Vero cells. Samples were incubated at 37° with gentle rotation for 2h, then infected supernatants were removed and replaced with 0.9% methylcellulose. After incubation at 37° for 5 days, methylcellulose was removed, replaced with methanol, and incubated at -80° for 1h. After removal of methanol, samples were stored at -80° until plaque development. Plaques were developed using a modified ELISA protocol. Briefly, cells were blocked for 1h at 37° with 25% Blotto (milk powder diluted in PBS), washed and incubated for 1h at 37° with goat anti-human RSV polyclonal Ab (Chemicon International). Cells were washed again and incubated for 1h with horseradish peroxidase-conjugated anti goat/sheep IgG (Serotec). Cells were washed and incubated at room temperature with chloronaphthol and viral plaques counted by eye.

Bronchoalveolar lavage chemokine measure. BALF was collected from sacrificed mice using an 18-gauge needle containing 1ml of PBS. Resulting cell suspensions were centrifuged and supernatants collected for chemokine analysis. CXCL10 and CCL5 were measured by Bioplex using antibody beads purchased from Biosource, and CCL20 was determined by standard sandwich ELISA, using antibodies obtained from R&D.

Dendritic cell transfer. Dendritic cells were obtained and differentiated from bone marrow. Briefly, bone marrow cells were flushed from the femurs and tibiae with sterile media. A single cell suspension was obtained by filtering cells though a 40µm nylon mesh filter. Cells were plated at a concentration of 5.0×10^6 cells/10mls in media

containing 10 ng/ml murine GM-CSF (R&D systems, Minneapolis, MN). Cells were cultured for 10 days with media replenished at days 3, 6 and 8 with fresh GM-CSF. For tracking experiments, differentiated DCs were pulsed with RSV for 24h, labeled with CFSE and 10^6 transferred intratracheally into animals. For immune assessment, $5x10^5$ DCs were transferred intratracheally and mice infected with RSV intranasally.

OVA-specific T cell transfer. Spleens were harvested from OTII animals and processed into a single cell suspension using a 40μm mesh filter. Red blood cells were lysed and resulting cells incubated with anti-CD4 magnetic beads (Miltenyi Biotec) to positively select CD4+ T cells. 10^6 CD4 T cells were intravenously transferred into animals, and bone marrow derived DCs pulsed for 2h with 1μg/ml of OVA peptide (323-339, Peptides International).were transferred intratracheally. After 4 days, animals were challenged with whole OVA administered intratracheally, and 6 days thereafter sacrificed for analysis.

T regulatory cell transfer. Spleens were harvested from Foxp3-GFP mice and processed into a single cell suspension using a 40μm mesh filter. Samples were enriched for CD4+ T cells by using biotin conjugated antibodies and anti-biotin magnetic beads (Miltenyi Biotec) to deplete erythrocytes, MHC Class II+ cells, CD8+ cells and B220+ cells. Foxp3+ cells were then isolated by FACS by selecting GFP (Foxp3)+ APC-CY7 CD4+ cells. $2x10^5$ Tregs were intravenously transferred into animals one day after infection with RSV.

RAG1-/- T cell transfer. WT or CCR7-/- T cells were positively selected from spleens of animals by MACS® cell separation (Miltenyi Biotec) using CD92.2 microbeads.

$5x10^6$ T cells were transferred intravenously and 21 days later, animals were infected with RSV.

In vitro co-cultures. Bone marrow-derived DCs were pulsed for 2h with either $4x10^4$ PFU RSV or 1μg/ml of OVA peptide (323-339, Peptides International). CD4+ T cells were isolated by positive selection using MACS® cell separation (Miltenyi Biotec) either from the lymph nodes of mice 8 days post-RSV infection, or from the spleens of naïve DO11.10 mice. DCs and CD4 T cells were co-cultured in fresh media at ratios of 1:20 DC:T for lymph node T cells and 1:10 DC:T for spleen DO11.10 T cells for 24 or 48 hours.

Statistics. Data was analyzed using Prism GraphPad software. Statistical significance was determined by one-way ANOVA with Newman-Keuls post test or Students T test. Significant differences were regarded as $p < 0.05$.

Appendix: Epigenetic Modifications in Human-Derived DCs

Introduction

Dendritic cells (DCs) undergo rapid phenotypic change upon stimulation of surface and intracellular Toll Like Receptors (TLRs), including upregulation of the lymph node homing receptor, CCR7, and production of cytokines such as IL-10, IL-12 and IFNβ. Pathogens are known to interfere with these signatures of DC maturation, and respiratory syncytial virus (RSV) in particular has been shown to antagonize TLR-mediated IFNβ production in dendritic cells. We thus sought to investigate the regulation of CCR7 and IFNβ in human DCs after activation by RSV and TLR agonists. We found that DCs stimulated with polyI:C and LPS, TLR3 and TLR4 agonists, respectively, significantly upregulated CCR7 after 6 and 24 hours. PolyI:C was the most potent upregulator of IFNβ at both 6 and 24 hours, with LPS upregulating this cytokine only at 6 hrs. Though other RSV-induced genes were activated, DCs stimulated with RSV failed to upregulate CCR7 or IFNβ. Analysis of activating (H3K4) and silencing (H3K27) histone modifications at the promoters of CCR7 and IFNβ showed, compared to unstimulated DCs, high H3K4 methylation of CCR7 and IFNβ upon PolyI:C stimulation, whereas LPS showed high H3K4 at CCR7, but high H3K27 at IFNβ. Methylation marks after RSV stimulation did not differ markedly from unstimulated DCs, suggesting that RSV may interfere with cellular machinery that controls gene expression, potentially delaying DC maturation.

Results and Discussion

Respiratory syncytial virus (RSV) represents a significant worldwide burden as the primary cause of bronchiolitis in infants and the elderly, a result of an inappropriate immune response. Furthermore, resolution of RSV infection is incomplete and re-infections occur frequently throughout an individual's life. An interesting dichotomy exists between the ability of RSV infection to elicit a pathologic immune response and mechanisms by which the virus evades immune detection (although these features may not be mutually exclusive). Clearly, further studies are required to understand the dynamics between virus and host in mediating proper immunity to RSV infection.

Previous work in our laboratory found an important role for dendritic cells (DCs) in altering the immune response to RSV infection. Mice that had received RSV-infected DCs down the airway and subsequently challenged with RSV mounted a predominant Th2 T cell response, whereas mice simply infected with RSV mounted a predominant Th1 T cell response (Lukacs et al, unpublished). Other studies have found RSV to directly interfere with the function of DCs by abrogating DC production of IFNβ, an important anti-viral cytokine (82, 83). RSV may also interfere with dendritic cell trafficking, as DCs were enhanced in the lungs of individuals up to 8 weeks after acute infection (46). The major trafficking receptor on DCs is chemokine receptor 7 (CCR7), which allows the DC to traffic into the lymph nodes upon activation by pathogenic stimuli. Together, these studies demonstrate a critical role played by dendritic cells in mediating the immune response to RSV, evidenced by the virus' ability to subvert key features of DC maturation.

Herein we examined the regulation of IFNβ and CCR7 in DCs upon infection with RSV to determine how the virus might modulate the maturation of DCs at the level

of chromatin. Epigenetic modification of genes has long been the mainstay of developmental biology, but has recently been found to play a role during immune cell differentiation. For example, DCs from septic mice were impaired in their ability to produce IL-12, and this was long after the septic episode, which was a result of a silencing modification at the promoter region of IL-12 (295). Our studies found a diminished expression of IFNβ and CCR7 in DCs during acute stimulation with RSV (Fig A1), as well as a histone methylation pattern that was unchanged from unstimulated cells (Fig A2), indicating that appropriate signaling to the transcriptional machinery upon stimulation by RSV is impaired. These data suggest an epigenetic mechanism by which RSV may impede the maturation of DCs for appropriate activation of T cells.

In addition to examining acute activation by RSV, these studies investigated secondary challenge of DCs both with RSV and LPS. The latter was intended to mimic bacterial co-infection, which is a serious problem for individuals infected with RSV and respiratory viruses in general (3). Furthermore, we were interested in how DCs respond to rechallenge with RSV, as long-term immunity does not develop in individuals, which may be a result of improper DC activation. Interestingly, when DCs were restimulated with RSV or LPS after primary RSV stimulation, a prominent silencing histone mark was observed at the promoters of IFNβ and CCR7 (Fig A3). These data suggest that acute infection with RSV may promote a state of inactivity in DCs, maintaining the DC in an immature state, but upon secondary infection, the virus actively shuts down these important genes.

While these studies are intriguing, a caveat is the complicating aspect of use of human cells for epigenetic analysis. Immune experience is incredibly variable between

individuals and so consistent results are a problem. Future studies will move to murine DCs and/or monocyte cell lines to verify the conclusions herein.

Materials and Methods

DC culture. Human peripheral blood mononuclear cells were obtained from blood of healthy human donors and isolated by Ficol gradient. Lymphocytes were depleted using biotinylated antibodies to CD3 and CD19, followed by anti-biotin conjugated magnetic beads (Milenyi). The negative fraction was further enriched for monocytes by positive selection using anti-CD14 magnetic beads (Miltenyi). Monocytes were cultured in 50ng/ml of GM-CSF and IL-4 for 6 days. At this time, DCs were stimulated with either 1ug/ml of LPS, 2.5ug/ml of PolyI:C, 0.5 MOI of RSV or left unstimulated.

RT-PCR. DCs were centrifuged and resuspended in 1ml of Trizol reagent (Invitrogen). RNA extraction was performed as described (Invitrogen). For gene analysis, approximately 1ug of RNA was reverse transcribed, and 1ul of cDNA was used in each analysis. Detection of cytokine/chemokine mRNA in each sample was determined using pre-developed primer/probe sets (PE Biosystems) and analyzed using an ABI Prism 7500 Sequence Detection System (Applied Biosystems). GAPDH was analyzed as an internal control and fold changes in gene expression levels were calculated by comparison to gene expression in unstimulated DCs, which was assigned a value of 1.

Chromatin Immunoprecipitation (ChIP). DC protein and DNA were crosslinked with formaldehyde added directly to culture. Cells were subsequently washed and lysed with SDS buffer. Samples were sonicated to fractionate the DNA into 200-1000bp fragments and debris removed by centrifugation. Supernatants were pre-cleared with protein A

agarose/salmon sperm DNA slurry, then samples were split and immunoprecipitated overnight with antibodies to trimethyl histone 3 lysine 4, trimethyl histone 3 lysine 27, and rabbit IgG controls. Antibody-histone complex was collected by incubation with protein A agarose/salmon sperm DNA slurry, then subjected to a number of salt washes to remove unbound protein/antibody. After elution of histone/DNA complex, crosslinking was reversed and protein digested with proteinase K. DNA was then purified by phenol chloroform according to standard procedure. Primers for the promoter regions of CCR7 and IFN-beta were developed using Primer Express, and samples were analyzed for presence of these gene regions by quantitative PCR. Enrichment of DNA for the promoter regions in each sample was determined by comparing the antibody-precipitated sample values to the total input DNA for each sample.

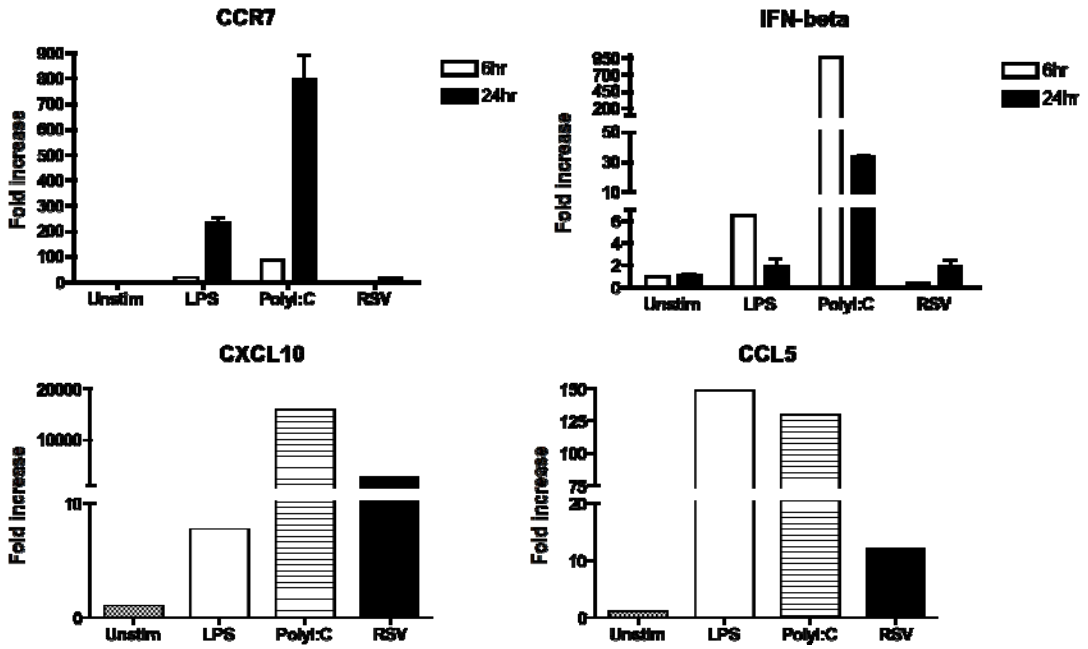

Figure A1. Dendritic cells stimulated with RSV do not upregulate IFN-beta and CCR7, but express RSV-associated chemokines.
Top two graphs: Expression of CCR7 and IFN beta was analyzed by RT-PCR at 6 and 24 hrs post-stimulation. PolyI:C and LPS stimulation upregulates CCR7 at both timepoints, whereas RSV-stimulated DCs showed very little upregulation at both timepoints. PolyI:C stimulation promotes high IFN-beta expression, whereas stimulation with LPS results only in early expression. RSV stimulation fails to upregulate IFN-beta. **Bottom two graphs:** RSV-stimulated DCs are able to express the chemokines CXCL10 and CCL5, which are associated with RSV-induced immunity.

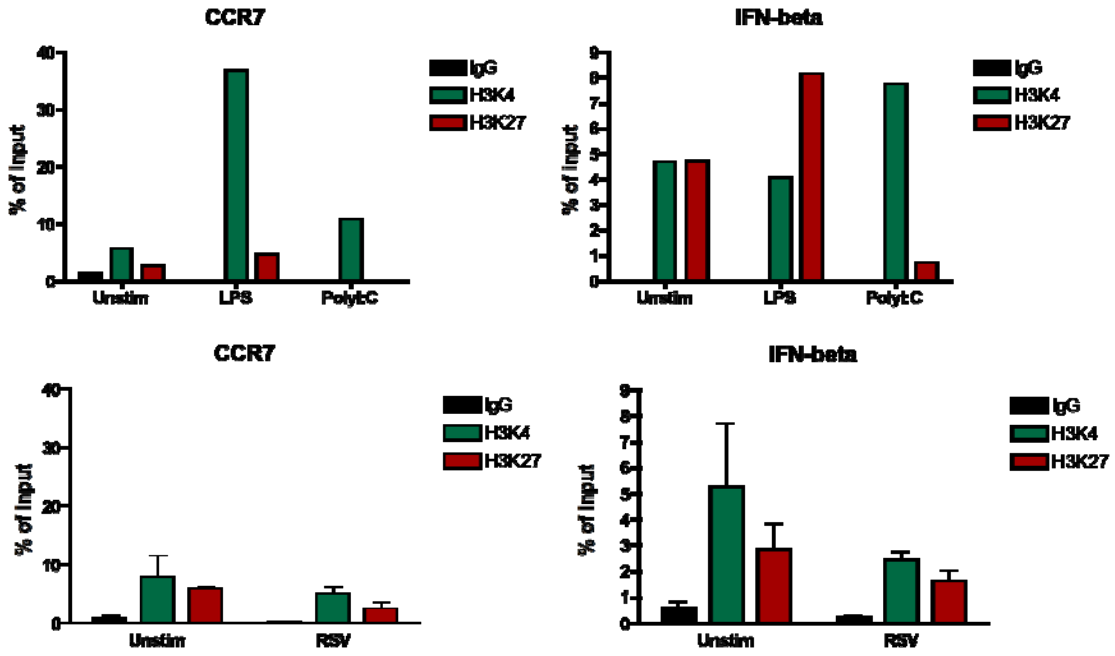

Figure A2. DCs stimulated with TLR agonists display histone marks consistent with RNA, whereas RSV-stimulated DCs show histone marks unchanged from that of unstimulated cells.

ChIP assay was performed to determine histone modifications at the promoter regions of CCR7 and IFN-beta in DCs 24h post-stimulation.. **Top two graphs:** Stimulation of DCs with PolyI:C shows predominant activating methylation marks at CCR7 and IFN-beta, whereas stimulation with LPS shows predominant activation at CCR7, but silencing at IFN-beta. **Bottom two graphs:** Stimulation of DCs with RSV does not alter the methylation status of CCR7 and IFN-beta, and is similar to DCs left unstimulated.

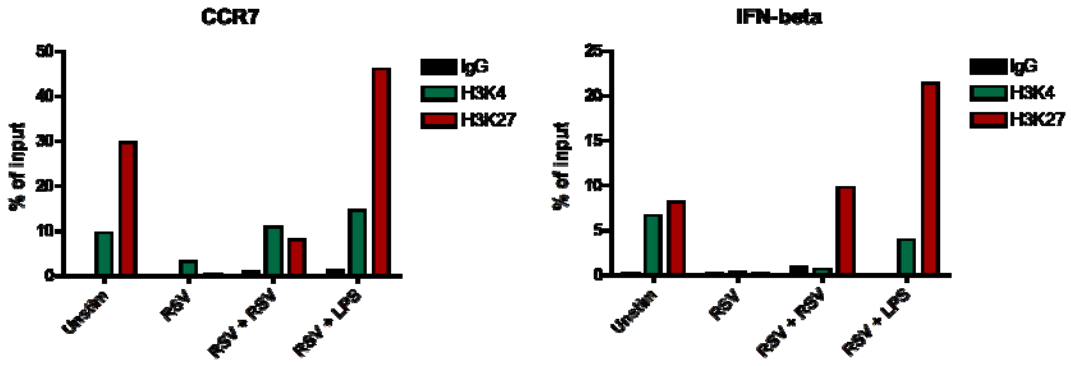

Figure A3. DCs restimulated with RSV or LPS after primary RSV stimulation show predominant silencing methylation mark at promoters of CCR7 and IFN-beta. DCs were restimulated with RSV and LPS 48hrs after primary stimulation, then analyzed 6h later by ChIP for histone marks. DCs restimulated with both RSV and LPS show predominant silencing methylation at IFN-beta, and DCs restimulated with LPS also demonstrate predominant silencing at CCR7.

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