Chapter 3

CD4 T-Cell Immunity in the Lung

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3.1 Introduction

CD4+ T helper cells are critical cells that mediate adaptive immune responses in the lung (Fig. 3.1). These cells are initially primed by antigen-presenting cells (APCs) that present peptide antigen in the context of class II major histocompatibility complexes engaged with the T-cell receptor (termed signal 1). Both APCs and T-cells express co-stimulatory molecules and receptors (termed signal 2) and this second signal is critical for both generating antigen-specific effector T-cells as well as memory cells. Antigen presentation without signal 2 can lead to T-cell anergy. CD4+ T-cell differentiation into specific effector lineages occurs under the influence of lineage-specific cytokines (signal 3) that can control both chromatin remodeling as well as the induction of lineage specific-transcription factors in CD4+ T-cells.

The profound role that CD4+ T-cells play in pulmonary host defense has clearly been demonstrated by the types of pulmonary infections that complicate congenital deficiencies in these cells or by their depletion in AIDS. Pulmonary infection with *Pneumocystis* was one of the first AIDS defining illnesses followed by bacteremic pneumococcal pneumonia (Centers for Disease Control (CDC) 1981; Gottlieb et al. 1981; Masur et al. 1981).

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Fig. 3.1 T-helper cell differentiation. Dendritic cells (DCs) present antigen in the context of class II MHC to naïve CD4⁺ T cells. Signal 1 consists of activation through the T-cell receptor followed by signal 2 which is mediated through co-stimulatory molecules such as ICOSL, CD28, and cytotoxic T lymphocyte-associated protein (CTLA-4). T-cell differentiation occurs under specific cytokine signals. IFN γ can induce T-bet via STAT1 signaling which in turn can regulate IL-12bR2 expression making Th1 cells responsive to IL-12p70. IL-4 induces Gata3 which augments IL-4 producing which supports the differentiation of Th2 cells. Th2 cells produce IL-4, IL-5, and IL-13 as their effector cytokines. TGF β and IL-6 can induce ROR γ T expression as well as activation of STAT3 that induces IL-23 receptor expression and the combination of these cytokines supports the differentiation and proliferation of Th17 cells. These cells produce the cytokines IL-17/IL-17F, IL-22, and IL-21. IL-21 can signal in an autocrine fashion to reinforce the differentiation of the Th17 lineage

3.2 CD4⁺ T Helper Subsets

3.2.1 *Th1*

Th1 cells were first described by Mossman et al. (1986) by their ability to express interferon-gamma (IFN- γ). Th1 cell development requires the transcription factors T-bet (Szabo et al. 2000) and these cells can differentiate from naïve T-cell precursors in the presence of Th1-polarizing cytokines such as IL-12p70 which is a heterodimeric cytokine consisting of IL-12p35 and IL-12p40 (Wolf et al. 1991). Th1 responses can also occur independent of IL-12 and type I interferons have been

shown to facilitate Th1 differentiation in certain situations (Longhi et al. 2009). IFN- γ once produced by differentiating Th1 cells can signal in an autocrine–paracrine fashion to further amplify Th1 differentiation and lineage commitment. IFN- γ signals via a receptor complex consisting of two IFN- γ R1 and two IFN- γ R2 chains which are widely expressed on myeloid-derived cells such as macrophages and dendritic cells as well as structural cells in the lung such as epithelial cells and fibroblasts (Gough et al. 2008). These receptors can activate Janus-associated kinases 1 and 2 which phosphorylate STAT1 which allows STAT1 homodimerization and translocation to the nucleus, followed by binding to gamma-activated sequences (GAS) that regulate gene transcription (Gough et al. 2008). IFN- γ regulates cellular immunity to many intracellular infections including *Mycobacterium tuberculosis*, *Listeria monocytogenes*, and *Salmonella typhimurium*. Patients with IL-12p40 mutations can develop BCG or *S. typhimurium* infection but some patients can be managed with antibiotics and theoretically they can respond to interferon (Picard et al. 2002). For example, patients with IFN- γ receptor mutations can develop disseminated infection with bacillus Calmette-Guerin (BCG) that is resistant to antibiotics and IFN- γ therapy (Dorman et al. 2004; Sologuren et al. 2011). Patients with IL-12p40 mutations can develop BCG or *S. typhimurium* infection but some patients can be managed with antibiotics and theoretically they can respond to interferon (Picard et al. 2002). Thus, there is strong evidence that this pathway is essential for human control of these intracellular pathogens.

3.2.2 Th2

Th2 cells differentiation requires the transcription factor GATA3 and STAT5 (Paul 2010), although initial differentiation and activation of these transcription factors can occur independently of IL-4. GATA3 binds to the *Il4* locus and IL-4 and signaling via STAT6 is critical for further TH2 proliferation and lineage commitment (Paul 2010). Th2 cells produce interleukin (IL)-4, IL-5, and IL-13 and mediate immunity against infections with helminths (Fig. 3.2). These cells also facilitate B-cell differentiation and antibody responses to T-cell dependent protein antigens (Willart and Hammad 2011). Deletion of *Gata3* in mice results in embryonic lethality but conditional deletion in T-cell confirms its essential role in Th2 differentiation and the expulsion of helminths from the gastrointestinal tract (Zhu et al. 2004). IL-5 is the principal growth factor that regulates eosinophilopoiesis and IL-5 deleted mice show marked reduction of peripheral and bone marrow eosinophils (Kopf et al. 1996; Fallon et al. 2002). Transgenic over-expression of IL-5 results in eosinophilia (Dent et al. 1990). IL-13 signaling via STAT6 in airway smooth muscle and in airway epithelium leads to airways hyperresponsiveness to methacholine (Wills-Karp et al. 1998; Grunig et al. 1998). Moreover IL-13 is a major factor in mucous production and goblet cell differentiation in the airway (Wills-Karp et al. 1998; Grunig et al. 1998). Th2 cell and their effector cytokines have been widely implicated in atopic diseases such as

Fig. 3.2 Th2 cells and immunity at the mucosa. Parasitic or helminth infection can induce TSLP and IL-25 in the epithelium which can support early IL-4 production leading to the differentiation of Th2 cells. IL-4 and IL-13 can support the induction of IgE as well as stimulate epithelial cells to increase mucous production. IL-5 induces eosinophilopoiesis. The combination of IgE-mediated degranulation of mast cells, the recruitment of eosinophils, and the changes in gene expression in epithelium can lead to host control of helminth infection

allergic rhinitis, atopic dermatitis, and asthma (Barnes 2008). Furthermore, IL-13 has been implicated in fibrotic processes in the lung in response to drugs such as Bleomycin (Belperio et al. 2002; Liu et al. 2004; Jakubzick et al. 2003). It has been recently recognized that Th2 cell priming can be driven by a number of cytokines produced by lung epithelium including TSLP, IL-25, and IL-33 (Willart and Hammad 2011).

3.2.3 *Th17*

The initial dichotomy of T-cell subsets however could not explain all of the infections complications of CD4⁺ T-cell deficiency. For example, CD4⁺ T-cells are essential for host resistance to *Pneumocystis* pneumonia, however, mice deficient in IL-4 (Garvy et al. 1997a), IFN- γ (Garvy et al. 1997b), STAT4, and/or STAT6 (unpublished observations) all clear *Pneumocystis* suggesting additional CD4⁺ T effector populations. IL-17 was cloned in 1993 (Rouvier et al. 1993) and the first IL-17 receptor (IL-17RA) was cloned in 1996 (Yao et al. 1995). IL-17 mRNA was largely restricted to CD4 memory cells and early studies

using intracellular cytokine staining showed that T-cell that produced IL-17 were divergent than those that produced IFN- γ but often co-expressed TNF and GM-CSF (Infante-Duarte et al. 2000). Pivotal studies published in 2005 showed that these cells develop independently of STAT4 or STAT6 and the canonical Th transcription factors T-bet or GATA3 demonstrating that Th17 cells are a distinct CD4+ T-cell lineage (Harrington et al. 2005; Park et al. 2005). Subsequently it was demonstrated that Th17 development requires STAT3, and two nuclear hormone receptors RORA and RORC for development from naïve CD4+ T-cells (Yang et al. 2008; Ivanov et al. 2006). It was initially believed that one of the critical instructional cytokines for Th17 differentiation was IL-23 (Aggarwal et al. 2003); however, IL-23 receptor is not expressed on naïve CD4 T-cells. Several groups showed that a critical first step in Th17 differentiation is stimulation with TGF- β and IL-6 which allows induction of IL-23R (Veldhoen et al. 2006; Bettelli et al. 2006; Mangan et al. 2006). Signaling via IL-23 allows terminal differentiation and expansion of Th17 cells (McGeachy et al. 2009). Another critical effector cytokine produced by Th17 cells is IL-22 which is controlled by IL-23 as well as the transcription factor aryl hydrocarbon receptor (Ahr) (Veldhoen et al. 2008). Th17 cells also produce IL-21 (Nurieva et al. 2007; Korn et al. 2007) which can function in an autocrine manner to further expand Th17 differentiation (Fig. 3.1).

3.2.3.1 T-Follicular Helper Cells

T_{FH} cells are a subgroup of CD4+ T-cells that are found in the B-cell follicle region in secondary lymphoid tissues such as bronchial associated lymphoid tissues or lymph nodes in the lung. These cells are critical for T-cell-dependent B-cell activation through the expression of CD40L and IL-21 (Crotty 2011). Their transcriptional program is distinct from other CD4+ T-cell lineages and they develop after stimulation with ICOS. These cells also require the transcription factor Bcl-6 for development as well (Crotty 2011).

3.2.3.2 T-Regulatory Cells

Tregs develop under the transcription factors Foxp3 and STAT5 and are critical for mediating tolerance to inhaled antigen in the lung and preventing or reducing allergic inflammation (Josefowicz et al. 2012). They can suppress the effector activity of many T-helper subsets and can be thymically derived (natural Tregs) or induced in the periphery (iTregs). An exhaustive review of these cells is beyond the scope of this chapter but the reader is referred to an excellent thorough review of these cells if they seek a more in-depth description of these cells (Josefowicz et al. 2012; Ray et al. 2010).

3.3 Other Sources of Th1/Th2/Th17 Effector Cytokines in the Lung

3.3.1 $\gamma\delta$ T-Cells

$\gamma\delta$ T-cells are resident in lung tissue and produce a variety of effector cytokine including IFN- γ , IL-4, IL-17, and IL-22 (Bonneville et al. 2010). In the context of pulmonary infection with bacteria these cells provide a substantial amount of early IL-17 and are regulated by IL-23 and IL-1 β (Lockhart et al. 2006; Shibata et al. 2007a; Sutton et al. 2009; Martin et al. 2009; Chen et al. 2011). As these cells express the $\gamma\delta$ T-cell receptor, it is unclear if these cells are responding directly to cytokine stimulation alone or whether they also require endogenous TCR-dependent signals as well in the lung. $\gamma\delta$ T-cells have recently been shown to limit pathology in RSV infection (Dodd et al. 2009). These cells have also been shown to produce IL-10 and can play a regulatory role in other pulmonary infections such as *Pneumocystis* infection (Steele et al. 2000).

3.3.2 NKT-Cells

NKT-cells are another source of multiple effector cytokines in the lung including IL-4, IFN γ , and IL-17 (Michel et al. 2007). One population that has extensively studied is a population that expresses an invariant T-cell receptor (iNKT cells) that recognizes a galactolipid Sphingomonas, alpha-galactosylceramide (Brossay et al. 1998; Burdin et al. 1998). These cells have been found to be elevated in the bronchial alveolar lavage fluid of patients with asthma (Akbari et al. 2006; Pettersson et al. 1985). These cells can also produce IFN γ in response to *Streptococcus pneumoniae* pulmonary infection (Nakamatsu et al. 2007). These cells also produce IL-17 in response to *Escherichia coli* LPS (Michel et al. 2007) as well as ozone (Pichavant et al. 2008). NK cells can develop under the control of IL-15 and express antiviral molecules such as IFN- γ as well as cytotoxic molecules (Steel et al. 2012).

3.3.3 Innate Lymphoid Cells

Another cell population that produces effector cytokines in the lung is innate lymphoid cell. These cells are defined by lacking lineage markers, a lack of T-cell receptors but require IL-7 signaling for their development. Thus these cells are present in RAG1 or RAG2^{-/-} mice but are lacking in RAG2, γ C double-deleted mice (Halim et al. 2012; Spits and Cupedo 2012). ROR γ T expressing cells are critical for the formation of secondary lymphoid tissues (via regulation of lymphotoxin expression)

and play critical roles in mucosal immunology in the gastrointestinal tract through the production of IL-17 and IL-22 (Ouyang et al. 2008). Type 2 ILCs produce IL-5 and IL-13 and participate in the clearance of helminths from the GI tract (Neill and McKenzie 2011). These cells appear to be regulated by IL-25 (IL-17E) as well as IL-33, a member of the IL-1 family. Recently it has been demonstrated that a population of ILCs prices IL-13 in response to IL-33 induced by viral infection and these cells mediate in part, viral-induced exacerbation of allergic disease in the lung (Kim et al. 2012). In addition to viruses, these cells can also be activated by protease allergens to drive eosinophilic airways inflammation as well as airways hyperresponsiveness (Halim et al. 2012). In this allergen setting the activation of these cells required IL-33 and TSLP (Halim et al. 2012). Thus these cells recapitulate many aspects of CD4+ T-cell immunity in that there are subsets that express similar effector molecules, yet these cells are activated early and their activation is independent of TCR stimulation.

3.4 Effector Mechanisms of CD4+ T-Cell Effector Cytokines in the Lung

3.4.1 *Type 1 Effectors*

As mentioned above, receptors for IFN γ are expressed on a variety of lung cells including alveolar macrophages, dendritic cells, fibroblasts, and lung epithelial cells. There are several mechanisms of action by which IFN- γ is thought to be critical for control of lung immunity against intracellular pathogens. The first is through macrophage priming and the induction of intracellular microbicidal activity of macrophages (Murray 1988). IFN- γ priming of macrophages results in significantly augmented TLR signaling (Schroder et al. 2006). IFN- γ also increases microbicidal activity in part, through the induction of inducible nitric oxide synthase which can increase the production of reactive nitrogen intermediates (Xie et al. 1992, 1993) as well as by increasing the production of reactive oxygen species. These activities may explain the therapeutic benefit of IFN- γ in patients with chronic granulomatous disease due to mutations in NADPH oxidase (Naderi et al. 2012; Segal et al. 2011; Fernandez-Boyanapalli et al. 2010). This increase in microbicidal activity has been termed classically activation of macrophages (Gordon 2003).

IFN- γ markedly upregulates class II MHC molecules as well as co-stimulatory molecules such as CD80 and CD86 which can augment antigen presentation to naïve T-cells. Of course, one of the IFN- γ 's first observed activities was its ability to suppress viral replication in many target cells including macrophages, fibroblasts, and lung epithelial cells (Hovanessian et al. 1980). This occurs in part though the induction of many anti-viral genes such as MxA (Ronni et al. 1995); however, other respiratory viruses such as SARS coronavirus are controlled by IFN γ via MxA independent mechanisms (Spiegel et al. 2004).

IFN- γ also induces chemokines such as CXCL9, CXCL10, and CXCL10 which are all ligands for CXCR3 which is expressed on Th1 cells and thus, IFN- γ can increase the recruitment of Th1 cells and this is critical for granuloma formation which is essential for control of many intracellular pathogens such as *M. tuberculosis* (Aly et al. 2007; Chakravarty et al. 2007). In fact both systemic and aerosolized IFN- γ have been investigated for the potential adjunctive treatment of TB. A recent meta-analysis showed that IFN- γ was well tolerated and associated with higher sputum sterilization rates (Gao et al. 2011); however, definitive randomized control trials are lacking to make firm conclusions on the efficacy of this cytokine.

3.4.2 Type 2 Effectors

Both IL-4 and IL-13 can activate STAT6 signaling in a variety of lung cells including alveolar macrophages, fibroblasts, airway smooth muscle, and airway epithelium. Activation of the STAT6 pathway leads to alternative macrophage activation characterized by the expression of arginase 1, YM1, YM2, and the macrophage mannose receptor (Gordon and Martinez 2010). IL-4 treatment of macrophages reduces their phagocytic ability although it increases the clearance of apoptotic neutrophils (Gordon and Martinez 2010). It has been suggested that AAMs have regulatory roles in helminth infection and can reduce immunopathology (Gordon and Martinez 2010). AAMs also have increased expression of Dectin-1 in addition to macrophage mannose receptor and thus they may have greater fungicidal activity. Recently it has been shown that *Francisella tularensis*, a virulent pathogen in the lung can subvert classical activation of macrophages and thus prolong its intracellular survival (Shirey et al. 2008).

IL-13 has profound effects on airway epithelium by increasing the expression of several mucin genes including *Muc5ac* and *Muc5b* as well as inducing goblet cell hyperplasia, by activation of STAT6 (Rose et al. 2000). These effects are critical for host defenses against helminths such as *Nippostrongylus brasiliensis* (Price et al. 2010) but contribute to pathology in allergy/asthma. During viral infection of the airways mucins can not only prevent viral spread but also may contribute to airway obstruction. Moreover IL-13 has recently been shown to increase the susceptibility of epithelial cells to infection with rhinovirus (Lachowicz-Scroggins et al. 2010).

3.4.3 Type 17 Effectors

Human bronchial epithelium express IL-17RA and IL-17RC which allow these cells to respond to IL-17A and IL-17F as well as IL-22R and IL-10R2, the receptors for IL-22 (Fig. 3.3) (McAllister et al. 2005; Aujla et al. 2008). Both IL-17A and IL-17F can induce ligands for CXCR2 such as IL-8 and granulopoietic growth

Fig. 3.3 Type 17 cytokines in mucosal immunity. Infections can activate dendritic cells and macrophages to produce IL-6, IL-23 as well as IL-1 β that can induce IL-17 and IL-22 production by several immune cells in the mucosa including innate lymphoid cells, NK cells, NKT cells, and $\gamma\delta$ T-cells. IL-17 and IL-22 can signal to the epithelium to augment G-CSF as well as ligands for CXCR2 that mediate the recruitment of neutrophils. IL-22 in conjunction with IL-17 augments antimicrobial peptides and epithelial repair function important for control of extracellular fungal pathogens. Upon vaccination with certain adjuvants Th17 cells through the production of IL-17 can also induce ligands for CXCR3 that increase the recruitment of IFN γ -producing Th1 cells that can also help control intracellular pathogen growth

factors such as G-CSF which can be augmented in the presence of TNF- α (McAllister et al. 2005; Jones and Chan 2002). Since IL-17A and IL-17F can be co-expressed in the same cell, it has been reported that these two IL-17 family members can form three cytokines including IL-17A homodimers, IL-17A/F heterodimers which has intermediate activity compared to IL-17A homodimers, and IL-17F homodimers which has the least potent activity (Wright et al. 2007). IL-17 receptors are also expressed on lung fibroblasts, pulmonary vascular endothelium, and bronchial smooth muscle. Using CXCL1 as a model CXCR2 ligand, it has been shown that a major effect of IL-17 signaling is increasing mRNA stability of this transcript (Hartupée et al. 2007; Sun et al. 2011) resulting in augmented protein production. A similar activity has also been reported for IL-17-mediated increases in G-CSF production (Cai et al. 1998). IL-17RA is also abundantly expressed on myeloid

cells; however, these cells express very little IL-17RC and thus IL-17A and IL-17F have limited activity on myeloid cells. It has been reported that IL-17 can enhance IL-12p70 in alveolar macrophages (Lin et al. 2009) as well CCL2, CCL3, GM-CSF, IL-1 β , and IL-9 in CD4+ T-cells (Ishigame et al. 2009). It has recently been shown that Th17 cells express IL-17RA and IL-17RE, the receptor for IL-17C and IL-17C, and increase the production of IL-17 by these cells (Song et al. 2011; Ramirez-Carrozzi et al. 2011; Chang et al. 2011). IL-17C can be expressed in lung epithelium (unpublished observations) and thus can serve as a feed forward mechanism by which the epithelium could influence interstitial T-cell responses. IL-17A can also augment apical bicarbonate anion transport in polarized NHBE cells and this activity may be important in mediated IL-17A's antimicrobial effect in the lung as bicarbonate anion has been shown to greatly augment the bioactivity of human defensins (Kreindler et al. 2009). IL-17RA is required not only for host resistance to the extracellular pathogens *Klebsiella pneumoniae* (Ye et al. 2001) but also for the intracellular pathogen *F. tularensis* (Lin et al. 2009). In the former scenario, IL-17 regulates G-CSF and neutrophil recruitment (Ye et al. 2001) and dominant sources in primary infection are lung $\gamma\delta$ T-cells (Chen et al. 2011). In the case of *F. tularensis*, IL-17 regulates IL-12p70 production by macrophages which are required for Th1 immunity which is ultimately the effector cell required for control of the pathogen (Lin et al. 2009).

IL-22 activates STAT3 in NHBE cells as well as increases their clonogenic potential in colony assays (Aujla et al. 2008). Moreover in mature epithelium IL-22 augments epithelial repair in response to mechanical injury (Fig. 3.3) (Aujla and Kolls 2009). IL-22 also induces antimicrobial genes in lung epithelium including lipocalin 2 (Aujla et al. 2008) and regenerating islet-derived protein three- γ (Zheng et al. 2008). Blockade of IL-22 during experimental *K. pneumoniae* lung infection results in rapid dissemination of bacteria from the lung and increased mortality due to bacteremia. In this model IL-22 is regulated by IL-23 and recombinant IL-22 can rescue IL-23-deficient mice (Aujla et al. 2008). IL-22 has also been shown to decrease lung leak in response to ventilator-induced lung injury (Hoegl et al. 2011). These data support a potential therapeutic role of IL-22 in diseases such as severe pneumonia or acute respiratory distress syndrome.

As stated above in primary infection, early sources of IL-17 and IL-22 can be from innate lymphoid cells (Crellin et al. 2010), NK or NKT cells (Crellin et al. 2010; Cella et al. 2009), or $\gamma\delta$ T-cells (Lockhart et al. 2006; Shibata et al. 2007b; Simonian et al. 2009). Classic Th17 cells can also be elicited in the lung in the setting of vaccination and in this setting they play roles in protection against a diverse set of organisms including both intracellular and extracellular bacteria as well as fungi. Using an ESAT-6 peptide Khader et al. showed that Th17 cells are primed in the lung prior to robust Th1 responses and that Th17 cells regulated local expression of ligands for CXCR3 (expressed on Th1 cells) and through this mechanism, Th17 cells could augment the recruitment of Th1 cells into the lung (Khader et al. 2007). Fungal-specific Th17 cells have also been shown to be critical for vaccine-induced protection against *Coccidioides posadasii*, *Histoplasma capsulatum*, and *Blastomyces dermatitidis* infection (Wuthrich et al. 2011). This protection was also

dependent on neutrophils. IL-17 has also been shown to mediate serotype-independent immunity using a whole cell polysaccharide vaccine from *S. pneumoniae* (Malley et al. 2006). Recently, Chen et al. showed that Th17 cells elicited by *K. pneumoniae* vaccination recognize conserved outer membrane proteins in the cell wall of the bacteria and these antigens could also provide serotype-independent immunity through Th17 cells that conferred heterologous protection against multiple serotypes of the organism (Chen et al. 2011). Again this protection was mediated by neutrophils. What is unclear from these studies to date are the specific aspects of Th17 function that are required for protection. For examples, what homing receptors are required to elicit these cells efficiently in the lung? Is only IL-17A required or is there a role for IL-17F or IL-17A/F heterodimers? Is IL-22 also important? What are the critical target cells that IL-17 is signaling in the lung to afford vaccine-induced immunity? What are the best Th17 adjuvants? Are there truly memory Th17 cells that can persist in the host or will these cells require periodic boosting to recall these responses? What is the role of plasticity in the function and fate of these cells?

3.5 Conclusions

CD4 T-cells play critical roles in lung immunity and when these cells are impacted by HIV or other modes of immunosuppression, the host is susceptible to many opportunistic infections by bacteria, fungi, and viruses. These cells will be critical targets to achieve therapeutic vaccines against intracellular pathogens such as *M. tuberculosis*. Furthermore these cells are now becoming attractive targets for other pathogens such as fungi and encapsulated bacteria. To this end, much work lies ahead to understand the generation of these cellular responses and how they can be manipulated therapeutically.

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