Immunity
Review

Lung Homeostasis: Influence of Age, Microbes, and the Immune System

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Pulmonary immune homeostasis is maintained by a network of tissue-resident cells that continually monitor the external environment, and in health, instruct tolerance to innocuous inhaled particles while ensuring that efficient and rapid immune responses can be mounted against invading pathogens. Here we review the multiple pathways that underlie effective lung immunity in health, and discuss how these may be affected by external environmental factors and contribute to chronic inflammation during disease. In this context, we examine the current understanding of the impact of the microbiota in immune development and function and in the setting of the threshold for immune responses that maintains the balance between tolerance and chronic inflammation in the lung. We propose that host interactions with microbes are critical for establishing the immune landscape of the lungs.

Introduction

Maturation of the pulmonary immune system occurs in an antigen-rich postnatal environment. Initially, the adaptive immune system has limited capacity to induce memory responses and this develops gradually. Although this early life immune programming likely has implications for lifelong immune health, there is little mechanistic evidence identifying underlying molecular pathways. It is well established that multiple viral infections in early life predispose to wheezing and asthma in childhood, yet certain bacterial exposures are thought to be protective. In addition, patients with chronic lung diseases experience disease exacerbations that are thought to be provoked by pathogens, which might otherwise be innocuous in a healthy person. Although the lungs are thought to be sterile in utero, postnatal exposures to viruses, bacteria, and fungi contained within the inhaled or aspirated environment not only form and shape the microbiota, but are vital for the education and maturation of the pulmonary immune system. Disturbance of microbes present at mucosal surfaces by inappropriate or serial antibiotic usage also affects immune development and might cause a microbe that is normally a commensal to become pathogenic.

The environment that a child grows up in also has a part to play in the education of the respiratory immune system because epidemiological studies show that premature birth, birth order, number of siblings, living with pets, living next to a major road, and parental smoking all increase the risks for developing lung disease in later life. Landmark studies showing that exposure to farmyard dust in early life is protective against development of allergies and asthma (Ege et al., 2011; von Mutius and Vercelli, 2010) indicate just how important our relationship with microbes is in shaping our immune systems. Experimental mouse models have underscored the importance of the pulmonary epithelium in mediating the protective effect of this farm dust (Schuijs et al., 2015). There is also evidence to show that the number and type of respiratory infections that a child encounters in early life affects pulmonary immune development and sets the trajectory for lifelong lung health. However, it is not clear that viral infections are universally bad while exposure to bacterial components is beneficial, because early colonization with *Streptococcus pneumoniae*, *Moraxella catarrhalis*, or *Haemophilus influenzae* is associated with an increased risk of later asthma (Bisgaard et al., 2007).

It is likely that the relationship between our pulmonary immune system and the microbiota is dynamic and changes with age and environmental exposures. Although respiratory diseases span the entire life course, asthma inception occurs in childhood while COPD and pulmonary fibrosis are commonly associated with older age groups. In addition, it is clear that there are significant changes in immunity associated with aging and that the elderly have an enhanced risk of pulmonary infections. Although little is known about how development of immune senescence affects pulmonary aging, it likely influences the ability to fight infections. Similarly, there is scant information regarding the healthy aging of the lung, because little investigation is carried out in healthy older people.

This review examines the relationship between host immune pathways and microbial communities at the pulmonary mucosa. We highlight the vital influence of these interactions on the education and maturation of the immune system as well as their implications for the development of chronic lung diseases. Although a great deal remains to be discovered concerning the relative contribution of the local pulmonary microbiota, as compared to that of the gut or skin, in determining generation of lung immunity, a greater understanding of how the pulmonary immune system is trained or matured by encounter with bacteria, viruses, or fungi is essential to determine novel strategies for disease prevention and treatment.

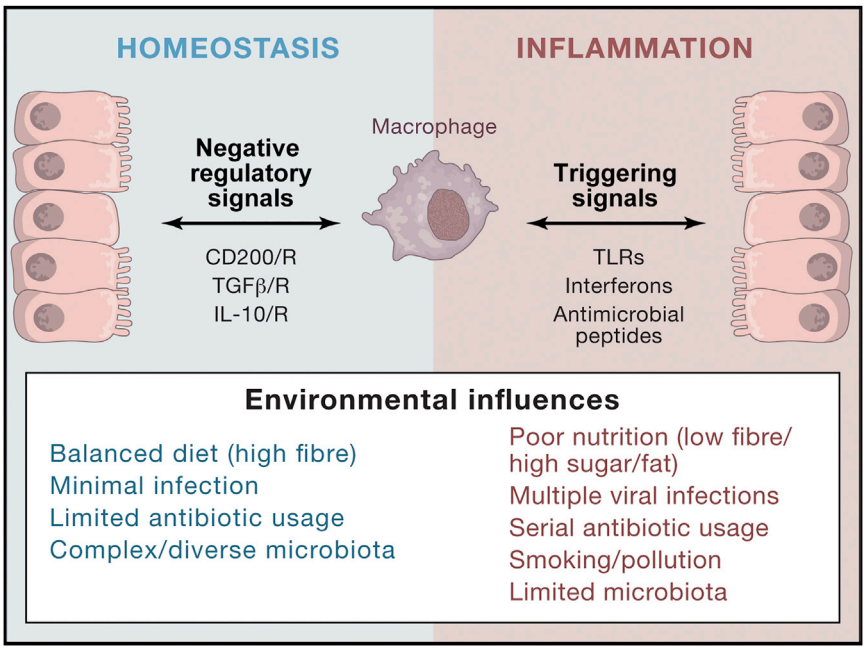

Figure 1. Maintenance of Homeostasis in the Respiratory Tract Is Tightly Regulated by a Series of Resident Cells and Immune Pathways

Macrophages that reside in the airway lumen together with the epithelial cells that line the airways sense external stimuli via a range of surface receptors. Engaging these receptors results in either negative or positive regulatory signals depending on the nature of the stimulus. All of these pathways are influenced by resident microbes, which reside in close proximity.

Pulmonary Immunity Is Maintained by a Network of Resident Leukocytes Interacting with Local Stromal Cells

A complex system of local immune pathways maintain homeostasis within the lungs, and epithelial-macrophages are an integral part of this. The resident epithelial cells and airway macrophages represent a specialized unit which facilitates maintenance of the steady state but their ability to sense the ever changing inhaled environment allows for prompt reactions to potential threats. The relationship between these local defense mechanisms and the microbiota is becoming apparent and their association is dynamic across the life course. However, disturbances in the relationship have consequences for development of efficient immunity.

The pulmonary epithelium provides a barrier between the outside environment and the internal tissues and provides vital first line host defense against noxious stimuli and pathogenic insults. Within the epithelium, ciliated columnar, mucus secreting goblet cells, tuft cells, and Club cells that secrete surfactant, adhere together to form a regulated, impermeable barrier due to the formation of tight junctions localized at the apical surface. Another protective feature of the airway epithelial layer is mucociliary action, which expedites clearance of particulate matter by scavenging and trapping particles and facilitates their removal by coughing. Mucus is produced at low levels in healthy airways and forms a protective layer that is vital for host defense but also for maintenance of immune homeostasis (Radicioni et al., 2016). In particular, defects in production of Muc5B are associated with severe respiratory illness (Roy et al., 2014). Respiratory viruses, air pollutants, and proteolytically active allergens that overcome the mucociliary barrier all have the capacity to damage the epithelium, disrupting tight junctions and increasing epithelial permeability. In addition to this barrier function, the pulmonary epithelium is immunologically active and is pivotal in the regulation of immune responses in the lung (Iwasaki et al., 2017).

Pulmonary epithelial cells are able to secrete a wide range of cytokines and chemokines, as well as being in intimate contact with cells of the immune system. Expression of cell surface receptors enable them to sense and react to environmental change. Lung epithelial cells express pattern-recognition receptors (PRR), such as the Toll-like receptors (TLRs) that recognize pathogen-associated molecular patterns (PAMPS) from viruses, bacteria, fungi, protozoa, and multicellular parasites (Lambrecht and Hammad, 2012). In addition they can react to stress and can secrete a range of anti-microbial mediators including lysozyme, defensins, collectins, and complement components (Iwasaki et al., 2017). Many of the molecules secreted by epithelial cells in response to danger have the ability to regulate immune reactions and recruit cells of the innate and adaptive immune system. Airway epithelial cells can also contribute to the local clearance of apoptotic cells, via apoptosis and secretion of anti-inflammatory cytokines (Juncadella et al., 2013). This function was dependent on intracellular signaling via the small GTPase Rac1. Specific deletion of Rac1 in bronchial epithelial cells led to enhanced inflammation following allergen exposure, with decreased secretion of IL-10 but increased expression of classical type 2 cytokines IL-4, IL-5, and IL-13. Specific deletion of epithelial Rac1 also led to an increased expression of IL-33 in epithelial cells and release into the lumen after allergen exposure. Direct administration of IL-10 to the airways was able to ameliorate this enhanced type 2 inflammation. These data identify how epithelial cells might regulate allergic inflammation via expression of Rac1, which controls the balance between IL-10 and IL-33. Therefore, the initiation and maintenance of inflammation at epithelial surfaces is induced by local mechanisms that have marked effects on the outcome of the immune response.

In health, the lungs are populated with a number of different macrophage populations, which display remarkable heterogeneity due to variations in origin and tissue residency, as well as environmental influences (Yona et al., 2013; Hashimoto et al., 2013; Guilliams et al., 2013). Those macrophages resident within the airway lumen (airway macrophages, AM) are long-lived cells derived from embryonic progenitors that colonize the airways soon after birth and self renew under homeostatic conditions.

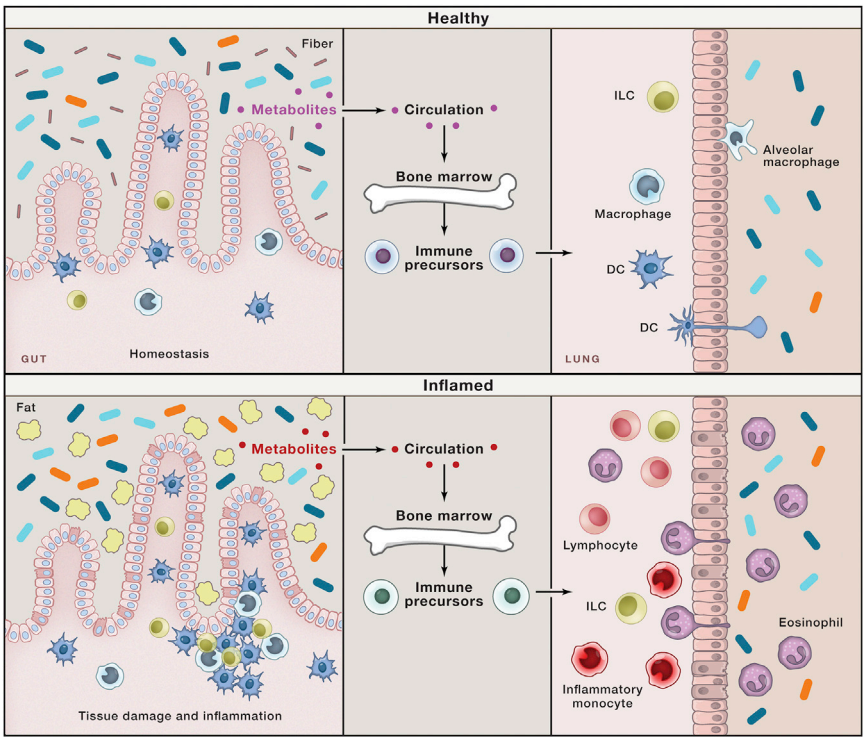

Figure 2. Homeostasis or Inflammatory Responses in the Airways Are the Sum of both Local and Distal Components of the Environment and Immune System

Host-microbe interactions, influenced by infection, inflammation, and diet, in the intestinal tract are powerful regulators of mucosal immunity. Metabolites produced in the intestinal tract, due to intake of dietary components (e.g., fat or fiber) and their fermentation by microbes, have local effects in the gut, but also enter the circulation where they have the potential to shape bone-marrow hematopoiesis promoting regulatory or proinflammatory responses. During pulmonary inflammation, newly recruited cells from the bone marrow seed the lung, and depending upon their nature, promote inflammation or resolution. Inflammation in the lung is also regulated by local signals, in part through the cross-talk with microbes which both shape, and are shaped by, local inflammation. Ultimately, it is the cross-talk between mucosal tissues, microbes, and their metabolites that sets the immunological tone of the airways.

AMs are distinguished from tissue dwelling interstitial macrophages (IM) via their particular repertoire of surface receptors (AM being F4/80^{hi}, CD11c ^{hi}, SiglecF^{hi}). Further populations of monocyte-derived macrophages are recruited to the lung during inflammatory conditions, but airway macrophages represent a first line of defense of the airways and maintain pulmonary immune homeostasis via their close interaction with other lung-resident cells, primarily pulmonary epithelial cells. Their broad program of surface receptors enables them to sense the environment and signal to local stromal cells in order to maintain immune homeostasis within the respiratory tract, but also to sense change within the inhaled environment. These signals can either be activating (TLR 2, 4, 6; IL-1R, IFNγR, and TNFR) or suppressive (CD200R, SIRPα, mannose receptor, TREM2, IL-10R, TGFβR) (Hussell and Bell, 2014). It is clear that close interaction and communication between airway epithelial cells and airway macrophages is vital for maintenance of immune homeostasis within the respiratory tract (Figure 1). A subset of airway macrophages communicate with epithelial cells via synchronized waves of calcium through connexion channels, which are activated via Akt to exert suppressive signals (Westphalen et al., 2014). Interruption of this close association led to stronger responses to inhaled LPS, with increased recruitment of neutrophils and secretion of pro-inflammatory cytokines. This local pulmonary environment is critical for providing phenotypic cues for the airway macrophages: pulmonary transplantation of bone-marrow-derived macrophages led to their transformation into an AM phenotype (Suzuki et al., 2014). Similarly, while macrophages from the yolk sac or fetal liver are able to recolonize the empty pulmonary niche and develop into functional tissue resident AM, mature AM from other tissue sites were not (van de Laar et al., 2016), illustrating the importance of the local environment.

Epithelial cells and AM also cooperate to facilitate clearance of cellular debris and particulate matter. Recent evidence indicates that macrophages are able to signal to epithelial cells to fine tune phagocytosis. Secretion of insulin-like growth factor (IGF1) from macrophages occurs following exposure to inflammatory cytokines or during phagocytosis of apoptotic cells. Binding of IGF1 to its receptor expressed on non-professional antigen-presenting cells, such as local epithelial cells, temporarily redirects their phagocytic capacity such that engulfment of apoptotic cells is reduced while uptake of microparticles is increased (Han et al., 2016). Disruption of IGF1 signaling by deletion of the IGF receptor in the epithelium enhances allergic airway inflammation and highlights the role of the airway macrophage in modulating epithelial functions.

Although epithelial macrophage interactions facilitate first line defense of the airways; if appropriate a second tier of immune responses coordinated by local, tissue-resident lymphoid cells is elicited. This will then control the initiation of effector responses that mediate repair and resolution, and ultimately restitution of homeostasis. Lymphocytes circulate through the lung via the lymph, patrolling for potential antigen encounter. Specialized subsets of T cells are recruited to the lung following encounter with antigen. For example, the chemokine receptors CCR4 and CCR8 promote migration of antigen-specific Th2 cells to the lung (Cho et al., 2016; Lloyd et al., 2000); however, the signals that mediate recruitment of specialized effector and memory cells, and crucially, those that facilitate their retention within the tissue have only recently been addressed. It is now clear that the lung harbors resident populations of both memory T cells and innate lymphoid cells, as well as other resident cells such as regulatory T cells and $\gamma\delta$T cells—all working collaboratively with the local dendritic cell network, thereby ensuring efficient triggering of both innate and memory responses.

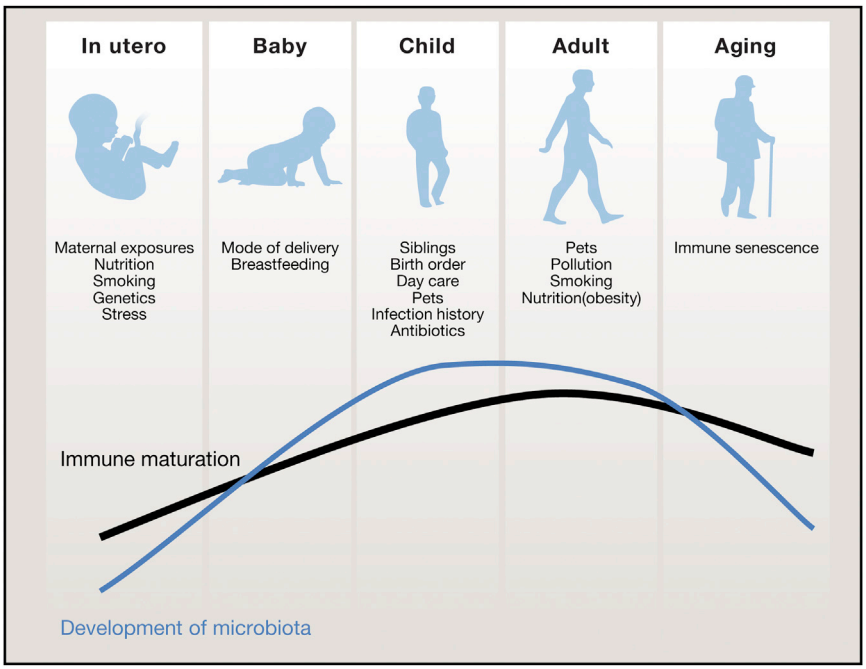

Figure 3. Both Immunity and the Microbiota Are Influenced by External Stimuli and Life Choices across the Life Course

Environmental factors during early life have long reaching consequences for immune development and function in later life and into old age. However, their relationship is dynamic and external factors can induce short- or long-term fluctuations in the microbiota which affect immune function.

It is clear that a subset of memory T cells remain in the lung following initial encounter with antigen and that these resident memory T cells (Trm cells) are sufficient to generate local inflammation, even in the absence of central memory T cells from secondary lymphoid organs (Park and Kupper, 2015). $CD4^+$ and $CD8^+$ Trm are present even in healthy lungs, expressing CD69 and a diverse TCR repertoire (Purwar et al., 2011; Sathaliyawala et al., 2013). Trm form an important component of immunity at barrier surfaces and provide rapid protective immune responses during secondary infections with respiratory viruses such as influenza or RSV. Indeed, in a human RSV challenge study, higher airway $CD8^+$ Trm correlated with reduced disease severity (Jozwik et al., 2015). The fact that Trm cells are able to facilitate the elimination of pathogenic virus even in the absence of antibody can be exploited for the generation of more effective vaccines (Jiang et al., 2012). Resident memory cells might also influence immune responses to inhaled allergens. Following influenza infection of mice $CD8^+$ memory cells in the lung can be reactivated in an antigen independent manner by cytokines such as IL-18, which led to their release of cytokines e.g., IFN-γ, that counterregulate subsequent Th2 responses, thereby protecting the mice against allergic airway inflammation (Marsland et al., 2004). Similarly, allergen-specific Type2 memory cells persist in the murine lung after exposure to house dust mite (Hondowicz et al., 2016). Locally derived, CD25-mediated IL2 signaling provides a comprehensive repertoire of migrational signals, including chemokine receptors that result in the generation and maintenance of these antigen-specific tissue resident T cells that can drive pathology on antigen re-encounter (Hondowicz et al., 2016).

The innate lymphoid cell family constitutes several phenotypically distinct groups of cells that lack the usual lineage markers that define classical lymphocytes, notably antigen-specific receptors and do not mediate antigen-specific responses (Diefenbach et al., 2014). Although they are distributed throughout both lymphoid and non-lymphoid organs their numbers are enhanced at barrier surfaces and they are thought to be vital for maintenance of homeostasis, regulation of immunity and tissue repair (Tait Wojno and Artis, 2016). Like effector T cells, ILCs have been classified according to the transcription factors that they express and the effector cytokines that they secrete (Spits et al., 2013). ILC2 cells are defined by the production of type2 cytokines IL-13, IL-5, and IL-4 as well as expression of the transcription factor Gata3. Indeed ectopic expression of Gata3 directs lineage negative ILCs toward an ILC2 phenotype and promotes de novo production of IL-13 and IL-5 (Klein Wolterink et al., 2013). ILC2s direct a seemingly diverse range of in vivo functions, being involved in protective immunity during helminth infections; development of allergic inflammation, tissue repair and sustaining metabolic homeostasis (Neill et al., 2010; Diefenbach et al., 2014; Tait Wojno and Artis, 2016).

ILC are thought to be tissue-resident cells. In an elegant series of experiments using mice with conjoined circulatory systems, the ILC2 found within the lung were demonstrated to be of host origin, indicating residency (Gasteiger et al., 2015). Moreover, virtually all of the ILC2 and ILC1 identified were situated within the lung parenchyma rather than the circulation. Interestingly, there were few ILC3 identified within the lung, the vast majority of ILC belonging to the ILC2 subset. Long-term tissue residency was shown to be maintained locally via self-renewal during homeostatic conditions. Somewhat surprisingly, even during episodes of inflammation such as during helminth infection, expansion was largely due to local proliferation rather than recruitment. A small proportion of recruited ILC2s were observed during chronic infection phase but this only represented 10% of the total ILC population (Gasteiger et al., 2015). It is notable that this was specific to the ILC2 subset since very few ILC3s were recruited and likely reflects the principal function of ILC2s in tissue repair. This might be a specific facet of the lung microenvironment since it was recently shown that ILC3 within the intestine can rapidly mobilize from cryptopatches during colitis to amplify inflammation (Pearson et al., 2016). Despite their residency in the lung, ILC2 accumulation can be facilitated by interaction with both local stromal cells as well as hematopoietic cells. Cytokines secreted by pulmonary epithelial cells such as IL-33 and TGF-β enhance the accumulation of ILC2 cells within the lung, particularly after encounter with allergen

(Denney et al., 2015; Mohapatra et al., 2016). The type 2 cytokines secreted by these ILC2 are thought to contribute to the inflammatory pathology typical of allergic asthma. Alternatively, they have been shown to contribute to tissue repair during acute influenza infection via the secretion of the EGFR ligand amphiregulin. The ability of ILC to sense their local environment is thought to be a cornerstone of the local pulmonary host defense. However, the mechanisms that balance their contribution to development of lung pathology versus facilitating tissue repair are not well understood. It may be that duration of immune signals is vital because most of the in vivo mouse studies have focused on acute inflammatory models. Although ILC2 have been described as the dominant subset within the mouse lung, in the human lung the frequency of ILC2 is lower than that of NKp44^{-}ILC3s (Barnig et al., 2013; Bal et al., 2016). Conversely, Cytof analysis of ILCs in human tissues under non-inflammatory conditions determined that ILC2 and ILC3 were rare in human lung, compared with ILC1 (Simoni et al., 2017). Interestingly, ILC phenotype in the lungs segregated with those of non-mucosal tissues, rather than with other mucosal tissues such as the gut. Although ILC numbers are small, it is well known that even very small populations of cells are able to evoke profound immune disturbance. The variations in findings probably reflect the differences between mice and human studies and indicate that cells in human tissues are influenced by prior exposures, whereas experimental mice are generally inbred, have a single exposure, and are immunologically naive at the outset. ILC2s have been described in the airways of patients during various diseases, including chronic rhinosinusitis with polyps (Shaw et al., 2013; Bal et al., 2016), asthma (Nagakumar et al., 2016; Smith et al., 2016) and pulmonary fibrosis (Hams et al., 2014). Lipids commonly found in inflamed lungs such as prostaglandins and lipoxins have been shown to regulate ILC2 function in allergic conditions ((Xue et al., 2014) (Barnig et al., 2013). Recent evidence indicates that the ILC profile within the lungs is not stable and is influenced by changes in environment. Smoke exposed mice subsequently infected with influenza exhibited an increase in proportions of ILC1 with a concomitant decrease in ILC2, likely driven by the change in cytokine milieu that involved an increase in IL-12 and IL-18 (Silver et al., 2016). Functional plasticity was also documented in human cells, with IL-12 able to convert ILC2 into ILC1. Moreover, the frequency of ILC1 in COPD patients correlated with disease severity and susceptibility to exacerbations (Silver et al., 2016) (Bal et al., 2016).

Regulatory T cells (Treg cells) are resident within lungs and are vital for maintenance of immune tolerance to airborne particles (Lloyd and Hawrylowicz, 2009). Depletion of naturally occurring CD4^{+}CD25^{+}FoxP3^{+}Treg cells facilitates allergic responses, while their transfer ameliorates symptoms, via the production of IL-10 (Lewkowich et al., 2005, Kearley et al., 2005;). In asthmatic patients, numbers of regulatory T cells increases after allergen specific immunotherapy and following treatment with corticosteroids (Karagiannidis et al., 2004; Radulovic et al., 2008). These induced regulatory T cells also express IL-10 and their phenotype maybe influenced by the local cytokine milieu, as well as infection status (Curotto de Lafaille et al., 2010; Krishnamoorthy et al., 2012; Morita et al., 2015). Accumulating evidence shows that these resident pulmonary regulatory cells are present from birth and their phenotype is directly influenced by the local microbiota (Gollwitzer et al., 2014). In particular, the formation of inducible Treg cells is linked with PD1-PDL1 expression, which is instigated by microbial exposure (Gollwitzer et al., 2014). Cross-talk between alveolar macrophages and T cells is also considered a mechanism through which inducible Treg cells are generated and allergic responses are dampened (Soroosh et al., 2013). Treg cell functionality can be shaped by local infections, as revealed in the context of early life RSV infection, where CD4^{+}FoxP3^{+} Treg cells have been reported to express GATA3 and Th2 cytokines, losing their suppressive activity, and in fact contributing to Th2-mediated pathology (Krishnamoorthy et al., 2012).

$\gamma\delta$T cells constitute a major T cell component of mucosal epithelial barrier tissues where they can respond to danger signals and facilitate orchestration of immune responses (Vantouroout and Hayday, 2013). In the lung, $\gamma\delta$T cells are thought to be important effector and regulator cells in innate host responses to pulmonary infections, but their precise role seems to be pathogen dependent (Nanno et al., 2007). There is also evidence that these cells have a regulatory role for pulmonary function, because mice lacking $\gamma\delta$T cells have increased reactivity to inhaled stimuli, indicating that they can regulate airway function, and implying that they are important for maintenance of airway tone (Lahn et al., 2002). Thus it has been postulated that $\gamma\delta$T cells downregulate airways hyperresponsiveness to allergen challenge, possibly by controlling the repair response of the airway epithelium to $\alpha\beta$T cell-mediated damage (Holt and Sly, 1999) (Murdoch et al., 2014). It is not known how the local environmental changes influence phenotypes; however, inhalation of innocuous bacteria has been shown to promote recruitment of $\gamma\delta$T cells and protect against airways hyperresponsiveness (Nembrini et al., 2011).

Under homeostatic conditions, the respiratory mucosa has an integrated network of dendritic cells largely comprising two conventional DC subsets and plasmacytoid DCs, each with distinct functions. In addition, upon inflammation, monocyte-derived inflammatory cells are recruited to the airways to facilitate the inflammatory response. A recent lineage tracing study indicates that the cells recruited to the airways following allergen challenge can be distinguished as either DCs or macrophages, and argues that monocyte-derived inflammatory cells are likely be ''macrophages'' as compared to the commonly referred to ''monocyte-derived dendritic cells'' (Wu et al., 2016). This repopulation of lung APCs could be a key mechanism through which the microbiota and systemic metabolome shapes the nature of pulmonary immune responses—the so called gut-lung axis (discussed below). Plasmacytoid DCs are predominantly located in the conducting airways and are characterized by the expression of CD11c, B220, PDCA-1, and the lack of CD11b (Neyt and Lambrecht, 2013). They are potent producers of type I interferons (IFN), particularly in response to viral infections (Asselin-Paturel et al., 2001); (Hornung et al., 2004; Lund et al., 2004), but also play immunoregulatory roles by influencing regulatory T cells (Martín-Gayo et al., 2010; Moseman et al., 2004) and tolerance to inhaled antigens (de Heer et al., 2004; Kool et al., 2009). The lung-resident conventional DCs can broadly be divided into two subsets—the CD11b^{+}CD103^{-} and CD11b^{-}CD103^{+} conventional DCs (Neyt and Lambrecht, 2013), although there are

continued efforts to further dissect subpopulations of these cell types. Although both subsets are located in the lung during homeostasis, they are phenotypically and functionally distinct. $CD11b^{-}CD103^{+}$ conventional DCs (cDC1) (Guilliams et al., 2014) are $CD11c^{+}$, MHC-II^{+}, Langerin^{+}, and $XCR1^{+}$ and located in the epithelial layer. Their dendrites can penetrate in between epithelial cells allowing them to capture antigens in the airway lumen and present these to T cells after migrating into the lung-draining lymph nodes. Comparatively, $CD11b^{+}CD103^{-}$ conventional DCs (cDC2) are $CD11c^{+}$, MHC-II^{+}, Sirp1α^{int}, and $CX3CR1^{+}$ and located in the lamina propria (Neyt and Lambrecht, 2013), without direct contact to the airway lumen. Although both conventional DC subsets are able to process and present foreign antigens, they play distinct roles during pulmonary immune responses. cDC1 migrate to the lung-draining lymph node to cross-present antigens to $CD8^{+}$ T cells during respiratory viral infections (Ballesteros-Tato et al., 2010; Ho et al., 2011). Comparatively, cDC2 migration into the lung-draining lymph nodes coincides with the peak of viral load in the lung tissue (Ballesteros-Tato et al., 2010), indicating a role for this subset in the latter stages of infection. During allergic airway inflammation, cDC2 are important for the recruitment of Th2-primed $CD4^{+}$ T cells into the lungs and their local re-activation (Medoff et al., 2009; Plantinga et al., 2013). However, not all of the cDC2 seen in those settings are conventional DCs;monocyte-derived inflammatory cells, likely to be macrophages (Wu et al., 2016) are recruited into the lungs during inflammatory responses and are difficult to distinguish phenotypically or functionally from cDC2 apart from the expression of Ly6C and FcεRI, which are both expressed on the surface of the newly recruited cells (Grayson et al., 2007; Neyt and Lambrecht, 2013). Notably, the majority of studies investigating the role of $CD11b^{+}$ DCs during lung inflammation do not discriminate between these two subsets. Overall, while there are clear overlaps, macrophage and dendritic cell functionality is split between different subpopulations and are some of the most critical determinants of the immunological tone of the airways.

Pulmonary Immunity Is Shaped by Interaction with Microbiota

Until recently, the airways have been considered as sterile under healthy steady-state conditions (Cotran et al., 1999). This historical perspective was largely based upon a failure to grow bacteria from lower airway samples using routine microbiological approaches and became dogma. However, a series of studies utilizing DNA sequencing approaches have now clearly described the microbiota of the lower airways, and importantly, shown that the constituents of this microbiota change depending upon the health status of the individual (Bernasconi et al., 2016; Hilty et al., 2010; Sze et al., 2012). The microbes present in the lower respiratory tract can also be cultured when appropriate protocols are utilized, revealing that this microbial load is not simply bacterial DNA or debris (Remot et al., 2017; Surette, 2014). In retrospect, why should the airways have been considered as sterile? The airways are topographically exterior to our body, have a surface area comparable to a tennis court and are constantly exposed to air which flows through the microbe-laden upper respiratory tract and oral cavity. In the respiratory research field now, there is little question that the airways harbor microbes in both health and disease—a review of the microbiome of the respiratory tract, including a discussion on the history and evolution of this field has recently been published (Dickson et al., 2016) and will not be discussed further in this article. However, particularly from the perspective of mucosal immunology, the relevance of the airway microbiota remains to be fully determined. It is clear that the airway microbiota is governed by distinct rules, as compared to the intestinal microbiota, for example. There is a bidirectional flow of air into and out of the airways, as compared to the unidirectional flow of material through the gastrointestinal tract. Furthermore, mucociliary clearance is a fundamental barrier mechanism assisting the steady removal of particles from the lower respiratory tract, and the alveoli are patrolled by AMs whose primary function is to phagocytose particulates and maintain homeostasis. As a consequence of these characteristics, and likely also due to the reduced energy sources available as compared to the GI tract, the biomass of the airway microbiota is low. Under healthy conditions, it remains unclear to what extent the airways are stably colonized, or whether the microbes are in a state of flux being constantly cleared and repopulated, most likely from the oral cavity. The microbiome of the healthy lungs fits a neutral community model, which indicates that all microbes are equally able to compete within the local environment; thus, the microbes could disperse there from other tissue sites, could populate the airways, or be removed, with equal potential (Venkataraman et al., 2015). Under healthy conditions, it appears that there is constant seeding and turnover of the lower respiratory tract microbiota, which can be characterized by both culture-dependent and -independent methods (Venkataraman et al., 2015); however, the lung habitat changes with age and health status and new habitats and niches form that select which bacteria can reside there (Marsland and Gollwitzer, 2014). Functional studies on the impact of the steady-state airway microbiome are limited. In humans, this is partly related to the significant barrier of getting longitudinal lower respiratory tract samples from individuals, due to the invasive nature of the bronchoscopy. As a consequence, the early studies in this regard are coming from the lung transplantation setting, where sequential bronchoscopies are part of the standard clinical follow-up for transplant recipients (Bernasconi et al., 2016). In addition, historically scientific approaches to host-microbe lung research have focused upon infections, and are only now starting to also consider interactions during homeostasis. Moreover, as a reflection of how recent this field is, studies are only now starting to employ in vitro approaches to address the impact of microbial communities on lung stromal and immune cells, and to couple their analysis of in vivo host cell function (e.g., transcriptomics and proteomics) with microbial sequencing. These approaches have recently found links between the constituents of the lower airways microbiota with macrophage phenotypes (Bernasconi et al., 2016), inflammatory cytokines profiles (Shenoy et al., 2017), and Th17 responses (Segal et al., 2016). In mouse studies, the low biomass of the airway microbiota makes characterization difficult, and limiting microbial colonization to the airways (for example with recolonization of germ-free mice), or specifically depleting the airway microbiota with antibiotics, are challenges still facing the field. However, there is emerging evidence for the functional impact of microbes in the airways and a discussion

of the microbiota in different settings is integrated in the following sections; indeed, a premise that now needs to be considered within the respiratory field is that the microbiota is a contributor to both health and disease in the airways.

Pulmonary Immune Homeostasis Is Disturbed by the External Environment

The inflammatory response against infection also shapes the habitat, and consequently, influences the colonization of certain microbes. Changes in barriers such as mucus, pH, antimicrobial peptides, in addition to recruitment of inflammatory cells, aid control of the infection but could have lasting consequences. An example is post-influenza bacterial pneumonia, whereby the viral infection leaves the host in an immunocompromised state, highly susceptible to bacterial infections. Even if a primary infection is swiftly cleared, it is apparent that the airways do not "reset" to their former state. Expression of surface markers such as TLRs on both macrophages and epithelial cells might not return to the same levels as before infection (Didierlaurent et al., 2008), but also transcriptional and epigenetic reprogramming likely contributes to this "trained immunity," which has consequences for any subsequent infection (Netea et al., 2016). This concept of "innate immune memory" stems from observations that organisms that lack adaptive immune systems, such as plants and invertebrates can generate resistance to infection, as well as vaccination studies that show protection from reinfection can occur when T and B cells are lacking, This antigen nonspecific innate immune memory depends on the nature and sequence of exposure to immune stimuli and might explain why secondary bacterial infections are relatively common following viral infections. The secondary infection could be due to a new exposure to a bacterial pathogen, such as Streptococcus or Klebsiella or alternatively, pathobionts already resident in the airways could bloom in this altered habitat and present as a secondary infection. Data supporting this concept comes from a recent study in humans where an experimental rhinovirus infection was given to healthy individuals or those with COPD (Molyneaux et al., 2013). Samples from people with COPD had a low abundance of Haemophilus influenza in their respiratory microbiota, and following infection with Rhinovirus, the H. influenza levels bloomed (Molyneaux et al., 2013). In a mouse model of COPD, inflammation caused by LPS exposure and tissue damage altered the microbiome, decreasing diversity and increasing the abundance of the genera Pseudomonas and Lactobacillus (Yadava et al., 2016), as reported in human COPD lungs (Sze et al., 2012).

The classical approach when considering "infections" is determining a single causative pathogen, and to directly target this pathogen with antiviral drugs or antibiotics. In fact, the presence of "pathogens," or perhaps more correctly "pathobionts"—which are members of the microbiota that stimulate the immune system even in the absence of overt inflammation, but can cause disease if they bloom and there is consequently bacterial dysbiosis—might be central to a balanced immune system. Within the context of lung transplantation, there is a spectrum of signals associated with the constituents of the microbiota (Bernasconi et al., 2016). At the simplest level, the microbes could be classed based upon their endotoxin structure as being of high or low stimulatory potential (Brix et al., 2015). At one end this spectrum there was a lack of diversity (dysbiosis) characterized by highly pro-inflammatory pathogens (e.g., Staphylococcus, Pseudomonas, Corynebacterium), and associated with this scenario were macrophages with a proinflammatory phenotype, as would classically be expected (Bernasconi et al., 2016). However, at the other end of the spectrum, was an increased abundance of less stimulatory bacteria (e.g., Streptococcus, Prevotella), and this was linked with macrophages having a "remodeling" phenotype. Analysis indicated that an "intermediate," or steady-state macrophage phenotype, was only reached when there was a balance between the high- and low-stimulatory bacteria (Bernasconi et al., 2016). Thus, bacteria typically considered pathogens might be important contributors to healthy homeostasis by providing tonic signals to the immune system setting responsiveness at an appropriate level.

Although little is known about the key requirements for host-microbe homeostasis in the airways during early life, there is clear evidence for a long-term detrimental effect of early life infections, particularly with viruses. The perhaps most extensively studied connection between early life exposures and asthma development is childhood respiratory viral infection. In particular, severe lower respiratory tract infections, especially with rhinoviruses (Jackson et al., 2008) and respiratory syncytial virus (RSV) (Henderson et al., 2005; Sigurs et al., 2005), have been linked with the development of asthma. Wu and colleagues have shown that children who are three to five months old during the winter virus season, where respiratory viruses are likely to strike, are at the highest risk of developing asthma during childhood (Wu et al., 2008). Children that are younger or older during the winter virus season have a diminished risk. Thus, although there clearly needs to be further research, there appears to be a tight window in early childhood during which environmental factors, particularly infections, put a child at risk for asthma. Comparatively, tonic exposure to environmental microbes, which do not result in a bacterial dysbiosis or infection, appears to have a protective effect against allergy. The classic epidemiological example is that of farm dust exposure, where high exposure, such as that found in farm houses which are structurally linked with stables containing cows and hay, is linked with a lower risk of allergies and asthma (Ege et al., 2011). A recent comparison of the incidence of asthma on Amish and Hutterite farms supported this association, where the high levels of dust and endotoxin present in Amish homes were linked to a low incidence of asthma (Stein et al., 2016). Data from experimental models suggests that this protective effect is mediated by microbial components, as inhalation of dust, certain bacteria, or their products, can protect mice against allergic airway inflammation (Hagner et al., 2013; Nembrini et al., 2011), in an MyD88/TRIF (Stein et al., 2016) and A20-dependent manner (Schuijs et al., 2015).

Epidemiological studies suggest that exposure to environmental pollutants (such as cigarette smoke and car exhaust) is a risk factor for development of allergic disease, particularly in babies and young children (Chen et al., 2015; Gauderman et al., 2007). Diesel exhaust particles (DEPs) have an adjuvant effect for allergen challenge in animals and human subjects (Acciani et al., 2013; Morita et al., 2015). Short-term low level exposure to DEPs promoted acute airway inflammation and AHR in allergic mice (Brandt et al., 2013). Although the molecular

mechanisms that result in increased immune responses are not known, it is thought that these environmental "triggers" initiate a series of reactions starting with the epithelium.

Metabolism and Nutrition: Key Mediators of the Gut-Lung Axis

Nutrition is a central parameter governing gut health; however, a growing body of literature also shows that diet impacts the systemic immune system and lung inflammation. An important player in this gut-immune-lung axis is the microbiota, which utilizes dietary components as energy sources and the resulting metabolic by-products can be potent immune modulators (Budden et al., 2017). One of the most studied dietary components is fiber, and indeed microbial fermentation of fiber is well established to lead to the release of short chain fatty acids (SCFA). Among the SCFAs, acetate, propionate, and butyrate are the most extensively described. They are produced in abundance in the caecum and are found in a molar ratio of 70:20:10, respectively. They play a crucial role in gut homeostasis, acting as an energy source for colonocytes (Roediger, 1980), and exert plethoric effects on gut morphology and function, such as fluid and electrolyte absorption (Binder and Mehta, 1989), mucin and hormones secretion, and colonic mucosal cell proliferation and differentiation (Desai et al., 2016; Scheppach, 1994; Willemsen et al., 2003). Not only are SCFAs essential for regulating a broad variety of processes in the gastrointestinal tract, they are also potent mediators of immune cell function, maturation, and fate (Atarashi et al., 2013; Le Poul et al., 2003; Vinolo et al., 2011). SCFAs are thought to elicit their immunoregulatory effects through two main mechanisms. One involves their interaction with cell surface receptors such as GPR41 and GPR43, both present on immune cells (Maslowski et al., 2009; Smith et al., 2013; Trompette et al., 2014); the other is via their capacity to inhibit Histone Deacetylase (HDAC) activity (Kim et al., 2007).

A recent study has shown that dietary fermentable fibers can influence allergic lung inflammation in mice by altering the gut microbiota leading to an increase in SCFA levels (Trompette et al., 2014). Mechanistically, SCFAs influenced the maturation state of dendritic cell progenitors in the bone marrow, rendering them less capable of instigating Th2 responses in the lungs. These effects were specifically mediated through the SCFA receptor GPR41, but not the related receptor, GPR43 (Trompette et al., 2014). Other studies have supported the beneficial effect of fibers against allergic lung inflammation. Indeed, the prebiotic oligosaccharide, galacto-oligosaccharide or a specific fructo-/galacto-oligosacharide mixture can prevent allergic inflammation in mice (Verheijden et al., 2015; Vos et al., 2007). Along these lines, a study from Choi et al. reported that Trichostatin A, a HDAC inhibitor, improved allergic airway inflammation in mice, supporting the concept that SCFAs could also alleviate asthma through their HDAC modulatory properties (Choi et al., 2005). Although fiber and SCFA have garnered the most recent attention, it is important to acknowledge that this represents only a fraction of the changes that occur to the metabolome following dietary, microbial, or inflammatory changes. Thus, this is a direction of future research that will shed further light on the gut-lung axis, and consequently some of the fundamental principles that determine lung homeostasis (Figure 2).

Critical Checkpoints Dictate Pulmonary Immune Maturation

The status of pulmonary immune homeostasis and the local microbiota is constantly shifting and remains dynamic across the life course, reacting to environmental change and circumstance but there are critical checkpoints—in utero, in early childhood, and during old age that have particular emphasis (Figure 3). Using a mouse model of reversible bacterial colonization, it was recently reported that exposure to *E. coli* only during pregnancy was sufficient to transfer bacterial components to the offspring, which led to changes in ILC3s (Gomez de Agüero et al., 2016). This carefully controlled system provided proof of concept that the maternal microbiota can influence aspects of the offspring's immune system; future studies are needed in order to ascertain the roles of diverse microbial communities, their components, and metabolites. Targeting the maternal environment, possibly through diet, is an attractive preventative approach for shaping immune development during the perinatal period. In line with this, a study has shown that feeding pregnant mice a fiber-rich diet, or simply the SCFA, acetate, was able to shape T regulatory cell response of the offspring, consequently protecting them against allergic airway responses (Thorburn et al., 2015). Similarly, supplementation of mothers with long chain polyunsaturated fatty acids in the third trimester of pregnancy reduced risk of persistent wheeze, asthma, or lower respiratory infections in their offspring (Bisgaard et al., 2016). These data highlight the opportunities to promote immune deviation even in utero; however, it will be important to determine the immune mechanism in humans.

An extensive collection of research has highlighted the postnatal period as critical for long-term pulmonary health. Epidemiological data indicates that babies born by caesarean-section have a higher risk of developing asthma than those born vaginally (Roduit et al., 2009). Linked with this are changes in the early life microbiota of children, which is linked to the mode of delivery (Biasucci et al., 2008; Dominguez-Bello et al., 2010). Direct evidence of the immunological implications for these microbial profiles are still lacking, however there are a number of studies indicating that alterations of the early life microbiota have consequences for lung diseases. First, in humans, early life antibiotic exposure is often considered a risk factor (Risnes et al., 2011), albeit controversial (Gagliardi et al., 2011), for subsequent development of wheeze and asthma. Supporting this, in a mouse model, treatment of mice with Vancomycin led to a drop in the diversity of the gut microbiota, which was linked with an exaggerated Th2-driven allergic airway inflammation (Russell et al., 2012). Microbial exposure from a farm environment, particularly raw milk and stable dust within the first year of life, has been epidemiologically linked (von Mutius and Vercelli, 2010), and experimentally shown (Stein et al., 2016; Schuijs et al., 2015), to protect against the development of allergic asthma. A separation between early life events that influence the gut followed by the immune system and lung (e.g., diet/raw milk), and those which directly influence the lung (e.g., dust) is difficult, and in all likelihood both pathways will be important. A recent report shows that the early life formation of the lung microbiota is a key step in the development of tolerance to aeroallergens via the transient induction of PD-L1 expression of DCs and subsequent peripheral Treg cell induction (Gollwitzer et al., 2014). Similarly,

Remot and colleagues have shown that colonization of the neonatal airways with specific strains of bacteria, isolated from the lung, had the capacity to either protect against or exaggerated allergic airway inflammation (Remot et al., 2017). A theme, which is gathering support and momentum in the field, is the so-called "Window of Opportunity." Broadly, this concept is that there is a defined post-natal period that is particularly potent at shaping the immune system, and thus susceptibility to or protection against lung diseases. In humans, the first year of life has historically been considered critical; however, recent studies have reported certain microbial profiles in children at 3 months of age, which associate with disease later in life (Arrieta et al., 2015). This concept is supported by data from animal models where there is a time window within the first 2–3 weeks of life, essentially pre-weaning, where microbial exposure is required for full immune maturation, most notably concerning iNKT cells (Olszak et al., 2012), control of IgE class switching (Cahenzli et al., 2013), and PD-L1-toleragenic pathways on DC (Gollwitzer et al., 2014).

Maturation of the immune system occurs throughout childhood in the antigen-rich postnatal environment. It is likely that pulmonary microbiota stabilizes early but will be influenced by subsequent encounter with pathogens or by inflammation. Asthma is one of the most common childhood diseases, and studies have shown that most school-age atopic asthmatics develop symptoms and exhibit reduced lung function during infancy. This early development of allergic symptoms occurs while the immune system and the lungs are still developing; therefore one could class age or developmental status as a risk factor for development of asthma. In fact, comparison between in vitro cultured bronchial epithelial cells obtained from children with asthma and those from healthy controls determined that there were intrinsic biochemical and functional differences (Kicic et al., 2006). Epithelial cells from asthmatics showed a less differentiated phenotype, an augmented release of anti-inflammatory mediators and interestingly, reduced levels of TGF-β1. Although some studies indicate an intrinsic defect in interferon signaling the findings are not universal (Baraldo et al., 2012; Edwards et al., 2013; Sykes et al., 2014). The resident pulmonary microbiota will likely affect these antiviral defense mechanisms and therefore the development of asthma (Iwasaki et al., 2017). Similarly, the airways are only colonized with macrophages in the immediate postnatal period, which is likely to be important for development of pulmonary immunity. Moreover, it is clear that children who have abnormal lung function in early life do not improve, which might lead to problems in later life (Tai et al., 2014). A significant proportion of patients over 40 presenting with chronic lung inflammation have symptoms of both asthma and COPD, indicating an underlying defect in immunity.

The elderly are particularly vulnerable to respiratory infections, and certain respiratory diseases such as COPD and IPF are generally found in older people. This might reflect the change in immune responses that area part of the normal aging processes. Key genes that control the aging process and modulate "healthy aging" include the IGF-1 signaling pathway, target of rapamycin (TOR), and AMP-activated protein kinase (AMPK) (López-Otín et al., 2013). Accumulated lifelong environmental exposures (the "exposome") contribute to increased ROS and a pro-inflammatory milieu ("inflammaging") leading to the expression of senescence-associated molecules, and culminate in a reduced capacity to fight infection (Thannickal et al., 2015). The elderly are also less responsive to vaccinations. Physical changes to the lung might also affect the balance of microbes within the aging lung. Elderly lungs generally have smaller tidal volumes and reduced respiratory rate together with decreased mucous production and composition, leading to reduced mucociliary clearance, which might alter the composition of the microbiome. The effect of aging on microbiome composition has not specifically been examined in aged populations versus young but it is altered in COPD and in IPF. Overall, pulmonary immunity in later life likely reflects genetic and epigenetic imprinting coupled with age related defects in innate and adaptive immune mechanisms that weaken host-defense, likely influencing host-microbe interactions.

Concluding Remarks

Significant advances in recent years have shed light on the interplay between the immune system and the environment in the respiratory tract. There are a number of themes that are gaining traction and further investigations are required in order for rational strategies to be designed for preventative and therapeutic interventions. One of the first concepts that need strengthening is that the lung environment changes with age, and as a consequence, the rules governing the nature of inflammation and disease are different throughout life. Early in life it is likely that tonic signals from microbes are imperative for directing the maturation of lung-resident cells, and these signals can have long-term consequences, through as-yet-undefined mechanisms. During this early life phase of immune development, severe infections can derail the maturation process, cause tissue damage and increase susceptibility to diseases such as asthma, later in life. Another concept is that the microbiota is ubiquitous throughout mucosal tissues and is an important determinant of immune development, homeostasis, and disease chronicity. The airway microbiota is clearly very low in biomass as compared to the gut; however, this shouldn't undermine the potential significance of these local host-microbe interactions. A deeper understanding of these interactions is likely to be critical for developing future prognostic tools and therapies. In addition, it is important that the field expands its view of the microbiota to include the virome and mycobiome, which are likely to be powerful regulators of the immune system. The lungs are particularly vulnerable to exposure to virus and fungus and as such the balance between pathogenesis and potential immune education by these agents is critical. The cross-talk between different tissues, such as the skin, gut, brain, and lung is an important future line of research. While localized events in the respiratory tract are central to immunity, during dynamic processes such as development of an immune response, cells are recruited from the circulation, lymph nodes, and bone marrow where their function has been shaped by those respective microenvironments. Progress in developing new therapies against respiratory diseases has been disconcertingly slow. Real progress will need a step change where we move from the generalized targeting of single molecules to interventions suited to the maturation stage of the immune system (age), and more broadly shaping the tissue environment by influencing inflammation,

microbes, and metabolites, to ensure maintenance, or restoration, of a healthy homeostasis.

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