Hypoxia, Innate Immunity and Infection in the Lung.

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Abstract

The mucosal surface of the lung is the key interface between the external atmosphere and the bloodstream. Normally, this well oxygenated tissue is maintained in state of sterility by a number of innate immune processes. These include a physical and dynamic mucus barrier, the production of microbiocidal peptides and the expression of specific pattern recognition receptors on alveolar epithelial cells and resident macrophages and dendritic cells which recognise microbial structures and initiate innate immune responses which promote the clearance of potentially infectious agents. In a range of diseases, the mucosal surface of the lung experiences decreased oxygen tension leading to localised areas of prominent hypoxia which can impact upon innate immune and subsequent infectious and inflammatory processes. Under these conditions, the lung is generally more susceptible to infection and subsequent inflammation. In the current review, we will discuss recent data pertaining to the role of hypoxia in regulating both host and pathogen in the lung during pulmonary disease and how this contributes to innate immunity, infection and inflammation.

Keywords: lung, hypoxia, innate immunity, inflammation, pulmonary disease

1 The healthy lung

1.1 Normal oxygen levels in the healthy lung

The pulmonary mucosal surface presents a first-line interface between the internal compartment of the body and the external atmosphere which provides the oxygen necessary for the vast majority of cellular metabolic activity and subsequently is key to cell, tissue, organ and organism survival. Efficient gas exchange in the healthy lung results in sufficient oxygenation of the blood to allow all tissues of the body receive adequate levels of oxygen to maintain normal physiologic rates of metabolic activity. At sea level, air contains approximately 21% oxygen (140 – 150mmHg). In a healthy human, the alveolar partial pressure of O_2 (pO_2) is 100 – 110mmHg (Jain et al. 2005; Tuder et al. 2007) (Figure 1). The large surface area of the mucosal surface of the lung (120 – 150 m^2) combined with a thin epithelial diffusion barrier allows optimum gas exchange at this surface. In the steady state, sufficient oxygen intake allows the maintenance of physiologic normoxia where oxygen supply to the tissues of the body equals or (more normally) slightly exceeds the metabolic demands.

1.2 Innate Immunity in the healthy lung

The pulmonary mucosa exhibits a large mucosal surface which is in direct contact with the external atmosphere (Tuder et al. 2007). Thus, like other mucosal surfaces such as in the gastrointestinal tract, the lung runs the constant risk of being directly exposed to invasive pathogenic organisms, environmental toxins and pollutants. However, multiple innate immune defence mechanisms have evolved in humans designed to combat the threat of inhaled particles / microbes and keep the lower respiratory tract in a normally sterile state.

Firstly, ciliated epithelium, overlaid with periciliary fluid and mucus, execute mucociliary clearance in the lower airways. Any inhaled micro-organisms or particles are trapped in the mucus, which is then propelled by cilia towards the oropharynx, where they are either swallowed or expectorated (Chilvers et al. 2000).

Secondly, multiple anti-microbial peptides, secreted by epithelial cells, resident alveolar macrophages and recruited leukocytes (Bartlett et al. 2008) provide a protective environment against inhaled microbes through multiple mechanisms. For example, lysozyme lyses gram-positive bacteria by hydrolysing $\beta_{1\text{-}4}$-glycosidic bonds of N-acetylmuramic acid and N-acetylglucosamine residues of peptidoglycan (Bartlett et al. 2008). Lactoferrin inhibits growth of bacteria by sequestering iron and by exerting direct antimicrobial activity (Ganz 2004). Human α- and β-defensins are broad-spectrum antimicrobial peptides which act against bacteria, fungi and viruses (Bartlett et al. 2008). Cathelicidins are another group of antimicrobial peptides which are activated by extracellular proteolytic cleavage of precursor molecules (Bartlett et al. 2008). The composition of the airway surface liquid provides an environment of low salt concentrations where the antimicrobial proteins are effective (Chilvers et al. 2000; Ganz 2004).

Thirdly, alveolar epithelial cells, resident dendritic cells (DC) and alveolar macrophages (AM) are the first line of defence against inhaled microorganisms in the lung. These cells recognise various microbial components via pattern recognition receptors (PRR). Ligation of these receptors with common microbial components results in the activation of innate immune and inflammatory signaling pathways resulting in synthesis and release of proinflammatory mediators such as interleukin-8 (IL-8), which drives the recruitment of neutrophils to the site of infection. These infiltrating neutrophils then phagocytose invading microbes and produce chemokines

to recruit and activate other immune cells to help in pathogen clearance. A further mechanism by which neutrophils destroy invading microbes is through the production and release of oxygen radicals (Thomas et al. 1988). In the normal state, upon the ultimate clearance of the inhaled microbe, inflammation is resolved by the induction of neutrophil apoptosis and clearance by macrophages (Chaudhuri et al. 2008; Maderna et al. 2009).

Dendritic cells (DC) play an essential role in linking innate and adaptive immune responses as they have the ability to activate T-cells (Chaudhuri et al. 2008). DC migrate into draining lymph nodes and present antigen fragments on major histocompatibility complex (MHC) molecules in the presence of co-stimulatory molecules to T-cells. Naïve T-cells can then differentiate to effector T-cells (von Garnier et al. 2009).

The recognition of the presence of microorganisms by cells at the pulmonary mucosal surface is through germline encoded pattern recognition receptors (PRRs) of the innate immune system. PRRs sense conserved microbial structures, called pathogen associated microbial patterns (PAMPs). Four classes of PRR are known: Toll like receptors (TLRs), NOD like receptors (NLRs), retinoic acid inducible gene (RIG) I-like receptors (RLRs) and C-type lectin like receptors (CLRs) (Takeuchi et al. 2010).

TLRs are the most well characterised class of the PRR. They are type I integral membrane glycoproteins consisting of extracellular leucin-rich repeats (LRR) for specific binding of PAMPs and cytoplasmatic Toll / Interleukin-1 homology (TIR) domains for downstream signalling. Ten TLRs have been identified in humans to date which recognise various microbial components (Kawai et al. 2010). TLR1, TLR2, TLR4, TLR5 and TLR6 are expressed on the cell surface and mainly recognize

microbial membrane components (Figure 2). Activation of these receptors typically leads to production of proinflammatory cytokines, primarily via the downstream nuclear factor (NF)-κB pathway (Figure 3).

TLR2 can form heterodimers with TLR1 or TLR6 which recognise bacterial and mycoplasmal lipopeptides, respectively. In addition, TLR2 can recognise various other pathogen components including lipoteichoic acid (LTA) from gram-positive bacteria, lipoarabinomannan from mycobacteria and zymosan from fungi (Kawai et al. 2010). TLR4 homodimers recognise lippopolysaccharide (LPS) from the surface of gram-negative bacteria. For LPS binding to TLR4 an accessory protein, myeloid differentiation factor 2 (MD2), is also necessary (Takeuchi et al. 2010). The main ligand for TLR5 is bacterial flagellin. TLR3, TLR7, TLR8, TLR9 are expressed in intracellular vesicles recognizing microbial nucleic acid with subsequent activation of type I interferons and proinflammatory cytokines. TLR3 binds to double stranded RNA, while TLR7 and TLR8 recognize single stranded RNA. TLR9 senses CpG DNA (bacterial-derived DNA with linear cytosine-guanine nucleotide sequences connected by a phosphodiester bond). The ligand for TLR10 is currently unknown (Kawai et al. 2010; Takeuchi et al. 2010).

Pulmonary epithelial cells express mRNA or protein for all 10 TLRs (Muir et al. 2004; Greene et al. 2005; Gribar et al. 2008). Importantly, the localisation of individual TLRs within polarized airway epithelial cells plays an important role in ligand-accessibility. For example, TLR2 is localised on the apical cell surface and is accessible to luminal pathogen components. In contrast, TLR5 is normally localised on the basolateral side of the epithelial cell, but is rapidly mobilised to the apical surface in response to flagellin (Adamo et al. 2004). As TLR4 requires the accessory molecule MD2 for LPS binding, the low level of MD2 in alveolar epithelial cells, limits the extent of the

response to LPS (Bartlett et al. 2008; Gomez et al. 2008). Other membrane associated pattern recognition receptors which are present in the membrane mostly of dendritic cells are the CLRs such as dectin 1 and dectin 2 which sense beta-glucans from fungi (Geijtenbeek et al. 2009).

NLRs, unlike TLRs, are typically localised in the cytoplasm. NOD1 and NOD2 recognize gram-negative bacteria g-D-glutamyl-meso-diaminopimelicacid (iE-DAP) and gram-positive bacterial peptidoglycans such as muramyldipeptide (MDP) respectively (Takeuchi et al. 2010). Like TLRs, NLRs activate proinflammatory cytokines via NF-κB. While NOD1 is ubiquitously expressed, NOD2 has been found mainly in leukocytes and epithelial cells in the lung (Gomez et al. 2008). Other pattern recognition receptors present in the cytoplasm include the RLRs which sense RNA viruses (Takeuchi et al. 2010). Alveolar macrophages and dendritic cells also express an array of PRRs (Figure 2).

In summary, in the healthy state, the pulmonary mucosa is a highly oxygenated tissue with an average pO_2 value in the region of 100-110mmHg. Furthermore, the pulmonary mucosa is armed with a battery of innate immune defence mechanisms which allow the maintenance of the tissue in a constantly sterile state.

2 The diseased lung

2.1 Oxygen levels in the diseased lung.

Despite (or perhaps because of) being an oxygen rich tissue in the healthy state, the pulmonary mucosa is susceptible to conditions where oxygen delivery is diminished (hypoxia). Pulmonary diseases associated with diminished ventilatory drive, airway obstruction, intra-alveolar exudates, edematous septal thickening, infection and inflammatory processes can inhibit oxygen diffusion and result in decreased mucosal

oxygenation with subsequent roll-on effects on gas exchange leading to decreased blood and tissue oxygenation. In fact, decreased partial pressure of oxygen in arterial blood is used as an indirect measurement of diminished oxygen diffusion for the majority of lung diseases including pneumonia, chronic obstructive airway disease, bronchiectasis and pulmonary fibrosis. Here we will briefly review evidence for the association of pulmonary hypoxia with a number of diseases of the lung.

Cystic fibrosis (CF) is an autosomal recessive disorder (Mogayzel et al. 2010) caused by mutations in the CF transmembrane conductance regulator (CFTR) gene, encoding a chloride channel key for the maintenance of normal hydration of the mucosal surface. As a consequence of the defective CFTR protein, hydration of the periciliary fluid is diminished, leading to impaired mucociliary clearance. The mucus layer adheres to the epithelial surface and mucus plaques develop. Mucus filled upper airway bronchi of CF patients taken at the time of bronchoscopy revealed pO_2 levels as low as 2.5 mmHg (Worlitzsch et al. 2002) indicating the presence of mucosal hypoxia. Experiments with isolated epithelial cells from patients with CF or primary ciliary dyskinesia suggest that the CF epithelium also contributes to the hypoxic mucus through increased O_2 consumption. It is also likely that oxygen consumption by bacteria and neutrophils as well as restricted O_2 diffusion through the mucus can contribute to the low pO_2 within CF mucus although this remains to be proven (Worlitzsch et al. 2002). Bronchiectasis is a chronic airway disease which shares several features with CF and is associated with destruction and dilation of the large airways, bronchi and bronchioles. Defects in mucociliary clearance lead to persistent bacterial colonisation, chronic mucosal inflammation and progressive tissue destruction. While direct pO_2 measurements in the lung of bronchiectasis

patients have not been made, it is likely that in any airway disease where significant mucus accumulation / pus formation occurs, mucosal hypoxia is encountered.

Chronic obstructive pulmonary disease (COPD) is a chronic respiratory disorder characterised by progressive airflow limitations and structural changes in the lung, leading to decreased oxygen transport with consequent alveolar hypoxia (Tuder et al. 2007). COPD is associated with local pulmonary inflammation as well as systemic inflammation (McNicholas 2009). The main cause of COPD is chronic exposure to tobacco smoke which contains reactive oxygen species and other toxic substances which leads to host cell damage through lipid peroxidation, protein carbonylation and the formation of DNA adducts (Hansel et al. 2009).

Pneumonia is characterised by acute inflammation with infiltration of neutrophils in and around the alveoli and terminal bronchi. The affected area of the lung may be consolidated by the resulting inflammation and oedema with subsequent decreases in ventilation. Patients with pneumonia and additional hypoxia have poorer clinical outcomes (Ayieko et al. 2006; Sanz et al. 2009).

2.2 Infection in the diseased lung

Bacterial infection is a common occurrence in the diseases of the lung. While the most frequently encountered pathogen in community-acquired pneumonia is *Streptococcus pneumonia* (*S. pneumonia*), the profile of bacteria causing infection in diseases such as CF, COPD and bronchiectasis is different and often involves gram-negative, non-lactose fermenting species (Martinez-Garcia et al. 2007). Among these *Pseudomonas aeruginosa* (*P. aeruginosa*) is a common opportunistic pathogen infecting patients suffering from CF, COPD and bronchiectasis and is associated with disease progression.

Thus in a range of pulmonary disease states, hypoxia and infection are co-incidental events at the mucosal surface of the lung. Recent work has identified that microenvironmental hypoxia may contribute to this process and the development of resultant inflammation which can cause tissue damage. A number of molecular mechanisms described below which are activated in response to hypoxia in pulmonary epithelial cells may contribute to infectious and inflammatory disease development.

3 Hypoxia sensitive signalling pathways.

Because hypoxia is a relatively common occurrence in both health and disease, it is perhaps not surprising that over the course of evolution, metazoans have developed the ability to respond to hypoxia with the induction of genes which encode proteins that promote adaptation to the initiating hypoxic insult. More than seventeen transcription factors have been demonstrated to show some degree of sensitivity to hypoxia (Cummins et al. 2005). These include a number of transcription factors shown to be important in the lung.

3.1 Hypoxia inducible factor (HIF)

The hypoxia inducible factor (HIF) is heterodimeric basic helix-loop-helix Per/ARNT/Sim transcription factor which is a master regulator of tissue oxygen homoeostasis. The HIF pathway has been recently extensively reviewed (Semenza 2007; Kaelin et al. 2008; Taylor 2008b). Briefly, HIF is a heterodimer consisting of an oxygen-sensitive α and a constitutively expressed β-subunit. When sufficient oxygen is available (normoxia), HIF-α is degraded and transcriptionally inactivated through the oxygen-dependent activity of a family of proline and asparagine hydroxylase enzymes. In hypoxia, decreased hydroxylase activity leads to HIF stabilisation /

transactivation and the induction of a transcriptional response (Figure 3). Target genes for HIF include those involved in hypoxic adaptation such as genes encoding proteins promoting metabolism, vascular tone, erythropoesis and angiogenesis.

Three isoforms of the α-subunit are known, HIF-1α, HIF-2α and HIF-3α. In alveolar epithelial cells of the lung, all HIF-α isoforms are expressed but can be regulated in different ways. HIF-2α and HIF-3α proteins remain stable in prolonged hypoxia, while elevation of the HIF-1α protein is more transient (Li et al. 2006; Clerici et al. 2009). It is thought that HIF activation in the lung can contribute to pulmonary disease through the activation of expression of the vasoconstrictor endothelin-1 (Semenza 2000; Semenza 2005).

3.2 Nuclear factor-κB (NF-κB)

NF-κB is a master regulator in innate immunity and inflammatory signalling. NF-κB regulates the expression of pro-inflammatory cytokines and chemokines including tumor necrosis factor (TNF) α, interleukin-(IL)-6, IL-8 and IL-1. The activation of such pathways promotes neutrophil, macrophage and DC recruitment to the site of infection and in a process that coordinates pathogen elimination. NF-κB is a transcription factor comprised of five members: p50, p65, c-Rel, Rel B, p52, which form homo- or hetero-dimers. NF-κB can be activated by two pathways, the canonical (IκB kinase (IKK) β dependent) and the non-canonical (IKKα dependent) pathway (Bonizzi et al. 2004). NF-κB can be activated by a variety of stimuli including PRR ligands and cytokines. It has also been demonstrated that NF-kB is responsive to hypoxia through the same oxygen sensing hydroxylase enzymes that regulate HIF (Cummins et al. 2006). Interestingly, NF-κB activity is necessary for effective mRNA expression of HIF-1α in basal conditions and during hypoxia (Rius et al. 2008). In the lung, excessive NF-κB activation has been linked to inflammatory aspects of

diseases including CF and COPD (Jacquot et al. 2008; Nichols et al. 2008; Edwards et al. 2009).

3.3 Cyclic AMP response element binding protein (CREB)

The cyclic AMP response element binding protein (CREB) is a transcription factor of the leucin zipper family and is typically activated by phosphorylation at proline 133 via protein kinase A (PKA) or calmodulin kinase (Mayr et al. 2001). CREB is also hypoxia responsive (Cummins et al. 2005). Recently, a study analysing hypoxia-responsive transcription factors in the lung revealed CREB to play an important role in the regulation of genes associated with lung disease (Leonard et al. 2008).

The presence of hypoxia typically worsens the course of acute and chronic lung diseases (Urquhart et al. 2005; Ayieko et al. 2006; Sanz et al. 2009). Hypoxia influences aspects of the innate immune system in immune cells as well as the alveolar epithelium and endothelium through impacting pathways likely including HIF, NF-κB and CREB.

4 Regulation of innate immunity and inflammation by hypoxia in host cells.

4.1 Myeloid Cells

Activation of HIF in myeloid cells can impact upon immunological activity and microbial clearance but also on the inflammatory activity of these cells and their capacity for causing tissue damage (Cramer et al. 2003). In macrophages, bacterial infection leads to activation of HIF-1α in an NF-κB-dependent manner through increased HIF-1α mRNA synthesis (Rius et al. 2008; Taylor 2008a). Both gram-positive and gram-negative bacteria induce HIF-1α (Peyssonnaux et al. 2005; Rius et al. 2008). LPS induced HIF-1α activity also occurs in dendritic cells (Jantsch et al. 2008) and in macrophages (Peyssonnaux et al. 2007; Sumbayev 2008). In one

model, the activation of HIF-1α in macrophages following LPS stimulation was proposed to be mediated by the generation of reactive oxygen species (ROS) via activation of phospholipase C1γ / proteinkinase C (PKC) α /β in a TLR4 dependent manner (Sumbayev 2008). Cytokine levels measured in HIF-1α deficient macrophages following LPS stimulation were decreased, compared to those detected in wild-type macrophages, suggesting that TLR4 dependent upregulation / stabilisation of HIF-1α contributes to cytokine production in macrophages (Peyssonnaux et al. 2007). The potential of macrophages for intracellular bacterial killing is also increased under hypoxic conditions in a HIF-dependent manner, further suggesting an essential role for HIF-1α in bactericidal processes in macrophages (Peyssonnaux et al. 2005; Acosta-Iborra et al. 2009).

The phagocytic capacity of human neutrophils is also enhanced in hypoxia (Walmsley et al. 2006). Neutrophils isolated from patients with van Hippel Lindau (VHL) disease showed an increase in bacterial killing under hypoxic conditions, consistent with aberrant HIF-1α signalling in this disease (Walmsley et al. 2006). In neutrophils, hypoxia significantly inhibits apoptosis and thus the inflammatory response is prolonged. This extended survival of neutrophils seems to be dependent on HIF-1α and NF-κB as HIF-1α deficient neutrophils and the use of NF-κB inhibitors showed decreased survival of neutrophils under hypoxia compared to wild-type neutrophils and untreated cells, respectively. Macrophage inflammatory protein (MIP)-1β in the supernatant of macrophages cultured in hypoxia was identified as a further hypoxic neutrophil survival factor (Walmsley et al. 2005).

The murine homolog of the human antimicrobial protein LL-37 (CRAMP) is altered by HIF-1α. CRAMP protein was drastically reduced in HIF-1α deficient neutrophils and increased in VHL deficient neutrophils. At the mRNA level CRAMP was also upregulated under hypoxic conditions (Peyssonnaux et al. 2005). In bone marrow-

derived macrophages (BMDM), an IKKβ dependent upregulation of the murine CRAMP RNA was detected after bacterial stimulation (Rius et al. 2008).

Bacterial exposure of macrophages induced nitric oxide (NO) production by inducible nitric oxide synthase (iNOS) in a HIF-1α dependent manner and NO was found to amplify TNFα release in macrophages, indicating that HIF-1α controls via the induction of iNOS cell killing by NO and also the inflammatory response by increasing TNFα release (Peyssonnaux et al. 2005).

Enhanced interferon γ (INFγ) secretion was observed in macrophages under hypoxic conditions. A functional HIF responsive element (HRE) within the INFγ promoter proposed a HIF-1α dependent up regulation of INFγ in an environment with low oxygen (Acosta-Iborra et al. 2009).

In hypoxia, TLR4 mRNA is increased in macrophages leading to an increased response to LPS. Chromatin immune precipitation (ChIP) and mutations in the HIF-1α binding site of the TLR4 promoter confirmed the presence of a HRE, suggesting a role for HIF-1α in the regulation of TLR4 under hypoxic conditions (Kim et al. 2010). Another screening for hypoxia responsive TLRs in murine DC showed elevated TLR2 and TLR6 mRNA in hypoxia. The upregulation of these two TLRs was confirmed in human DC, a monocytic cell line, in endothelia, intestinal epithelia and in colon, liver and lung of hypoxic mice. Both receptors comprise HREs in their promoters and functional studies confirmed HIF-1α as a coordinator of TLR2 and TLR6 induction under low oxygen concentrations (Kuhlicke et al. 2007).

Expression of co-stimulatory molecules and cytokines under inflammatory conditions is necessary for activation of DC. Under hypoxic conditions, LPS stimulated DC showed increased expression of the stimulatory molecules MHCII, CD80 and CD86 as well as increased levels of the pro inflammatory cytokines TNFα and IL-6 and also T-cell proliferation was enhanced under these conditions. A knock down of HIF-1α

reduced the expression of the factors necessary for maturation, and subsequently the potential of DC to stimulate proliferation of allogenic T-cells was decreased, indicating that HIF-1α is an important immune modulator at the intersection between innate and adaptive immunity (Jantsch et al. 2008).

Alveolar macrophages also initiate systemic inflammation at low oxygen (Gonzalez et al. 2007; Chao et al. 2009). Release of monocyte chemoattractant protein (MCP)-1 by alveolar macrophages is increased in hypoxia and causes mast cell degranulation, providing evidence that MCP-1 is an inflammatory mediator (Chao et al. 2009).

In summary, activation of the HIF pathway by microenvironmental hypoxia in myeloid cells enhances immunological activity and likely contributes to more effective clearance of invading microbial pathogens. This may occur through a range of mechanisms including increased cytokine production, bacterial killing, myeloid cell survival, phagocytosis, NO production and TLR expression. A negative consequence of this may be enhanced inflammatory activity which can contribute to tissue damage in lung disease. Thus, the delicate balance between microbial clearance and tissue damage is sensitive to environmental hypoxia via the HIF pathway and may be effected in diseases of the lung.

4.2 Alveolar epithelial cells

As well as the activation of HIF and NF-kappaB described above, the family of CREB transcription factors was shown to be specifically upregulated in the lung by hypoxia (Leonard et al. 2008). Within the lung, alveolar epithelial cells and microvascular endothelial cells respond differently to adapt to an environment with low oxygen. While CREB and activating transcription factor (ATF) 1 activity were increased in epithelial cells, no significant change was observed in endothelial cells in hypoxia. Endothelin-1 (ET-1), a vasoconstrictor involved in regulation of vascular tone was

increased in endothelial cells but decreased in epithelial cells. VEGF and IL-6 secretion was increased in both cell types (Signorelli et al. 2010).

CFTR is important to maintain epithelial homeostasis. Hypoxia decreases CFTR on mRNA and protein level in epithelial cells and decreases transepithelial transport functions. It is a robust effect as diminished CFTR mRNA was observed also in mice airways and in lungs of patients with lung diseases (Guimbellot et al. 2008). The decreased expression of CFTR at low oxygen has been suggested to be HIF-1α dependent (Zheng et al. 2009). Different mutations result in varied levels of functional CFTR activity. Furthermore, in a hypoxic environment additional repression of CFTR could contribute to respiratory failure in CF patients (Guimbellot et al. 2008).

Sodium-potassium adenosine triphosphatase (Na^+, K^+ ATPase) is located at the basolateral membrane and contributes to sodium transport, alveolar fluid resorption and the clearance of edema. It has been reported that hypoxia induces mitochondrial ROS production leading to endocytosis and degradation of Na^+, K^+ ATPase possibly thus contributing to impaired clearence of pulmonary edema. Furthermore hypoxia induced ROS have been reported to be responsible for hyperphosphorylation and disassembly of keratin intermediate filaments, an important cytoskeleton component of epithelial cells (Zhou et al. 2008).

Enhanced transcellular translocation of gram-positive bacteria mediated by platelet-activating factor receptor (PAFR) was observed in intestinal epithelial cells under hypoxic conditions. In hypoxia expression at the transcriptional level and distribution of the receptor was altered. Reduced translocation rates in HIF-1α deficient cells and the identification of a HIF-1α binding site in the promoter of the PAFR revealed that a HIF-1α dependent upregulation of PAFR leads to increased bacterial translocation via the intestinal epithelium (Keely et al. 2010). Although this study was concentrating

on the intestine, the PAFR also plays a role in chronically inflamed airways (e.g. COPD). A study investigating the effects of a corticoid (fluticasone propionate) on PAFR expression and invasion potential of *Streptococcus pneumoniae* (*S. pneumoniae*) and *Haemophilus influenzae* (*H. influenzae*) showed that reduction of PAFR expression in alveolar epithelial cells results in decreased invasion capacity of the above mentioned bacteria (Barbier et al. 2008).

In summary, at a number of levels, hypoxia and immune signalling pathways are connected in the lung. This increases the likelihood that a background level of environmental hypoxia as is experienced in a range of pulmonary disorders has a significant impact upon the innate immune and inflammatory processes that occur in the diseased lung. These effects will be reflected by altered levels of bacterial clearance in the hypoxic disease lung as well as dysfunctional immune responses leading to the exacerbation of inflammation.

5 Influence of hypoxia on bacteria

We have hypothesized above, based on our knowledge of the effects of hypoxia on lung cells that hypoxia exacerbates infection and inflammation in the diseased lung. Although the impact of hypoxia on human cells has been investigated extensively, our knowledge of the effects of a hypoxic microenvironment on invading pathogens is limited. In contrast to humans, microbes have evolved to adapt to various oxygen concentrations either permanently as obligate anaerobes or temporarily as facultative anaerobes. Adaptation to environmental oxygen is especially important for pathogens, which want to be successful in infecting mucosal surfaces with high oxygen levels or in penetrating into tissues and cells with lower oxygen levels.

Influences of decreased oxygen levels have been investigated on bacterial, viral and fungal pathogens. Among bacterial pathogens capable of causing lung infections in humans, the predominant species that have been studied under decreased oxygen levels are *P. aeruginosa*, *Mycobacterium tuberculosis* (*M. tuberculosis*) and *Staphylococcus aureus* (*S. aureus*).

P. aeruginosa is an opportunistic pathogen, which is frequently isolated from patients with hospital acquired or ventilator-associated pneumonia. It can cause infective exacerbations of chronic lung diseases, like COPD and bronchiectasis, and is the major pulmonary pathogen in CF lung disease. Chronic infection of the CF lung with *P. aeruginosa* is associated with increased morbidity and mortality (Spilker et al. 2004). Decreased oxygen partial pressures have been documented by *in vivo* measurements within thick mucous secretions of the CF lung (Worlitzsch et al. 2002). Although inflamed tissues are generally assumed to have low oxygen and glucose levels (Zinkernagel et al. 2007), it has not been documented whether hypoxic microenvironments as found in the CF lung exist in other infectious pulmonary diseases. It is generally assumed that in scenarios where alveoli are filled with inflammatory exudates oxygen diffusion is diminished and surrounding tissues are hypoxic.

P. aeruginosa is an aerobic bacterium that can grow under anaerobic conditions using nitrate, nitrite, or nitrous oxide as terminal electron acceptors, or can ferment arginine by substrate level phosphorylation (Hassett 1996). Sufficient concentrations of these electron acceptors have been documented in the CF lung (Worlitzsch et al. 2002). In the last decade the effects of complete lack of oxygen (anaerobiosis) on metabolism and virulence of *P. aeruginosa* have been extensively investigated. Whether changes observed during anaerobiosis and discussed in the following

paragraph apply in a similar way to hypoxic bacteria needs to be determined in future studies.

Under anaerobic conditions both the *P. aeruginosa* reference strain PAO1 and environmental *P. aeruginosa* strains form mucoid colonies by increasing alginate production, a viscous exopolysaccharide involved in biofilm formation (Hassett 1996; Worlitzsch et al. 2002). Growth in biofilms has been documented for *P. aeruginosa* in the CF lung (Singh et al. 2000). Optimal biofilm growth under anaerobic conditions is regulated by cell-cell communication known as quorum sensing (Yoon et al. 2002). Quorum sensing factors not only control transcription of genes important for anaerobic biofilm growth, but also virulence genes encoding flagellin and pilin (Yoon et al. 2002). Among 1,030 CF *P. aeruginosa* isolates investigated, 39% had lost flagella mediated motility and 35% were mucoid (Mahenthiralingam et al. 1994)

Similar to *P. aeruginosa, S. aureus* increases polysaccharide expression and biofilm formation under anaerobic conditions, but fails to do so under hypoxic conditions (Cramton et al. 2001). This highlights the fact that bacteria can respond differently to decreased and to absent oxygen availability. The capability to grow as biofilms has been shown for other potential pulmonary pathogens (*S. pneumoniae, H. influenzae*) in the context of chronic middle ear infections (Hall-Stoodley et al. 2009). Apart from CF other respiratory diseases associated with bacterial biofilm formation are diffuse panbronchiolitis and bronchiectasis with *P. aeruginosa* infection. Over the last years significant information has accumulated to suggest that bacteria deeply embedded into biofilms are exposed to hypoxic / anaerobic environments (de Beer et al. 1997).

Production of alginate and biofilm growth protect against host immune responses and confer increased antimicrobial resistance to the organisms. Mucoid *P. aeruginosa* is more capable to resist nonopsonic phagocytosis and oxygen radicals released by

human neutrophils (Leid et al. 2005). Similarly loss of flagellum mediated motility increased the organisms resistance to nonopsonic phagocytosis by macrophages (Mahenthiralingam et al. 1994). In addition several studies have shown that *P. aeruginosa* isolates grown under anaerobic or biofilm conditions were significantly more resistant towards antibiotics, thus complicating treatment for multi-drug resistant isolates (Hill et al. 2005).

More than 10 years ago Hassett (1996) observed that during sustained anaerobic growth mucoid isolates are incapable to revert to their non-mucoid counterparts, but reversion of the mucoid to the non-mucoid phenotype occurred in isolates growing statically in aerobic conditions and therefore facing an oxygen gradient (Hassett 1996). Surprisingly reversion to the non-mucoid phenotype was associated with gain of motility through flagellum synthesis, and regained flagellin expression was an independent adaptive response to the growth conditions rather than an effect of mutations in transcription factors (Wyckoff et al. 2002). The authors speculated that flagellin biosynthesis under these circumstances is switched on in response to sensing an oxygen gradient and in an attempt to reach areas with higher oxygen levels and optimal energy generation (Wyckoff et al. 2002). The movement of bacterial cells towards or from oxygen to an area with optimal oxygen concentration for its metabolism is called Aerotaxis. Aerotaxis has been shown for *P. aeruginosa* in vitro and two potential inner-membrane bound oxygen sensors, Aer and Aer-2 were identified in *P. aeruginosa* (Hong et al. 2004). Activation of Aer is thought to lead to transcription of genes controlling flagellum biosynthesis.

Further understanding of the complex interplay between *P. aeruginosa* and its surrounding oxygen concentrations could reveal new drug targets for treatment of these multi-resistant organisms in chronic pulmonary infections.

One of the biggest challenges in infectious diseases in the 21st century is posed by the spread of *M. tuberculosis*, especially multi-drug or extensive-drug resistant *M. tuberculosis*. It is estimated that one third of the world's population is latently infected with *M. tuberculosis* and 10% of latently infected immunocompetent patients will develop active disease. *M. tuberculosis* persists in its latent form as 'dormant' organisms within host granulomas. Apart from immune effector functions granuloma formation is thought to inhibit bacterial growth by limiting oxygen and nutrient supply. A hypoxic microenvironment has been shown for granulomas elicited *in vivo* in guinea pigs, rabbits and nonhuman primates (Via et al. 2008). Hypoxia along with other stimuli, nitric oxide, carbon monoxide and intracellular location in macrophages, can trigger a transcriptional response leading to a slower replicative or bacteriostatic stage (Rustad et al. 2009). Huge advances in understanding this transcriptional response have been made in the last decade. Further analysis of the *M. tuberculosis* transcriptional response to hypoxia might reveal their importance for the establishment of latent disease and might reveal new drug targets to kill bacteria more efficiently in this metabolic state.

Fungal infections of the lung are less common and are typically found in immunocompromised patients. Transcriptional responses to hypoxia have been analyzed in *Candida albicans (C. albicans)* and *Cryptococcus neoformans* (*C. neoformans*). Whereas in *C. albicans* glycolytic gene transcripts are upregulated in response to hypoxia, *C. neoformans* upregulates genes involved in respiratory metabolism. Two hypoxia sensitive pathways have been identified in *C. neoformans*, one homologues to the mammalian sterol-response element binding protein (SREBP) cholesterol biosynthesis regulatory pathway and the other a two-component-like pathway involving a fungal-specific hybrid histidine kinase family member, Tco1.

(Chun et al. 2007). Mutants in both pathways revealed diminished ability to proliferate in host tissue and to cause disease in mice and had lower antifungal resistance. (Chun et al. 2007). Similarly in *Aspergillus fumigatus* SREBP is required for virulence and resistance to antifungal drugs (Willger et al. 2008).

Strikingly not only hypoxia itself, but hypoxia elicited epithelial cell responses can influence the virulence of human pathogens. Intestinal epithelial cell hypoxia and reoxygenation activated expression of the virulence factor PA-I lectin / adhesion in *P. aeruginosa* via release of soluble *factors* (Kohler et al. 2005).

6 Hypoxia in inflammation- friend or foe

In host myeloid and epithelial cells, hypoxia plays an important role in the promotion of innate immune and inflammatory pathways through the activation of HIF-dependent gene expression. Furthermore, an extensive degree of crosstalk between HIF and NF-κB underpins this response. While this is favourable for the host in the context of microbial killing and clearance, it also exacerbates the collateral damage caused to tissue through inflammation. Thus the degree and duration of hypoxia is likely a key determinant as to how it impacts upon disease progression.

In the invading microbe hypoxia appears to alter not only bacterial metabolism but expression of virulence genes as well. Although decreased expression of virulence genes should make bacteria less harmful for the host, it could allow for evasion of host immune responses and lead to potential persistence of the organism in the host. The enormous capability of invading pathogens to adapt to microenvironmental changes therefore poses an additional challenge on the host immune system to clear infectious organisms in hypoxic tissues.

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Figure Legends:

Figure1: *Comparison of microenvironments of healthy and diseased lung.*

Alveoli of the healthy lung are represented on the left hand panel. Resident cells included are epithelial cells, resident macrophages and dendritic cells. In the healthy state, alveoli are sterile and at an ambient oxygen tension of approximately 100-110 mmHg. In diseases such as cystic fibrosis, chronic obstructive pulmonary disease and bronchiectasis, the inflamed mucosa contains infiltrating neutrophils as well as expanded numbers of macrophages and dendritic cells. Furthermore the alveolar space is less well oxygenated due to the accumulation of mucus and pus. Invading pathogens cause sustained inflammation in the lung.

Figure 2: *Pattern recognition receptor expression in resident alveolar cells*.

Alveolar epithelial cells (EC), alveolar macrophages (MO) and dendritic cells (DC) are the primary components of the alveolar mucosal surface. These cells can recognize microbial structures on invading pathogens through pattern recognition receptors (PRR). Sub-cellular localization and expression of the different PRR subtypes are outlined. In polarized epithelial cells TLR2 and 4 are localized apically while TLR5 is found on the basolateral membrane and TLR3,7,8,9 are in intracellular endosomes. Resident alveolar macrophages and dendritic cells also express various PRR.

Figure 3: The interplay of inflammation and hypoxia in lung diseases.

In chronic inflammatory conditions of the lung, hypoxia and infection with subsequent inflammation are co-incidental occurrences. The presence of hypoxia leads to

activation of HIF which can contribute to innate immunity and inflammation through the expression of genes involved in the regulation of innate immunity and infection. Furthermore, the presence of microbes and hypoxia in the inflamed lung leads to activation of NF-kappaB which impacts upon the expression of genes involved in innate immunity and inflammation both directly and through the amplification of the HIF pathway.