INFLAMMATION IN CHRONIC GRANULOMATOUS DISEASE AND MODULATION OF HUMAN DENDRITIC CELL FUNCTIONS BY BURKHOLDERIA CENOCEPACIA AND BURKHOLDERIA MULTIVORANS

by

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Abstract

Inflammation and infection are integral to the human diseases cystic fibrosis (CF) and chronic granulomatous disease (CGD). Inflammation was examined in peripheral blood mononuclear cells (PBMCs) from CGD patients. Reactive oxygen species (ROS) generated by the phagocytic NADPH oxidase have been implicated in the activation of the NF-κB, a transcription factor required for proinflammatory cytokine production in response to inflammatory stimuli. Patients with CGD, an immunodeficiency characterized by the inability to produce ROS, frequently develop inflammatory complications indicative of exaggerated inflammatory responses. In the present study, human and murine CGD leukocytes displayed a hyperinflammatory phenotype with increased production of proinflammatory cytokines in response to Toll-like receptor agonists. The major steps involved in NF-κB activation were also intact in human CGD cells. ROS were therefore not required for NF-κB activation and ROS production may instead dampen inflammation. The interaction of *Burkholderia cepacia* complex (BCC) bacteria with primary human monocyte-derived dendritic cells (DCs) was also explored as a model of infection. *B. cenocepacia* and *B. multivorans*, the most clinically important BCC members, are serious opportunistic pathogens infecting CF and CGD patients. The present study investigated whether these pathogens could modulate normal functions of DCs, important phagocytic cells that act as orchestrators of the immune response. DCs co-incubated for 24 h with *B. cenocepacia*, but not *B. multivorans*, had reduced expression of co-stimulatory molecules when compared with BCC lipopolysaccharide-matured DCs, as determined using flow cytometry. *B. cenocepacia*, but not *B. multivorans*, also induced necrosis in DCs after 24 h, as determined by annexin V and propidium iodide staining. DC necrosis only occurred after phagocytosis of live *B. cenocepacia*; DCs exposed to heat-killed bacteria, bacterial supernatant, or those pre-treated with cytochalasin D then exposed to live bacteria remained viable. The intracellular lifestyle of BCC bacteria was
also examined using transmission electron microscopy. After 6 h of co-incubation with DCs, *B. cenocepacia* occupied the phagosome while *B. multivorans* resided in the cytoplasm. The ability of *B. cenocepacia* to modulate DC functions may contribute to its pathogenicity. Understanding the sophisticated mechanisms of infection and inflammation may lead to better treatments for CF and CGD.
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List of Abbreviations

AP-1  activator protein-1
ATPase  adenosine triphosphatase
BCC  Burkholderia cepacia complex
CBA  cytometric bead array
CD  cluster of differentiation
CF  cystic fibrosis
CFTR  cystic fibrosis transmembrane conductance regulator
CGD  chronic granulomatous disease
CL  chemiluminescence
DCs  dendritic cells
DNA  deoxyribonucleic acid
DPI  diphenyleneiodonium
EDTA  ethylenediaminetetraacetic acid
ELISA  enzyme-linked immunosorbent assays
EMSA  electrophoretic mobility shift assay
ERK-1/2  extracellular response kinase-1/2
EthD-1  ethidium homodimer
ΔF508  deletion of phenylalanine at codon 508
FACS  fluorescent-activated cell sorting
FCS  fetal calf serum
FITC  fluorescein isothiocyanate
GM-CSF  granulocyte-macrophage colony stimulating factor
GTPases  guanosine triphosphatases
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>IDO</td>
<td>indolamine 2,3-dioxygenase</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>interferon-γ</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>iNOS</td>
<td>inducible nitric oxide synthase</td>
</tr>
<tr>
<td>KRG</td>
<td>Krebs-Ringer phosphate buffer with glucose</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>MAP kinase</td>
<td>mitogen activated protein kinase</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MOI</td>
<td>multiplicity of infection</td>
</tr>
<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor-κB</td>
</tr>
<tr>
<td>p65i</td>
<td>p65 inhibitor peptide</td>
</tr>
<tr>
<td>PBMCs</td>
<td>peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PE</td>
<td>phycoerythrin</td>
</tr>
<tr>
<td>PGN</td>
<td>peptidoglycan</td>
</tr>
<tr>
<td>PHA</td>
<td>phytohaemagglutinin</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumour necrosis factor-α</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
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Co-authorship Statement

The second chapter of my thesis was a collaborative effort. As second author of our publication, I made a significant contribution to all aspects of the research program. I designed, performed, and analyzed the initial set of experiments looking at inflammation in CGD leukocytes, which formed the basis of the paper. Working independently or as part of a team, I also isolated PBMCs, prepared nuclear and cytoplasmic extracts, and performed some Western blotting and EMSA experiments. I was not directly involved in the mouse experiments, which were all done in Sweden. I designed, performed, and analyzed most of the additional experiments added during revisions or required by reviewers, including those outlined in Appendices 5 and 6, and the phenotyping of CGD and normal PBMCs. I also made an important contribution to manuscript preparation by revising and editing the manuscript.
1. Introduction

One of the fascinating aspects of the study of human disease, despite a bewildering and diverse array of causes, manifestations, treatments and outcomes, is the integral role often played by infection and inflammation. The study of these unique processes is therefore vital to the understanding and treatment of disease. The innate immune system represents a critical component of the host response to infection by microbial pathogens and is responsible for much of the inflammation generated in response to infectious agents, other noxious substances, or defects within the host. Phagocytic cells are key cellular mediators of the innate immune system; therefore, it is important to understand their role in infection and inflammation.

Chronic granulomatous disease (CGD) and cystic fibrosis (CF) are two discrete human diseases with unique genetic causes. However, both CF and CGD patients often endure excessive inflammation and serious lung infections by opportunistic bacterial pathogens within the *Burkholderia cepacia* complex. There is compelling evidence that phagocytes play an important role in both CGD and CF. In order to gain insight into these diseases, this thesis will explore the inflammatory response in CGD and the interaction of *B. cepacia* complex bacteria with human dendritic cells (DCs).

The purpose of this initial chapter is to introduce CGD and CF, with special emphasis on the role of inflammation and infection. The phagocytic system will also be summarized. I will then describe the *B. cepacia* complex, focusing on its clinical features, virulence factors, ability to subvert host cells, and the emerging field of genomics. This literature review will lead to a discussion of the main themes and hypotheses of the thesis. One of the outstanding questions in CGD research, whether or not superoxide radicals influence inflammatory cell signaling, will be

introduced. I will also describe DCs as a model to explore the dynamic interplay between *B. cepacia* complex and host phagocytic cells.

### 1.1 Cystic fibrosis

Cystic fibrosis (CF) is the most common life threatening genetic disease among Caucasians, occurring at a frequency of about 1 in 2500 newborns (Gibson *et al.*, 2003; Davies *et al.*, 2007), with a median survival age in Canada of 37 years (Canadian Cystic Fibrosis Foundation, 2002). An autosomal recessive disorder, CF is caused by mutations in a single gene on chromosome 7 that encodes the cystic fibrosis transmembrane conductance regulator (CFTR) (Rommens *et al.*, 1989). Over 1500 mutations in the CFTR have been described, but the most common mutation is deletion of phenylalanine at codon 508 (ΔF508), which occurs in about 70% of patients with CF (Gibson *et al.*, 2003; Davies *et al.*, 2007).

The CFTR is a member of the adenosine triphosphate-binding cassette protein family and acts as a cyclic adenosine monophosphate-regulated chloride ion channel (Rowe *et al.*, 2005). Defective CFTR leads to the systemic production of thick dehydrated mucus, affecting multiple organ systems and resulting in: pancreatic insufficiency, intestinal obstruction, infertility and lung disease; the last represents the most significant cause of morbidity and mortality in CF patients (Gibson *et al.*, 2003; Rowe *et al.*, 2005; Davies *et al.*, 2007). Abnormal mucus secretions impair mucociliary clearance in the lungs, an essential mechanism for removing inhaled particles and microbes, allowing opportunistic microbial pathogens to colonize the otherwise sterile lower airways. Micro-organisms are typically acquired in an age-dependent sequence, with early airway infections caused by *Staphylococcus aureus* and *Haemophilus influenzae*, followed by later infections with organisms such as *Pseudomonas aeruginosa*, *B. cepacia* complex, *Stenotrophomonas maltophilia*, *Achromobacter xylosoxidans*, *Aspergillus sp.*, and nontuberculous mycobacteria (Gibson *et al.*, 2003). CF lung disease also manifests as intense inflammation, with excessive infiltration of neutrophils (Rowe *et al.*, 2005; Downey *et
The microenvironment of the CF lung creates barriers both physical (thick dehydrated mucus, microbial biofilms) and chemical (an ion dysregulation which may inhibit antimicrobial peptide function and create an oxidant-antioxidant imbalance) (Speert, 2002; Gibson et al., 2003; Matsui et al., 2005), thus preventing neutrophils from eradicating CF pathogens. In aborted attempts to target pathogens, neutrophils release cytokines, and toxic compounds such as proteases and superoxide, which injure surrounding tissue and signal the influx of more innate immune cells (Elizur et al., 2008; Downey et al., 2009). The net result of these repeating cycles of infection and inflammation is progressive lung damage and ultimately death (Gibson et al., 2003).

The hope of correcting the underlying CFTR defect or of improving its function has been the impetus for much CF research. However, CF gene therapy remains elusive, and potentiators of the defective CFTR are still in early clinical trials. Therefore, treating infection and inflammation in CF lung disease remains at the forefront of therapeutic strategies for this disease. Clinical trials into corticosteroid (Auerbach et al., 1985; Eigen et al., 1995) and ibuprofen therapy (Konstan et al., 1995; Konstan et al., 2007) show promise, although the side effects may outweigh the anti-inflammatory benefits (Elizur et al., 2008; Ratjen, 2008). Antibiotic therapy is essential to delay onset of chronic bacterial colonization, for routine use as maintenance therapy, and to treat pulmonary exacerbations. However, widespread antibiotic resistance amongst many CF pathogens and the varying abilities of antibiotics to penetrate the congested CF lung impair the efficacy of these drugs (Gibson et al., 2003).

### 1.2 Chronic granulomatous disease

Chronic Granulomatous Disease (CGD) is a rare primary immunodeficiency, with an estimated incidence in the United States of between 1 in 200,000 and 1 in 250,000 live births (Winkelstein et al., 2000). This disease is caused by mutations in genes encoding components of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, a plasma membrane-
phagosomal-associated enzyme complex found in neutrophils, other granulocytes, monocytes, macrophages, dendritic cells, and B lymphocytes (Babior, 1999) which is responsible for generating the respiratory burst (Dinauer et al., 2000; Segal et al., 2000; Bylund et al., 2005a). The NADPH oxidase is composed of five main subunits: the membrane bound gp91phox and p22phox, which together form a heterodimeric flavoprotein known as cytochrome b558, and the cytosolic components p40phox, p47phox and p67phox, which exist as a complex that upon activation migrates to the membrane and associates with cytochrome b558 to form the active enzyme (Nauseef, 2008). Rac1 and Rac2 GTPases seem to play important roles in the assembly of the phagocyte oxidase in mononuclear cells and neutrophils, respectively (Fang, 2004). Seventy percent of patients have the X-linked recessive form of CGD, resulting from mutations in the gene encoding gp91phox. Autosomal recessive forms of the disease arise from genetic mutations in p22phox, p47phox and p67phox, which are found in two, 12, and three percent of patients, respectively (Winkelstein et al., 2000). Defects in gp91phox, p22phox and p67phox are associated with the most severe form of CGD while mutations in p47phox tend to cause mild disease (Dinauer et al., 2000; Bylund et al., 2005a). Patients with mutations in gp91phox, p22phox and p67phox are diagnosed with CGD at an earlier age, have more infections, and significantly higher mortality rate than patients defective in p47phox (Winkelstein et al., 2000). This is believed to be due to the fact that p47phox-deficient patients can produce residual amounts of superoxide.

The basic defect in superoxide production has profound implications for CGD patients. Without one of their most potent phagocytic killing mechanisms, CGD patients are vulnerable to recurrent infections, which manifest most commonly as pneumonia (in 79% of patients), abscesses (in 68% of patients), lymphadenitis (in 53% of patients), osteomyelitis (in 25% of patients), and sepsis (in 18% of patients) (Winkelstein et al., 2000). Fungal pathogens of CGD patients include Aspergillus spp., in particular A. fumigatus and A. nidulans, Paecilomyces spp., Candida albicans, Penicillium spp. and various genera with the phaeohyphomycete group (Segal
et al., 2000; Winkelstein et al., 2000). CGD patients are often infected with catalase-positive bacteria, including the Gram-positive organisms *S. aureus* and *Nocardia spp.*, and the Gram-negative organisms *B. cepacia complex, Serratia marcescens, Klebsiella spp.*, and *Chromobacteria violaceum* (Segal et al., 2000; Winkelstein et al., 2000). The failure to prevent, manage and resolve fungal and bacterial infections adequately is the most serious complication of CGD.

In addition to infections, CGD patients also exhibit enhanced inflammatory responses, leading to the frequent formation of tissue granulomas. Granulomas are usually created when groups of immune cells organize around a persistent foreign invader, such as a microbe, and generate a sustained inflammatory assault that does not resolve (Johnston, 2001; Bylund et al., 2005a). This typical presentation sometimes occurs in CGD patients. However, CGD granulomas are frequently sterile, and uncontrolled inflammation often occurs without any sign of infection (Segal et al., 2000). Gastrointestinal and genitourinary obstructions result from the formation of granulomas in the hollow viscera. CGD patients also present with other non-infectious sequelae including skin ulceration, pathological healing of surgical wounds, inflammatory bowel diseases similar to Crohn’s disease, and autoimmune diseases akin to systemic lupus erythematosus (Segal et al., 2000).

Successful resolution of CGD is ultimately aimed at replacing defective cells with healthy cells. Bone marrow transplantation is curative for CGD patients, but the high rates of associated morbidity and mortality preclude its routine use (Segal et al., 2000). Researchers have also investigated hematopoietic stem cell gene therapy in the hopes of generating a stable population of myeloid precursors with a functional NADPH oxidase that could reconstitute a biologically significant proportion of normal phagocytes (Dinauer et al., 2000; Segal et al., 2000). Patients with a mixed chimeric myeloid population should be essentially healthy, with perhaps some residual increased susceptibility to autoimmune diseases like lupus, as is seen with maternal
carriers of X-linked CGD (Dinauer et al., 2000; Winkelstein et al., 2000). More research is needed to improve the transduction efficiency, stability, and differentiation potential of re-introduced corrected myeloid precursors in order for this therapy to move beyond clinical trials (Segal et al., 2000).

While potential cures remain elusive, CGD is managed by a combination of acute and prophylactic therapies, treatment of acute infections, and therapy for inflammatory complications (Bylund et al., 2005a). The most common drugs used prophylactically in CGD are the antibiotic trimethoprim sulfamethoxazole and the antifungal agent itraconazole. Interferon-γ (IFN-γ) which enhances the bactericidal capacity of macrophages and is required to kill certain intracellular bacterial infections (Rosenberger and Finlay, 2003), is also used prophylactically to treat CGD patients and appears to diminish serious infections, including those caused by B. cepacia complex (Marciano et al., 2004). Its mechanism of action in CGD is unclear, and some experts do not believe that IFN-γ plays a salutary role in CGD. IFN-γ has been reported to increase nitric oxide production by neutrophils, but it is unclear whether this minor difference has any relevance to neutrophil killing (Ahlin et al., 1999).

Successful treatment of CGD is hampered by the fact that patients often present with mild symptoms and few signs of overt disease or are asymptomatic, even when suffering from life-threatening infections with organisms such as Aspergillus spp. Thus, heightened vigilance is required and antimicrobial agents are often prescribed upon a hint of infection and tailored to the most likely infectious agents when the suspected pathogen is not readily identified (Johnston, 2001; Bylund et al., 2005a). Corticosteroids are often prescribed to treat inflammatory conditions related to this disease, often leading to rapid improvement for CGD patients (Dinauer et al., 2000; Bylund et al., 2005a). A novel therapeutic strategy which involves bypassing the redox-sensitive indolamine 2,3-dioxygenase (IDO), the rate-limiting enzyme for tryptophan catabolism in the kynurenine pathway, has recently been proposed (Segal et al., 2009). An IDO
blockade caused by the absence of the ROS co-factor in CGD mice leads to T cell skewing toward an inflammatory Th17 phenotype, impairment of tolerogenic T regulatory cell development, and mortality in CGD mice challenged with *Aspergillus fumigatus* (Romani et al., 2008; Segal et al., 2009). However, administration of interferon-γ and exogenous L-kynurenine, a metabolite downstream of IDO, augments production of anti-inflammatory T regulatory cells, leading to improved outcomes in murine models of CGD (Romani *et al.*, 2008; Segal *et al.*, 2009).

The clinical picture in both CF and CGD is characterized by recurrent cycles of infection and/or inflammation. Clearly, more research is needed to understand the processes of infection and inflammation in CF and CGD, which should inspire innovative treatments for these life-threatening diseases.

### 1.3 Phagocytic cell biology

Phagocytes play an integral role in the infectious and inflammatory processes seen in CF and CGD; therefore, it is important to provide some background context for ensuing discussions. The human phagocytic system is an essential component in both innate and adaptive immune responses; it is comprised of neutrophils, monocytes, macrophages and dendritic cells. These highly specialized cells bind and internalize microbes through the sophisticated process of phagocytosis. The ultimate goal of the phagocytic system is to kill or control infectious microbes and to orchestrate an appropriate immune response to eradicate the infection. Because the lung is a common site of infection in CF and CGD patients, I will introduce the phagocytic system by describing its role in the healthy and infected lung.

Neutrophils play a crucial role in infection and inflammation. They are the most abundant leukocyte in the peripheral blood and are rapidly recruited to the site of infection along a chemotactic gradient (Bylund *et al.*, 2005a). Though they lack the broad array of non-opsonic phagocytic receptors found on mononuclear cells, they are still capable of non-opsonic
phagocytosis and extremely adept at opsonic phagocytosis (Allen, 2003). Individual neutrophils can rapidly phagocytose hundreds of different particles in the presence of complement and antibodies (Li et al., 2002). Most importantly, neutrophils are supremely potent bactericidal phagocytic cells (Allen, 2003; Bylund et al., 2005a). Oxygen-dependent and independent mechanisms are both employed to eradicate invading microbes. Neutrophils have abundant granules loaded with antimicrobial peptides and catalytic enzymes that, upon activation, can fuse with the plasma membrane for extracellular release or can fuse with the phagosomal membrane to target intracellular microbes. Oxidative killing by neutrophils is intricate and effective. When activated by phagocytosis or soluble agonists, the NADPH oxidase catalyzes the transfer of electrons from cytoplasmic NADPH across the membrane to molecular oxygen, forming the superoxide anion. This weakly bactericidal species is rapidly dismutated to hydrogen and hydrogen peroxide. Hydrogen peroxide is more stable and also diffuses across biological membranes. When hydrogen peroxide reacts with myeloperoxidase, an enzyme located in azurophilic granules, the highly toxic hypocholorous acid (or bleach) is formed (Roos et al., 2003; Bylund et al., 2005a). Reactive oxygen species may also enhance microbial killing indirectly through activation of phagosomal proteases (Reeves et al., 2002). In neutrophils, the membrane bound cytochrome b$_{558}$, composed of gp91$^{phox}$ and p22$^{phox}$ subunits of the NADPH oxidase, resides within intracellular granule membranes and within the plasma membrane. Therefore, upon activation and assembly of the complete NADPH oxidase, the respiratory burst can be generated intracellularly or extracellularly (Karlsson and Dahlgren, 2002). Neutrophil phagosomes also recruit the vacuolar-ATPase, which activates acidophilic proteases to complete microbe killing (Savina and Amigorena, 2007). The sheer speed of granule mobilization and the ability to generate large amounts of various toxic reactive oxygen species (ROS) make neutrophils swift and proficient killers (Allen, 2003).
Neutrophils are short lived cells, with a half-life of about one day in the peripheral blood or tissue (Akgul et al., 2001); as such, their necrotic death may be responsible for much of the pathology associated with excess inflammation in diseases like CF (Simon, 2003). The bactericidal mediators they secrete are toxic to host cells, so there is always some collateral damage during bacterial killing. However, following activation and bacterial killing, neutrophils normally undergo apoptosis, or programmed cell death, and are efficiently phagocytosed and safely degraded by other host cells, usually macrophages (Savill et al., 1989). Disruption of this cycle can lead to neutrophil necrosis, where neutrophils continually lyse and release all their degradative components into the extracellular milieu, resulting in chronic inflammation (Savill et al., 1989).

Monocytes are the major mononuclear phagocyte of the blood. They have a much longer lifespan than neutrophils, and migrate more slowly to sites of infection or inflammation (Dale et al., 2008). Abundant opsonic and non-opsonic phagocytic receptors are constitutively expressed by monocytes. The respiratory burst of monocytes is less robust than that of neutrophils (Dale et al., 2008), but stronger than that of macrophages or DCs (Vulcano et al., 2004). Unlike neutrophils, monocytes have tremendous capacity to enhance production of granule proteins and to generate cytokines and chemokines through de novo protein synthesis (Dale et al., 2008). Monocytes also have the ability to differentiate into pulmonary macrophages and DCs, although the exact details of this process and the specific monocyte sub-populations which may act as precursors for particular cell types in vivo are still unclear (Gordon and Taylor, 2005; Randolph et al., 2008). It is believed that, under steady state conditions, pulmonary DCs arise from monocyte precursors, whereas macrophages are replenished from local proliferation of resident cells. However, monocytes do differentiate into both macrophages and DCs under conditions of inflammatory or infectious insults (Gordon and Taylor, 2005; Randolph et al., 2008)
Macrophages and dendritic cells are resident phagocytic cells in the lung. They mediate bacterial uptake, processing and presentation, vital functions that are often subverted by bacterial pathogens in order to gain access to this privileged intracellular niche (Cutler et al., 2001; Pieters, 2001; Gordon and Read, 2002; Palucka and Banchereau, 2002; Rosenberger and Finlay, 2003).

Macrophages, “the journeymen of the immune system”, are vital mediators of the early response to infectious assault (Rosenberger and Finlay, 2003). They are abundant in tissues such as the lung, both interstitially, beneath mucosal surfaces of the upper airway, and in the lumen of the lower airway, as specialized alveolar macrophages. Inhaled particles are rapidly phagocytosed by macrophages that use a diverse array of non-opsonic and opsonic receptors. Reactive oxygen and nitrogen intermediates can be generated within infected macrophage phagolysosomes (Fang, 2004). Though very efficient at phagocytosis, macrophages are not as effective as neutrophils at killing bacteria. Most of the NADPH oxidase appears to be assembled on the plasma membrane and not the phagosomal membrane (Linehan and Holden, 2003), and they are effete in their production of ROS. There are conflicting published data, but macrophages have been reported to lack myeloperoxidase, and therefore fail to generate the most toxic ROS, hypochlorous acid (Allen, 2003; Fang, 2004). However, macrophage phagosomes do rapidly mature into phagolysosomes, acquiring lysosomal degradative enzymes and the vacuolar-ATPase, which enhances their degradative capacity (Savina and Amigorena, 2007). Phagosomal maturation is slower in the macrophage than the neutrophil, giving the internalized microbe more time to adapt to the intracellular environment and perhaps avoid being killed (Allen, 2003). Upon activation with IFN-γ, macrophages induce transcription of the inducible nitric oxide synthase (iNOS) which catalyzes the formation of nitric oxide radicals, capable of interacting with ROS to generate bactericidal species such as nitrogen dioxide and peroxynitrate.
Macrophages are therefore more potently microbicidal than resting cells.

Macrophages are also potent secretors of cytokines and chemokines that recruit other inflammatory cells, such as neutrophils, monocytes and macrophages, to the site of infection. They are capable of presenting phagocytosed and degraded antigen via the major histocompatibility complex (MHC) to lymphocytes of the adaptive immune system, particularly at localized sites of infection. These versatile cells are vitally important in innate immunity as rapid initiators of the immune response and collaborators with more specialized phagocytes in microbial killing and antigen presentation (Rosenberger and Finlay, 2003).

Dendritic cells are crucial mediators between innate and adaptive immunity. Immature dendritic cells act as sentinels in the tissue; they are very efficient in antigen capture through macropinocytosis, receptor-mediated endocytosis, and phagocytosis (Banchereau et al., 2000). They express a broad repertoire of opsonic and non-opsonic phagocytic receptors and are capable of binding and internalizing microbes (Banchereau et al., 2000; Cutler et al., 2001). DC phagosomal maturation differs from macrophages and neutrophils. Proteases are abundant in DC phagosomes, but reduced recruitment of lysosomes leads to lower activity of the vacuolar-ATPase, resulting in lower proteolytic activity (Trombetta et al., 2003; Savina and Amigorena, 2007). The NADPH oxidase is also less active in DC phagosomes, making the quantity and rate of ROS production much lower in dendritic cells than in neutrophils or macrophages (Vulcano et al., 2004; Savina and Amigorena, 2007). Upon capture of soluble antigen or bacteria, immature dendritic cells migrate to secondary lymphoid organs while undergoing phenotypic and functional changes that transform them from antigen capturing cells to mature antigen processing and presenting cells. DCs are the supreme antigen presenting cell, with much higher expression of major histocompatibility and co-stimulatory molecules than other phagocytic cells and the unique and critical capacity to activate naïve T cells, inducing the appropriate immune response.
response to deal with the invading microbe (Banchereau and Steinman, 1998; Banchereau et al., 2000; Kapsenberg, 2003). Reduced proteolysis in DC phagosomes is thought to better preserve antigens for antigen presentation (Banchereau and Steinman, 1998; Trombetta et al., 2003; Savina and Amigorena, 2007), but this may have unfortunate consequences for the ability of DCs to control intracellular pathogens. DC maturation does enhance both phagosomal acidification and ROS production (Trombetta et al., 2003; Vulcano et al., 2004; Savina and Amigorena, 2007), but the relevance of these events for intracellular bacterial pathogenesis is not yet known.

1.4 *Burkholderia cepacia* complex

The *B. cepacia* complex (BCC) is a group of closely related Gram negative β-proteobacteria that have diverse roles as environmentally beneficial agents, phytopathogens, and opportunistic human pathogens. The original member of this complex, *B. cepacia*, was first described as the etiological agent of sour skin rot in onions (Burkholder, 1950). Environmentally, *B. cepacia* complex bacteria are also capable of bioremediation, biocontrol, and plant growth promotion (Mahenthiralingam et al., 2005). Due to their tremendous metabolic capacity, these bacteria can break down phthalates, herbicides, and chlorinated hydrocarbons, including trichloroethylene, a common groundwater toxin (Parke and Gurian-Sherman, 2001; Coenye and Vandamme, 2003). BCC bacteria also prevent fungi from attacking young seedlings and from causing fruit molding, mainly through the production of anti-fungal compounds. These bacteria can also fix nitrogen, which boosts growth of important food crops such as rice, wheat, and maize (Parke and Gurian-Sherman, 2001). However, the ecological potential of the BCC has been overshadowed by its emerging role as opportunistic human pathogens.

BCC causes serious infections in patients with CGD and CF (Isles et al., 1984; Corey and Farewell, 1996; Hutchison and Govan, 1999; Winkelstein et al., 2000; Johnston, 2001; Speert, 2002). BCC is the most virulent Gram negative bacterium infecting CGD patients, as
bacteriemia can develop following lung infections with this pathogen (Winkelstein et al., 2000; Speert, 2002). CF patients are also susceptible to bacterial lung infections with a few characteristic pathogens. *Pseudomonas aeruginosa* is the most prevalent CF pathogen. However, CF patients infected with BCC bacteria have a higher proportional hazard of death than those infected with *P. aeruginosa* or infected with neither (Corey and Farewell, 1996; Hutchison and Govan, 1999; Chaparro et al., 2001). A retrospective review of the Toronto lung transplant program demonstrated that CF patients colonized with BCC bacteria had a much lower one-year survival following double lung transplantation than uncolonized CF patients (67% vs. 92%) (Chaparro et al., 2001). Approximately 20% of BCC-infected CF patients develop “cepacia syndrome”, characterized by rapid pulmonary deterioration, septicaemia, and death, a complication which is not associated with *P. aeruginosa* infections (Hutchison and Govan, 1999).

The BCC is comprised of 17 distinct species (Table 1.1) (Vanlaere et al., 2009), most of which have been isolated from patients (Mahenthiralingam et al., 2002). However, two species in particular, *B. multivorans* and *B. cenocepacia*, are the two most clinically significant members of the BCC and display distinct pathogenicity in CF. Prior to rigorous isolation of CF patients colonized with BCC, *B. cenocepacia* comprised 80% of BCC clinical CF isolates in Canada (Chaparro et al., 2001; Speert et al., 2002). However, the global epidemiology of the BCC may be shifting, as reports from the United States and the United Kingdom demonstrate that *B. multivorans* is now the most prevalent BCC species isolated from CF patients (Reik et al., 2005; Govan et al., 2007). *B. cenocepacia* is associated with a higher mortality rate than *B. multivorans* and, unlike most strains of *B. multivorans*, is readily transmissible among patients, resulting in multiple epidemics (Chaparro et al., 2001; Speert et al., 2002).

BCC are clearly dangerous pathogens for these vulnerable patients. A combination of bacterial and host factors likely play a role in their pathogenesis. The following section will
review the ability of these bacteria to colonize the human lung, abrogate host defenses, and infect CF and CGD patients, highlighting the virulence factors that have been described, *in vitro* and *in vivo* models that have been used to study BCC pathogenesis, and the emerging field of BCC genomics.

Table 1.1 *Species within the BCC and their clinical significance in CF (adapted from (Mahenthiralingam et al., 2005)).*

<table>
<thead>
<tr>
<th>Organism</th>
<th>Previous designation</th>
<th>Importance in CF</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. cepacia</em></td>
<td>Genomovar I</td>
<td>Low prevalence in CF</td>
<td>(Vandamme <em>et al.</em>, 1997; Mahenthiralingam <em>et al.</em>, 2002; Vandamme <em>et al.</em>, 2003)</td>
</tr>
<tr>
<td><em>B. multivorans</em></td>
<td>Genomovar II</td>
<td>Second most common BCC species in CF, some isolates transmissible</td>
<td>(Vandamme <em>et al.</em>, 1997; Mahenthiralingam <em>et al.</em>, 2002)</td>
</tr>
<tr>
<td><em>B. cenocepacia</em></td>
<td>Genomovar III</td>
<td>Most common BCC species in CF, causes poor prognosis, highly transmissible, many epidemic strains described</td>
<td>(Vandamme <em>et al.</em>, 1997; Mahenthiralingam <em>et al.</em>, 2002)</td>
</tr>
<tr>
<td><em>B. stabilis</em></td>
<td>Genomovar IV</td>
<td>Low prevalence in CF</td>
<td>(Vandamme <em>et al.</em>, 1997; Mahenthiralingam <em>et al.</em>, 2002)</td>
</tr>
<tr>
<td><em>B. vietnamiensis</em></td>
<td>Genomovar V</td>
<td>Low prevalence in CF</td>
<td>(Vandamme <em>et al.</em>, 1997; Mahenthiralingam <em>et al.</em>, 2002)</td>
</tr>
<tr>
<td><em>B. dolosa</em></td>
<td>Genomovar VI</td>
<td>Rarely causes infections in CF, transmissible, some epidemic strains described in limited geographic areas</td>
<td>(Coenye <em>et al.</em>, 2001; Mahenthiralingam <em>et al.</em>, 2002; Biddick <em>et al.</em>, 2003; Vermis <em>et al.</em>, 2004)</td>
</tr>
<tr>
<td><em>B. ambifaria</em></td>
<td>Genomovar VII</td>
<td>Rarely causes infections in CF</td>
<td>(Mahenthiralingam <em>et al.</em>, 2002)</td>
</tr>
<tr>
<td><em>B. anthina</em></td>
<td>Genomovar VIII</td>
<td>Rarely causes infections in CF</td>
<td>(Vandamme <em>et al.</em>, 2002)</td>
</tr>
<tr>
<td><em>B. pyrocinia</em></td>
<td>Genomovar IX</td>
<td>Rarely causes infections in CF</td>
<td>(Vandamme <em>et al.</em>, 2002)</td>
</tr>
<tr>
<td><em>B. ubonensis</em></td>
<td>Genomovar X</td>
<td>No reported infections in CF</td>
<td>(Vanlaere <em>et al.</em>, 2008)</td>
</tr>
<tr>
<td><em>B. latens</em></td>
<td></td>
<td>Rarely causes infections in CF</td>
<td>(Vanlaere <em>et al.</em>, 2008)</td>
</tr>
<tr>
<td><em>B. diffusa</em></td>
<td></td>
<td>Rarely causes infections in CF</td>
<td>(Vanlaere <em>et al.</em>, 2008)</td>
</tr>
<tr>
<td><em>B. arboris</em></td>
<td></td>
<td>Rarely causes infections in CF</td>
<td>(Vanlaere <em>et al.</em>, 2008)</td>
</tr>
<tr>
<td><em>B. seminalis</em></td>
<td></td>
<td>Rarely causes infections in CF</td>
<td>(Vanlaere <em>et al.</em>, 2008)</td>
</tr>
<tr>
<td><em>B. metallica</em></td>
<td></td>
<td>Rarely causes infections in CF</td>
<td>(Vanlaere <em>et al.</em>, 2008)</td>
</tr>
<tr>
<td><em>B. contaminans</em></td>
<td>Taxon K</td>
<td>Rarely causes infections in CF, some epidemic strains described</td>
<td>(Vanlaere <em>et al.</em>, 2009)</td>
</tr>
<tr>
<td><em>B. lata</em></td>
<td>Taxon K</td>
<td>Rarely causes infections in CF</td>
<td>(Vanlaere <em>et al.</em>, 2009)</td>
</tr>
</tbody>
</table>
1.5 Antibiotic Resistance

BCC bacteria are intrinsically resistant to many antibiotics, most notably β-lactams, polymixin B, and aminoglycosides (Mahenthiralingam et al., 2005). Penicillin G can in fact be metabolized as the only source of carbon (Beckman and Lessie, 1979). Most isolates are also resistant to chloramphenicol, trimethoprim, and tetracycline, and resistance to quinolones has also been reported (Nzula et al., 2002; Mahenthiralingam et al., 2005). This organism utilizes multiple antibiotic resistance mechanisms, including reduced bacterial outer membrane permeability (Moore and Hancock, 1986; Parr et al., 1987; Aronoff, 1988; Vinion-Dubiel and Goldberg, 2003; De Soyza et al., 2008), production of inactivating enzymes such as β-lactamases (Chiesa et al., 1986; Trepanier et al., 1997), modification of drug targets such as production of a trimethoprim-resistant dihydrofolate reductase (Burns et al., 1989), and multidrug efflux pumps (Burns et al., 1996a; Fehlner-Gardiner and Valvano, 2002; Wigfield et al., 2002; Nair et al., 2004; Guglierame et al., 2006).

1.6 Virulence factors that may aid in host colonization

1.6.1 Adhesins

BCC produce five morphologically distinct pili (Goldstein et al., 1995), but only one type, the 2 to 4 µm long, peritrichously arranged cable pilus, is associated with epidemic strains (Sun et al., 1995). B. cenocepacia strains which co-express cable pili, along with an associated 22 kDa adhesin, bind to cytokeratin 13, an intermediate filament protein highly expressed by CF airway epithelial cells (Sajjan and Forstner, 1993; Sajjan et al., 2000). These adhesins also mediate binding to mucin (Sajjan and Forstner, 1992) and buccal epithelial cells (Sajjan et al., 2003), binding and translocation across squamous epithelial layers (Sajjan et al., 2003), and prevention of autoaggregation among bacterial cells (Tomich and Mohr, 2003).
A *B. cenocepacia* lectin called BclA has recently been identified (Lameignere et al., 2008). BclA binds to mannoses, specifically oligomannose-type N-glycan structures present on human glycoproteins. However, the role of BclA in adherence *in vitro* or *in vivo* has yet to be established (Lameignere et al., 2008).

1.6.2 Flagella

Two types of flagella have been described in the BCC which differ in size and restriction fragment length polymorphism patterns (Hales *et al.*, 1998). *B. cenocepacia* mutants lacking flagella had significantly reduced invasion of undifferentiated human A549 epithelial cells, though bacterial adherence was similar for flagellated and non-flagellated bacteria centrifuged onto cell monolayers (Tomich *et al.*, 2002). Mice exposed to non-flagellated *B. cenocepacia* mutants also had increased survival in a mouse agar bead model as compared to those inoculated with wild type bacteria (Urban *et al.*, 2004). Mice infected with mutant and wild type bacteria had similar percent weight loss, bacterial loads in the lungs and spleen, and lung infiltration of immune cells. However, there was elevation of KC, a murine interleukin-8 homologue, in bronchioalveolar lavage fluid and serum of mice infected with wild type *B. cenocepacia*, suggesting that flagella contribute to pro-inflammatory cytokine production induced by *B. cenocepacia* (Urban *et al.*, 2004). This has been confirmed by recent studies describing a prominent role for toll-like receptor 5, the receptor for flagella, in the lung epithelial pro-inflammatory response to *B. cenocepacia* (Blohmke *et al.*, 2008; de C. Ventura *et al.*, 2008). Interestingly, there is also upregulation of many flagellar genes when *B. cenocepacia* is grown in CF sputum (Drevinek *et al.*, 2008).

1.6.3 Siderophores

Iron is essential for bacterial growth, but is tightly sequestered in lactoferrin, transferrin and heme proteins in the human body. Bacterial pathogens often produce siderophores, low molecular weight ferric iron chelators, to facilitate the acquisition and transport of iron. The
BCC produce four siderophores: ornibactins, salicylic acid, pyochelin, and cepabactin. Ornibactin is essential for virulence of *B. cenocepacia* in the rat agar bead model of infection (Visser *et al.*, 2004).

1.6.4 Exopolysaccharide production and biofilm formation

BCC produce at least five types of exopolysaccharides (Chiarini *et al.*, 2004; Conway *et al.*, 2004) and the biosynthetic cluster of cepacian, the most common type of exopolysaccharide, has been characterized (Moreira *et al.*, 2003) In liquid media, exopolysaccharide appears to be stably produced upon entry into stationary phase (Richau *et al.*, 2000). Mucoid BCC have been widely found, indeed the first nine species within the BCC can produce exopolysaccharide (Zlosnik *et al.*, 2008). Production of *B. cenocepacia* exopolysaccharide is correlated with reduced association with human macrophages and neutrophils and persistence in an intranasal mouse model (Chung *et al.*, 2003; Conway *et al.*, 2004). Along with reduced association with neutrophils, *B. cenocepacia* exopolysaccharide also inhibits neutrophil chemotaxis and scavenges reactive oxygen species (Bylund *et al.*, 2005c). The cepacian *bce* gene cluster is induced by mannitol, raising questions about the therapeutic benefit of using inhaled mannitol in CF to increase hydration of airway secretions (Bartholdson *et al.*, 2008; Reid and Bell, 2009).

Exopolysaccharide is clearly a virulence factor for BCC associated with persistence of the bacteria in the host. However, it has recently been demonstrated that *B. cenocepacia* is frequently non-mucoid (Bartholdson *et al.*, 2008; Zlosnik *et al.*, 2008). Members of the *B. cenocepacia* ET12 lineage contain an 11-base pair deletion within the *bceB* gene, rendering them non-mucoid (Bartholdson *et al.*, 2008). Other *B. cenocepacia* isolates can convert from mucoid to non-mucoid during chronic infection, raising the possibility that loss of exopolysaccharide production may be associated with increased disease severity (Zlosnik *et al.*, 2008). Attempts to correlate clinical data of lung function with mucoid production of serial BCC clinical isolates from a group of 100 CF patients are ongoing in our laboratory.
Mucoid production may aid in the formation of biofilms, microbial communities that can help bacteria persist inside the host. Isolates from *B. cepacia*, *B. multivorans*, *B. cenocepacia*, *B. stabilis*, and *B. vietnamiensis* all produce biofilms *in vitro* (Conway *et al.*, 2002) and the BCC can create mixed species biofilms with *P. aeruginosa* (Tomlin *et al.*, 2001). Biofilm formation in the BCC is associated with increased resistance to ceftazidime and ciprofloxacin (Desai *et al.*, 1998).

### 1.6.5 Quorum sensing

Quorum sensing is the process of bacterial cell-to-cell communication, and is mediated by the production of freely diffusible small molecules, acyl-homoserine lactones. Quorum sensing systems are widely distributed among the BCC (Lutter *et al.*, 2001; Venturi *et al.*, 2004). The CepRI system was first identified in *B. cenocepacia* (Lewenza *et al.*, 1999), and its homologues exist in *B. cepacia*, *B. multivorans*, *B. stabilis*, and *B. vietnamiensis* (Venturi *et al.*, 2004). *B. vietnamiensis* also produces an additional quorum sensing system, BviIR (Lutter *et al.*, 2001; Malott and Sokol, 2007). In *B. cenocepacia*, quorum sensing regulates biofilm formation, siderophore production, protease and lipase activity, and swarming motility. Polygalacturonase activity of *B. cepacia*, which is responsible for onion maceration, is also regulated by CepIR (Venturi *et al.*, 2004). Production of the antifungal agent pyrrolnitrin, produced by several BCC species, is also regulated by this quorum sensing system (Schmidt *et al.*, 2009). CepIR was required for both slow and fast killing of the nematode *Caenorhabditis elegans* by *B. cenocepacia* (Köthe *et al.*, 2003). The *B. cenocepacia* CepIR also contributes to the severity of respiratory infections in both rat agar bead and intranasal CF mouse models (Sokol *et al.*, 2003). An additional quorum sensing system, CciIR, has also been discovered in *B. cenocepacia* and is associated with increased inflammation and significant lung pathology in the rat agar bead model of infection (Baldwin *et al.*, 2004). The clinical relevance of quorum sensing is also suggested
by the detection of acyl-homoserine lactones in the sputum of CF patients infected with BCC (Venturi et al., 2004).

1.7 Evasion of host defences by *B. cepacia* complex

1.7.1 Resistance to non-oxidative killing

One of the primary roles of phagocytes is to destroy invading microbes. Neutrophils, in particular, are profoundly microbicidal, using both oxidative and non-oxidative mechanisms. BCC bacteria display an intrinsic resistance to cationic peptides and are therefore impervious to non-oxidative phagocytic killing (Speert et al., 1994). This has obvious and devastating implications for CGD patients, where oxidative phagocytic killing is inherently defective. Neutrophils from CGD patients are unable to kill bacteria from the *B. cepacia* complex, explaining in part why these bacteria are so virulent in patients with CGD.

The predilection of BCC bacteria for CF is not well understood. Mutant CFTR alters salt transport in the respiratory epithelium, leading to abnormally thickened mucus and bacterial infection of the lung. Another hallmark of CF is an exaggerated inflammatory response, characterized by a sustained influx of neutrophils in the lungs. There is little indication that neutrophils from CF patients are inherently defective in oxidative killing as they are fully competent to produce ROS *in vitro* upon stimulation with soluble agonists (Brockbank et al., 2005). However, the harsh environment of the CF lung may itself impair the killing ability of neutrophils. There may be a physical barrier - abnormally thickened mucus of the CF airway may impede neutrophil motility and impair both bacterial capture and killing (Matsui et al., 2005). More significantly, an oxidant/antioxidant imbalance has been reported in the CF lung, which may compromise oxidative killing (Speert, 2002; Bylund et al., 2005b). Impaired oxidative killing due to either defects in the phagocytes themselves (i.e. CGD) or to a redox imbalance in the local environment (i.e. CF) may play an important role in BCC pathogenesis. When one also considers this pathogen’s innate ability to withstand non-oxidative killing, it is
clear why BCC bacteria are such a formidable opponent of phagocytes from CF and CGD patients.

BCC bacteria may not simply rely on defects or local impairment of oxidative killing. *B. cepacia, B. multivorans,* and *B. cenocepacia* produce superoxide dismutase, catalase, alkyl hydroperoxide reductase subunit C (AhpC), and a melanin pigment which scavenge superoxide *in vitro* (Zughaier et al., 1999b; Lefebre and Valvano, 2001; Chung and Speert, 2007). Some isolates of *B. cenocepacia* also express heme binding proteins in their outer membrane which may help detoxify ROS (Smalley et al., 2001). In addition, the exopolysaccharide produced by *B. cenocepacia* is very effective at scavenging ROS produced by both neutrophils and the xanthine-xanthine oxidase cell free system and can also inhibit neutrophil chemotaxis *in vitro* (Bylund et al., 2005b). The *in vivo* role of these virulence determinants in inhibiting ROS remains to be established, but they are potentially pertinent factors which could enable BCC bacteria to persist within the CF lung.

1.7.2 **Resistance to complement**

The complement system is composed of several serum proteins which, through classical or alternative pathways, can assemble on the bacterial surface to directly lyse potential pathogens or opsonize them to facilitate phagocytosis. The ability to resist complement mediated killing is presumably not important in the establishment of an infection in opsonin-poor sites such as the lung, but may be crucial when these pathogens invade the bloodstream. Resistance to complement-mediated killing has been reported in isolates from *B. cenocepacia, B. multivorans, B. stabilis,* and *B. vietnamiensis* (Butler et al., 1994; Speert et al., 1999; Savoia et al., 2008), including a non-mucoid clinical isolate of *B. cenocepacia* but not its mucoid derivative (Conway et al., 2004). The other principal function of the complement system is to opsonize bacteria for uptake. No specific inhibition of complement-mediated uptake of BCC bacteria has been described. Toxic compounds released from neutrophils or from other CF pathogens may be able
to cleave and inactivate complement opsonins *in vitro* (Terheggen-Lagro et al., 2005), but the *in vivo* relevance of this observation is unclear, except maybe in the very late stages of the disease, when damage to the lung is severe.

### 1.7.3 Intracellular pathogenicity

*B. pseudomallei* is a primary human intracellular pathogen that is closely related to the BCC. This causative agent of melioidosis can invade and survive within human epithelial and fibroblast cell lines, human and murine monocyte-macrophage cell lines, primary murine alveolar macrophages, and primary human peripheral blood neutrophils and mononuclear cells (Pruksachartvuthi *et al.*, 1990; Jones *et al.*, 1996; Kespichayawattana *et al.*, 2000; Utaisincharoen *et al.*, 2001; Kespichayawattana *et al.*, 2004). Intracellular bacterial replication has also been demonstrated *in vitro* using antibiotic protection assays (Jones *et al.*, 1996; Kespichayawattana *et al.*, 2000; Kespichayawattana *et al.*, 2004). These bacteria lyse phagosomal membranes, escape into the cytoplasm, and induce actin polymerization into a comet-like tail on one pole of the bacterium which may facilitate cell-to-cell spreading (Harley *et al.*, 1998; Kespichayawattana *et al.*, 2000; Stevens and Galyov, 2004). One aspect of *B. pseudomallei* pathogenesis appears to be unique among intracellular bacteria: its ability to induce cell fusion leading to the formation of multinucleated giant cells (Wong *et al.*, 1995; Kespichayawattana *et al.*, 2000; Suparak *et al.*, 2005).

Though not as substantial as *B. pseudomallei*, evidence for intracellular pathogenicity in *B. cepacia* complex bacteria is beginning to accumulate. A microscopic examination of lungs from BCC-infected CF patients undergoing lung transplantation revealed BCC bacteria in inflamed peribronchiolar and perivascular areas and in sites of acute pneumoniae and abscesses, but also associated with bronchiolar epithelium, between adjacent epithelial cells, and within alveolar septae and macrophages (Sajjan *et al.*, 2001). *B. cenocepacia* can invade murine respiratory epithelial cells and pulmonary macrophages *in vivo* and induce a strong inflammatory
response (Chiu et al., 2001; Chu et al., 2002) while B. multivorans can persist in the murine lung within alveolar macrophages (Chu et al., 2002; Chu et al., 2004) and in the spleen (Speert et al., 1999) without causing overt inflammation and disease, which suggests that this organism may be occupying a privileged intracellular niche.

Virulence factors for intracellular survival and persistence of B. cenocepacia in rodent infection models include flagella (Urban et al., 2004), the siderophore orniabant (Visser et al., 2004), a type III secretion system (Tomich et al., 2003), exopolysaccharide (Chung et al., 2003; Conway et al., 2004), the B. cenocepacia pathogenicity island (Baldwin et al., 2004; Mahenthiralingam et al., 2005), and a periplasmic HtrA protease (Flannagan et al., 2007).

These in vivo observations prompted researchers to investigate the intracellular pathogenicity of BCC bacteria in in vitro epithelial models. BCC bacteria can invade cultured undifferentiated and both primary and immortalized differentiated human airway epithelial cells in vitro (Burns et al., 1996b; Martin and Mohr, 2000; Cieri et al., 2002; Sajjan et al., 2002; Schwab et al., 2002; Duff et al., 2006; Mullen et al., 2007; Moura et al., 2008; Pirone et al., 2008), though extensive variability within and between species has been reported (Cieri et al., 2002; Moura et al., 2008; Pirone et al., 2008). B. cenocepacia may be more invasive of epithelial cells than B. cepacia, B. multivorans, B. stabilis, and B. vietnamiensis (Cieri et al., 2002), although equivalent or greater internalization by undifferentiated and differentiated epithelial cells of B. multivorans than B. cenocepacia (Schwab et al., 2003; Moura et al., 2008) and slower transmigration of B. cenocepacia than B. cepacia, B. multivorans and B. stabilis (Duff et al., 2006) have also been reported. Environmental B. cenocepacia isolates are also generally less able to invade unpolarized human A549 epithelial cell lines than clinical isolates (Martin and Mohr, 2000; Pirone et al., 2008). It has also been noted that the overall level of invasiveness in the BCC bacteria is generally rather low compared to other bacterial pathogens (Cieri et al., 2002; Moura et al., 2008) and is greatly influenced by the choice of epithelial cell
model; i.e., well-differentiated epithelial cell layers containing ciliated and mucus-producing cells show much less invasion than unpolarized epithelial cells (Sajjan et al., 2002). However, *B. cenocepacia* does invade murine respiratory cells in vivo (Chiu et al., 2001) and there is a good correlation between in vitro epithelial cell invasion and in vivo murine splenic persistence (Cieri et al., 2002), suggesting that the ability to invade epithelial cells may play a role in BCC pathogenesis.

The mechanism of BCC uptake and invasion of epithelial cells is not well understood. *B. cenocepacia* flagella, and a lipase produced by *B. cenocepacia* and *B. multivorans*, are involved in epithelial cell colonization (Tomich et al., 2002; Mullen et al., 2007). Actin rearrangement has been associated with uptake of *B. multivorans* and both viable and heat-killed *B. cenocepacia* (Schwab et al., 2003; Sajjan et al., 2006). *B. multivorans*, *B. cenocepacia*, *B. stabilis*, and *B. vietnamiensis* have generally been localized to membrane-bound endosomes within epithelial cells (Burns et al., 1996b; Sajjan et al., 2002; Schwab et al., 2002; Sajjan et al., 2006), although one study has also reported occasional *B. cenocepacia* bacteria in the cytoplasm (Sajjan et al., 2002). Other researchers have reported translocation of bacteria through epithelial cells by disruption of tight junctions and paracytosis (Schwab et al., 2002; Kim et al., 2005) and by inducing cytotoxicity which compromises the integrity of the epithelial cell monolayer (Schwab et al., 2002). An in vitro study of primary well-differentiated airway epithelial explants from non-CF and CF patients undergoing lung transplantation documented extracellular growth of *B. cenocepacia*, followed by epithelial cell invasion, compromised transepithelial resistance and extensive cell damage in CF but not control cells (Sajjan et al., 2004). Bacteria were found within the cytoplasm and between adjacent CF epithelial cells, but did not invade non-CF cell monolayers (Sajjan et al., 2004), which contradicts a previous study showing invasion of primary human non-CF differentiated airway epithelium (Schwab et al., 2002). In addition, live *B. cenocepacia* enters the CF IB3 epithelial cell line and survives within a modified vacuole that
acquires the late endosomal marker lysosome-associated membrane protein-1 but not cathepsin D, the autophagosomal marker monodansylcadaverine and the endoplasmic reticulum marker calnexin (Sajjan et al., 2006). Heat-killed bacteria, in contrast, enter the normal endosomal pathway in CF IB3 cells, residing in a vacuole which fuses with lysosomes, acidifies, and degrades the bacteria (Sajjan et al., 2006). The endosomal trafficking of BCC bacteria in control epithelial cells was not examined in this study, so it is not known whether this is unique to CF epithelial cells. In summary, BCC bacteria exhibit varying capacities to invade epithelial cells and may be more adept at invading and surviving with CF epithelium, although many details remain to be clarified.

Another model that has been used to examine intracellular pathogenicity in BCC is the amoeba, a free-living soil protozoan with uptake and endosomal processing capacities similar to phagocytic cells (Cosson and Soldati, 2008). Environmental and clinical isolates of *B. cepacia*, *B. multivorans*, *B. cenocepacia*, *B. stabilis*, and *B. vietnamiensis* can survive within *Acanthamoebae sp.* (Marolda et al., 1999; Landers et al., 2000). In addition, live *B. vietnamiensis* persists without replication within an acidified vacuole that does not fuse with lysosomes (Lamothe et al., 2004).

The ability to survive within mononuclear phagocytes requires bacteria to adapt or mitigate the harsh intracellular environment specifically evolved to kill bacteria. BCC bacteria are poorly internalized by human and murine macrophage cell lines (Saini et al., 1999; Martin and Mohr, 2000; Lamothe et al., 2007; Sajjan et al., 2008a). However, *B. stabilis* and *B. vietnamiensis* can persist within normal murine macrophage cell lines (Saini et al., 1999) and *B. cenocepacia* survives within normal murine and human macrophage cell lines (Martin and Mohr, 2000; Lamothe et al., 2007; Sajjan et al., 2008a) and within a CF murine macrophage cell line (Lamothe and Valvano, 2008). There have been conflicting reports concerning the ability of BCC bacteria to replicate within macrophages (Martin and Mohr, 2000; Lamothe et al., 2007;
Lamothe and Valvano, 2008; Sajjan et al., 2008a). Although some groups have reported that \textit{B. cenocepacia} can replicate intracellularly (Martin and Mohr, 2000; Sajjan et al., 2008a), another group has maintained that the high level of antibiotic resistance among BCC makes it difficult to distinguish intracellular from residual extracellular bacterial growth (Lamothe \textit{et al.}, 2004). This fact, combined with the ability of BCC bacteria to induce macrophage cell death (Hutchison \textit{et al.}, 1998; Melnikov \textit{et al.}, 2000; Punj \textit{et al.}, 2003; Nair \textit{et al.}, 2004), makes demonstration of intracellular replication by BCC inconclusive.

In murine macrophages, live \textit{B. cenocepacia} delay phagosomal maturation, as evidenced by an approximate five hour delay in phagosome acidification, the acquisition of the late endosomal marker lysosome-associated membrane protein-1, and phagosome-lysosome fusion, as compared to the rapid kinetics of phagosomal maturation when heat-killed bacteria were employed (Lamothe \textit{et al.}, 2007). The virulence factors of \textit{B. cenocepacia} that appear to contribute to this phagolysosomal fusion delay include the RpoN sigma factor (Saldías \textit{et al.}, 2008) and MgtC, a protein involved in survival under low magnesium conditions (Maloney and Valvano, 2006). This \textit{B. cenocepacia}-induced phagosomal maturation defect is even more enhanced in cultured macrophages derived from CF mice but not from their littermate controls (Lamothe and Valvano, 2008). This difference is surprising due to the fact that macrophages are not believed to express CFTR. These researchers did report detection of CFTR gene expression by real-time polymerase chain reaction, although they were unsuccessful in demonstrating protein expression by Western blotting (Lamothe and Valvano, 2008). A recent study has caused debate as to the constitution of the modified \textit{B. cenocepacia}-occupied vacuole. Phagosomes of human cultured macrophages infected with \textit{B. cenocepacia} acquire lysosomal protease cathepsin D and the endoplasmic reticulum marker calnexin but also fail to acidify (Sajjan \textit{et al.}, 2008a), suggesting that \textit{B. cenocepacia}-occupied vacuoles are not simply delayed in maturation, but may divert to an alternative pathway which involves the endoplasmic
reticulum. The plant tissue watersoaking type IV secretion system has been implicated in this process (Sajjan et al., 2008a). Clearly, more research is needed to elucidate the details of phagosomal trafficking in B. cenocepacia-infected macrophages.

### 1.7.4 Pro-inflammatory potential

The ability of BCC bacteria to induce pro-inflammatory cytokines has been investigated using mononuclear phagocytic cells, a significant source of cytokines and chemokines. B. cepacia complex bacteria may directly contribute to the excessive inflammation observed in CF and CGD patients as their lipopolysaccharide (LPS) appears to be more pro-inflammatory and has a higher endotoxic potential than LPS from other CF pathogens, as measured by the release of pro-inflammatory cytokines in human and murine monocytic-macrophage cell lines and primary monocytic cells (Shaw et al., 1995; Zughaier et al., 1999a; Hutchison et al., 2000; Shimomura et al., 2001; Gronow et al., 2003). Murine models of B. cepacia infection have also implicated other bacterial factors in the induction of inflammation [e.g. quorum sensing molecules (Sokol et al., 2003), flagella (Urban et al., 2004; de C. Ventura et al., 2008), and products secreted from the type III secretion system (Tomich et al., 2003)]. In addition, the human CF respiratory epithelial cell line IB3-1 produces more interleukin-6 than the CF-corrected C38 airway cell line when stimulated with heat-killed B. multivorans and B. cenocepacia (Blohmke et al., 2008). Recently, it has also been demonstrated that well-differentiated primary epithelial cells from CF patients and a CF bronchial epithelial cell line secrete more interleukin-8 than non-CF cells when exposed to a B. cenocepacia isolate from a cepacia syndrome patient (Sajjan et al., 2004; Sajjan et al., 2008b). The bacteria bind and activate tumour necrosis factor receptor 1, leading to increased interleukin-8 production (Sajjan et al., 2008b). Thus far, this has only been demonstrated in a single virulent isolate of B. cenocepacia and is not shared by another member of the same ET12 lineage (Sajjan et al., 2008b).
1.7.5 Modulation of apoptosis and necrosis

Phagocytes that have ingested BCC bacteria can undergo programmed or necrotic cell death. *B. cenocepacia* cable pili have been implicated in lung epithelial cell death (Nair et al., 2004). The bacteria secrete a haemolysin which induces apoptosis in a murine macrophage cell line (Hutchison et al., 1998) and human neutrophils. Production of ATP-utilizing enzymes, azurin and cytochrome c551 also stimulate cytotoxicity and apoptosis in a murine macrophage cell line (Melnikov et al., 2000; Punj et al., 2003). BCC bacteria secrete a protease which causes tissue damage in rats upon intratracheal instillation (McKevitt et al., 1989) and the production of at least one zinc metalloprotease by *B. cenocepacia* is required for virulence (Kooi et al., 2006). It is not clear that these virulence factors are produced at sufficient concentrations *in vivo* to induce similar toxic effects. By stimulating either apoptosis or necrosis in antigen presenting cells, *B. cepacia* complex bacteria may modulate the development of adaptive immunity and contribute to inflammation which ultimately may damage the host. The *in vivo* relevance of these processes and the factors which contribute to generation of either apoptosis or necrosis have yet to be determined.

Our laboratory has recently explored the induction of apoptosis and necrosis in neutrophils (Bylund et al., 2005d). Neutrophils phagocytosing a wide spectrum of pathogens typically undergo apoptosis (Kobayashi et al., 2003), followed by phagocytosis by macrophages, and are thus disposed of in a safe and non-pathologic manner (Savill et al., 1989). Indeed, non-opsonic uptake of *B. cenocepacia* leads to apoptosis in normal neutrophils *in vitro* (Bylund et al., 2005d). However, *B. cenocepacia* challenge of CGD neutrophils caused significant necrosis. Release of toxic components from infected necrotic neutrophils may therefore contribute to the pathology of CGD (Bylund et al., 2005d).

Induction of epithelial cell death by members of the BCC has also been reported. *B. cepacia*, *B. multivorans*, *B. cenocepacia*, *B. stabilis*, and *B. vietnamiensis* all induce cell death in
human undifferentiated epithelial cells (Moura et al., 2008), while B. cenocepacia co-expressing
cable pili, with the associated adhesin, cause cell death in primary squamous epithelial cells
(Sajjan et al., 2002). B. multivorans and B. cenocepacia also provoke cytotoxicity which
compromises the integrity of the human differentiated epithelial cell monolayers (Schwab et al.,
2002). CF primary and cultured well-differentiated epithelium are also susceptible to the
cytotoxic effects of B. cenocepacia, particularly at higher multiplicity of infections (Sajjan et al.,
2004; Sajjan et al., 2006). While there are scattered reports of cytotoxicity and cell damage
within the BCC bacteria-epithelial cell literature, a functional analysis of the actual type of cell
death induced by BCC has not been performed.

1.8 BCC genomics

Our knowledge of the genetic organization and constituents of BCC has reached a critical
milestone, with the recent publication of the B. cenocepacia J2315 genome (Holden et al., 2009)
and the on-line availability of draft sequences of B. cenocepacia strains AU1054 and HI2424, B.
viethamensis strain G4 (ATCC 53617), B. ambifaria strain AMMD, and B. contaminans strain
383 (ATCC 17660) (Mahenthiralingam et al., 2005; Holden et al., 2009). All of these BCC
species have multiple chromosomes and genomes of 6 to 9 Mb, which are among the largest of
all Gram-negative bacteria (Mahenthiralingam et al., 2005). The B. cenocepacia J2315 genome
is 8,056 Mb and has an average GC content of 66.9% (Mahenthiralingam et al., 2005). It is
composed of three circular chromosomes of 3,870,082; 3,217,062, and 875,977 base pairs and a
plasmid of 92,661 base pairs (Holden et al., 2009). These replicons encode 3,537; 2,849; 776,
and 99 predicted coding sequences, respectively (Holden et al., 2009). There is a segregation of
gene arrangement, as chromosome 1 encodes mainly housekeeping genes, such as those
functioning in cell division and metabolism, while chromosomes 2 and 3 contain proportionally
more genes involved in accessory functions, such as horizontal gene transfer, protective
responses, and genes of unknown function (Holden et al., 2009).
There is evidence of some similarities within the *B. cenocepacia* J2315 genome and other draft BCC genome sequences. Approximately 80% of *B. cenocepacia* J2315 putative virulence genes were orthologous matches to the *B. cenocepacia* strains AU1054 and HI2424, while approximately 74% have orthologs within the *B. contaminans* strain 383 sequence (Holden *et al.*, 2009). The functions and contribution to virulence of many of these factors remain to be established, but they may represent common mechanisms by which the BCC colonizes the rhizosphere and establishes infection in both plants and CF and CGD patients.

It is instructive to compare the *B. cenocepacia* J2315 genome with that of *B. cenocepacia* AU1054 and HI2424. *B. cenocepacia* J2315 is a member of a RecA group III-A while *B. cenocepacia* strains AU1054 and HI2424 are members of RecA group III-B, two distinct phylogenetic clusters within this species (Mahenthiralingam *et al.*, 2000). Twenty-one percent of the DNA in the *B. cenocepacia* J2315 genome is uniquely different when compared to the other in *B. cenocepacia* strains. These regions of difference include seventy-nine insertion sequence elements and fourteen putative genomic islands (areas of %G+C content or dinucleotide frequency anomalies or containing mobile genetic elements) (Holden *et al.*, 2009). The only well characterized genomic island is the *B. cenocecepacia* island (CCI), a 44 kb region with 41 predicted coding sequences. The CCI encodes putative genes involved in resistance to arsenic and antibiotics, ion and amino acid transportation, fatty acid metabolism, the CciIR quorum sensing system, putative stress response coding sequences, and the *B. cepacia* epidemic strain marker (BCESM) (Baldwin *et al.*, 2004; Holden *et al.*, 2009). This island is more common in *B. cenocepacia* III-A than III-B lineages; indeed, it was not found in *B. cenocepacia* AU1054 and HI2424 (Holden *et al.*, 2009). Mutations of CCI virulence factors have also demonstrated its association with persistence and inflammation in the rat agar bead model of infection (Baldwin *et al.*, 2004). Despite the similarities within BCC genomes, it is clear that *B.
*cenocepacia* J2315 contains many unique genetic components which may contribute to its significant ability to cause disease in CF patients.

Although many BCC genomes, including the clinically significant *B. multivorans*, await sequencing and annotation, the recent explosion in BCC genomics will contribute greatly to clarifying the role of different members of the BCC in CF and CGD pathogenesis and aid in identifying particular virulence factors that play key roles in this process.

1.9 Major thesis themes and hypotheses

1.9.1 Inflammation in CGD

One of the most intriguing aspects of CGD is the hyper-inflammatory phenotype that is observed in both CGD patients (Segal *et al.*, 2000) and in mouse models of CGD (Jackson *et al.*, 1995; Pollock *et al.*, 1995; Morgenstern *et al.*, 1997). Inflammation is mediated by the production of pro-inflammatory cytokines, which is usually initiated following ligation of toll-like receptors recognizing conserved microbial patterns (Kopp and Medzhitov, 2003). A signaling cascade is then induced, ultimately activating the transcription factor NF-κB, which translocates to the nucleus and induces transcription of pro-inflammatory cytokine genes (Natoli *et al.*, 2005). NF-κB is thought to be redox regulated (Kabe *et al.*, 2005) and there is evidence that phagocyte NADPH-oxidase-generated ROS are directly involved in NF-κB activation (Flohe *et al.*, 1997; Fan *et al.*, 2003; Asehnoune *et al.*, 2004; Sadikot *et al.*, 2004). Deficient ROS production may therefore decrease NF-κB activation, leading to diminished inflammation. These reports are in direct contradiction to the clinical observations of CGD patients (Segal *et al.*, 2000) and even CGD mouse models (Jackson *et al.*, 1995; Pollock *et al.*, 1995; Morgenstern *et al.*, 1997). Therefore, this thesis will investigate the hypothesis that lack of ROS does not decrease activation of NF-κB and, in fact, enhances production of pro-inflammatory cytokines.
1.9.2 Dendritic cells and BCC

Infection with BCC bacteria obviously has multiple effects on phagocytosing cells, ranging from abrogation of phagocytic killing with resulting intracellular parasitism to induction of inflammation, apoptosis or necrosis. Clearly, host factors, and in particular mediators of innate and adaptive immunity, must play a role in these diverse fates in vivo. An exploration of the immune response to BCC is therefore warranted to understand more completely the phagocyte-bacteria interaction and the ultimate outcome of infection.

One of the most glaring gaps in this literature is the role of dendritic cells (DCs) in the pathogenesis of BCC. DCs play a crucial and unique role in the immune response, and there is increasing evidence for bacterial subversion of these processes (Cutler et al., 2001; Palucka and Banchereau, 2002). Because DCs migrate from the sites of infection to secondary lymph nodes to present antigen, they may also serve as systemic vectors (a sort of Trojan horse) for persistent intracellular bacteria. This concept is tantalizing in light of the proclivity of B. cepacia complex organisms to cause fatal necrotizing pneumonia and sepsis in susceptible individuals (Hutchison and Govan, 1999). This thesis will therefore explore the hypothesis that B. multivorans and B. cenocepacia modulate the normal functions of primary human monocyte-derived DCs, to subvert antibacterial mechanisms and persist intracellularly.
1.10 References


2. Enhanced inflammatory responses of chronic granulomatous disease leukocytes involve ROS-independent activation of NF-kappa B.¹

2.1 Summary

Reactive oxygen species (ROS) generated by the cellular NADPH oxidase are crucial for phagocytic killing of ingested microbes and have been implicated as signaling molecules in various processes. For example, ROS are thought to be involved in activation of the transcription factor NF-κB, central for mediating production of proinflammatory cytokines in response to inflammatory stimuli. Several studies have demonstrated that inhibitors of the NADPH oxidase interfere with NF-κB activation and production of proinflammatory cytokines. Curiously, patients with chronic granulomatous disease (CGD), an immunodeficiency characterized by an inability to produce ROS, are not only predisposed to severe infections, but also frequently develop various inflammatory complications indicative of exaggerated inflammatory responses. Here, we show that human CGD leukocytes display a hyperinflammatory phenotype with increased production of proinflammatory cytokines in response to stimulation with Toll-like receptor agonists. The hyperinflammatory phenotype was also evident in mononuclear cells from CGD mice (gp91<sup>phox−/−</sup>), but not in control cells in the presence of NADPH oxidase inhibitor diphenyleneiodonium, probably reflecting NADPH oxidase-independent effects of the inhibitor. Furthermore, we show that the major steps involved in NF-κB activation were intact in human CGD cells. These data indicate that ROS were nonessential for activation of NF-κB and their production may even attenuate inflammation.

2.2 Introduction

Chronic granulomatous disease (CGD) is a very unusual, primary immunodeficiency affecting approximately 1/250,000 births; the affected individuals are hypersusceptible to a narrow range of bacterial and fungal pathogens, most notably *Staphylococcus aureus*, *Burkholderia cepacia* complex and various *Aspergillus* species (Winkelstein et al., 2000). The underlying defect is caused by one of several mutations in genes that encode different subunits of the NADPH oxidase, which is responsible for production of ROS that are necessary for full antimicrobial activity of phagocytes. In addition to being hypersusceptible to infection, CGD patients are also predisposed to a number of different inflammatory complications. It has been argued that these complications are secondary to failure to completely eradicate various infections, but in fact many of the inflammatory symptoms appear to be without any obvious infectious antecedent and the inflammatory sites are often sterile (Gallin and Buescher, 1983; Segal et al., 2000; Bylund et al., 2005a). Two murine CGD models exist, each of which is characterized by an inability to eradicate infecting microbes and also by exuberant inflammatory responses upon experimental challenge (Jackson et al., 1995; Pollock et al., 1995; Morgenstern et al., 1997). These observations in humans and mice lacking a functional NADPH oxidase suggest that ROS play a critical role in inflammatory regulation. Furthermore, ROS have been implicated as signaling molecules of importance in a variety of cell signaling systems. Given the fact that ROS are, per definition, highly reactive and can alter a number of key organic structures (DNA, proteins, lipids, etc), their involvement in signaling appears to be logical, although the details are obscure.

The best characterized proinflammatory signaling pathway resulting in transcription of inflammatory genes, involves activation of the transcription factor NF-κB. This pathway is typically activated through ligation of genome-encoded receptors such as the TLR that recognize conserved microbial signatures (Kopp and Medzhitov, 2003). The NF-κB family is composed of
five different members/subunits- p50, p52, p65, c-Rel and RelB (Ghosh et al., 1998). In resting cells, NF-κB heterodimers are present in the cytoplasm, where they are bound to the inhibitory protein I-κBα. Upon activation, I-κBα is phosphorylated and eventually degraded by ubiquitination, releasing the NF-κB subunits that become available for nuclear translocation. Once within the nucleus, NF-κB binds to specific NF-κB consensus sequences in the genome and initiates gene transcription (Natoli et al., 2005). Activation of NF-κB is generally regarded as a proinflammatory event and this transcription factor is considered to be subjected to redox regulation (Kabe et al., 2005). Recent reports imply that NADPH oxidase-derived ROS are directly involved in activation of NF-κB (Flohe et al., 1997; Fan et al., 2003; Asehnoune et al., 2004; Sadikot et al., 2004). These published reports, suggesting that lack of ROS production leads to decreased NF-κB activation and subsequently to decreased inflammatory responses, are difficult to reconcile in the context of the gross inflammatory phenotype displayed by both CGD patients (Gallin and Buescher, 1983; Segal et al., 2000) and mice (Jackson et al., 1995; Morgenstern et al., 1997).

We investigated the role of NADPH oxidase-derived ROS in the context of NF-κB driven cytokine production using PBMCs from CGD patients with mutations in gp91phox (X-linked CGD). We found that CGD cells exhibited a hyperinflammatory phenotype and produced significantly more IL-6 and TNF-α than control cells upon stimulation with a variety of substances, including the bacterial-derived TLR agonists LPS and peptidoglycan (PGN). We also show that NF–κB signaling in response to LPS was intact in human CGD cells, indicating that ROS derived from the NADPH oxidase were nonessential for NF-κB activation. Instead, the lack of a functional NADPH oxidase was associated with exaggerated production of proinflammatory cytokines, which implies a role for ROS in dampening inflammation.
2.3 Materials and methods

2.3.1 Patients and isolation of cells

Peripheral blood was obtained from healthy volunteers and four CGD patients, all male gp91phox-deficient patients (X-linked CGD). None of the patients were infected at the time of the experiments and blood from one patient was analyzed in detail; white blood cell count, red blood cell count, as well as differential counts for granulocytes, lymphocytes, monocytes, eosinophils and basophils were all within normal range (not shown). All of the donors provided informed consent and the study was conducted according to UBC Clinical Research Ethics protocol C04-0193.

PBMCs were isolated by mixing peripheral blood at a ratio of 1:1 with RPMI 1640 supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, and 1 mM sodium pyruvate (R10 media; all from Invitrogen, Burlington, Ontario, Canada) in an endotoxin free bottle. This solution was then overlayed on Ficoll-Paque Plus (Amersham Pharmacia Biotech, Baie D' Urfe', Quebec, Canada) and centrifuged (450 g) at room temperature. The resulting PBMC layer, consisting mainly of lymphocytes (T cells, B cells and NK cells) and monocytes, was washed twice in phosphate buffered saline (PBS) and the cells resuspended in R10 media. In some instances, the PBMC preparations were further characterized by flow cytometry and cellular compositions were always within the normal range as reported (Tollerud *et al.*, 1989; Peters and al-Isma'il, 1994).

2.3.2 Cytokine assays

PBMCs in R10 media at 2x10⁶/ml were equilibrated in 96-well plate and incubated at 37°C in 5% CO₂ incubator for 30 minutes before starting the experiments. Stimulation was achieved by the addition of the following: R10 (unstimulated); 100 ng/ml ultra-pure *E. coli* LPS (Invivogen, San Diego, CA) in the presence of 1% human AB serum (Sigma Chemical, St. Louis, MO) as a source of LPS-binding protein; 10 µg/ml PGN (Invivogen); 250 ng/ml IL-1β
(Research Diagnostics, Flanders, NJ) or 10 µg/ml phytohaemagglutinin (PHA; Sigma). After 20 hours of culture, supernatants were collected and stored in -80°C until evaluated by enzyme-linked immunosorbent assays (ELISA) (BD Biosciences, Mississauga, Ontario, Canada) according to the manufacturer’s instructions. When DPI (Sigma) was used, the cells were preincubated with this agent (10^{-5}M) for 45 min before stimulation. When MG-132 (Calbiochem, San Diego, CA) was used, the cells were pretreated with this proteasome inhibitor (5 µM) for 30 min before stimulation.

In certain experiments, PBMCs were preincubated with 100µM of p65i, 100µM of control peptide [NF-κB p65 (Ser276) Inhibitory Peptide set; Imgenex], or medium 60 minutes before stimulation. After 24 hours, the supernatants were collected and frozen at -80°C. These samples were analyzed using the Cytometric Bead Array (CBA) human inflammation kit (BD Biosciences) as outlined in the manufacturer’s instructions. Following data acquisition on a BD FACSCalibur (BD Biosciences) flow cytometer, the concentration of each cytokine in the samples and standards was determined using BD CBA software.

2.3.3 Stimulation and preparation of cellular extracts

PBMCs in R10 media at 10^7/ml were equilibrated at 37°C in 5% CO₂ for 30 min before starting the experiments after which 100 ng/ml LPS in the presence of 1% human AB serum, as a source of LPS-binding protein, was added. At the indicated time points, the samples were placed on ice, washed in ice-cold PBS and cytoplasmic and nuclear extracts were obtained using the NE-PER kit (Pierce, Rockford, IL) in the presence of Complete mini, ethylenediaminetetraacetic acid- (EDTA-)free protease inhibitor cocktail (Roche diagnostics, Basel, Switzerland) at 4°C. Protein content was determined using BCA Protein Determination Kit (Pierce), and the samples were aliquoted and stored at -80°C.
2.3.4 Western blotting

Equal amounts of cytoplasmic or nuclear extracts were separated on 12% SDS polyacrylamide gels, transferred to PVDF membranes, and immunoblotted. Primary antibodies against phospho-I-κBα, I-κBα and NF-κB p50, and secondary horseradish peroxidase- (HRP-) linked antibodies were obtained from Cell Signaling Technology (Beverly, MA). Visualization was conducted using CL peroxidase substrate (Sigma) and blots were routinely treated with stripping buffer (Pierce) before reprobing. Anti-phospho-antibodies were always used on un-stripped membranes. After blotting was finished, the membranes were stripped again and Coomassie Blue stained to ascertain equal loading.

2.3.5 NF-κB p50 ELISA

Equivalent amounts of nuclear extracts from control or CGD PBMCs were analyzed for NF-κB subunit p50 content by StressXpress NF-κB p50 ELISA Kit (Stressgen Bioreagents, Victoria, BC, Canada) according to manufacturer's instructions. Luminescence was detected with SpectraFluor Plus Multifunction Microplate Reader (Tecan Systems, CA).

2.3.6 Electrophoretic mobility shift assay (EMSA)

Nuclear extracts (5 µg), from resting and LPS-stimulated cells, were mixed with buffer (10 mM Tris, pH 7.5, 100 mM KCl, 5 mM MgCl₂, 1 mM DTT, 0.75% glycerol, and 2 mM EDTA), 1.8 µg of poly (dI-dC), in the presence or absence of 50 µg/ml unlabeled NF-κB probe and incubated for 20 min at room temperature. Biotin-labeled NF-κB probe (0.25 µg/ml, probe set from Panomics, Redwood City, CA) was added to all samples, which were incubated for 20 min at room temperature before addition of loading buffer and separation on 7% native polyacrylamide gels in a running buffer of 0.5 x TBE buffer (50 mM Tris pH 8.0, 45 mM boric acid, 0.5 mM EDTA). The reactions were then transferred to nylon membranes, UV-cross-linked and visualized using the LightShift chemiluminescent EMSA kit (Pierce) according to manufacturer’s instructions.
2.3.7 **Mouse strains**

For stimulation experiments, 8-week-old female wild-type (CB57BL/6J) and NADPH oxidase deficient (Cybb\textsuperscript{-/-}) mice, which lack the fragment of chromosome X that encodes gp91\textsubscript{phox} (X-linked CGD) were purchased from B&K Universal AB (Sollentuna, Sweden) and maintained in the animal facility of the Department of Rheumatology and Inflammation Research, Göteborg University. The experiments were performed with the approval of the Ethical Committee of Göteborg University.

2.3.8 **In vitro stimulation of murine spleen mononuclear cells**

Spleens were removed from mice and placed in sterile PBS on ice. Using a cell strainer (BD Falcon 70-µm nylon), the cells were filtered into 10 ml of sterile, cold PBS. The suspension was centrifuged (250 \times g, 5 min, 4°C) and the pellet was resuspended in 10 ml of cold, sterile ammonium chloride pH 6.8 to lyse residual erythrocytes. The cells were then washed three times with the sterile PBS solution and counted. This procedure repeatedly generated preparations consisting mainly of lymphocytes (85%) and monocytes/macrophages (10%) as assessed by differential cell counting and flow cytometric analysis of size and granularity (not shown). No significant differences in cell composition were noted between spleen cells from WT and CGD mice. Cells were seeded onto 24-well plates (2\times10^6/well) in 1 ml of Iscove’s modified Dulbecco’s medium (Sigma) and incubated with formalin-killed \textit{S. aureus} (LS-1 strain; MOI 1:25), 2 µg/ml \textit{S. aureus} lipoteichoic acid (Invivogen) or with 3.3 µM of a completely phosphorothioated CpG motif, containing oligodeoxynucleotide (5’-TCCATGACGTTCCTGCT-3’), which was synthesized by Scandinavian Gene Synthesis AB (Köping, Sweden). After 24 h of incubation, the supernatants were harvested and frozen at -70°C for future analysis. Cells were analyzed for viability by flow cytometry using the fluorescent nuclear dye ethidium homodimer (EthD-1; 1 µM; Molecular probes) that is impermeable to viable cells. The BD CBA Mouse Inflammation Kit (BD Biosciences) was used
to measure the levels of IL-6 and IFN-γ according to manufacturer’s instructions. Analysis was performed on a FACSCalibur using the BD CellQuest- and BD CBA softwares (BD Biosciences).

2.3.9 Production of ROS

A luminol-enhanced chemiluminescence (CL) system was used with a Mithras LB940 (Berthold technologies; Bad Wildbad, Germany) plate reader and disposable 96-well plates containing 220-µL reaction mixtures. Each well contained $2 \times 10^5$ spleen cells, HRP (4 U/mL) and luminol ($2 \times 10^{-5}$ M) in Krebs-Ringer phosphate buffer (KRG, pH 7.3) containing glucose (10 mM), $\text{Ca}^{2+}$ (1 mM), and $\text{Mg}^{2+}$ (1.5 mM). The cells were transferred to $37^\circ$C, stimulated with CpG (3.3 µM), light emission recorded continuously and data are expressed as relative light units.

2.4 Results

2.4.1 CGD leukocytes produce increased amounts of proinflammatory cytokines after stimulation.

We stimulated PBMCs from CGD patients and healthy controls with a battery of well characterized proinflammatory substances, including ultra-purified *E. coli* LPS, and measured the release of cytokines IL-6 and TNF-α after 20 hours of culture. IL-6 production from CGD cells was significantly increased compared to control cells, for all stimulators used except for IL-1β (Fig. 2.1A); IL-6 production in response to this stimulus was very low in general compared to the responses to, e.g. LPS or peptidoglycan (PGN). Control cells produced only minute amounts of IL-6 in response to IL-1β and CGD cells, again, displayed an enhanced response. The hyperresponsiveness of CGD cells was also apparent in terms of TNF-α production (Fig. 2.1B), indicating that the lack of ROS did not inhibit the production of proinflammatory cytokines, but instead led to exaggerated proinflammatory responses.
Figure 2.1  Hyper inflammatory phenotype of CGD cells. PBMCs from healthy controls (open bars) and CGD patients (filled bars) were cultured in the presence of medium (unstimulated), human AB serum (1%), LPS (100 ng/ml in the presence of 1% serum), PGN (10 µg/ml), IL-1β (250 ng/ml) or PHA (10 µg/ml) for 20 hours after which the cell-free supernatants were analyzed by ELISA. CGD cells responded to stimulation by producing more IL-6 (A) and TNF-α (B). In a separate series of experiments, control PBMCs were preincubated in the presence (filled bars) or absence (open bars) of DPI (10^-5 M) for 45 min before stimulation as above. After 20 hours of incubation, cell free supernatants were analyzed for TNF-α content by ELISA; DPI inhibited cytokine production (C). Shown are the mean ± SD of three to four independent experiments. Asterisks denote statistically significant differences between control and CGD cells (P <0.05).

Several previous reports have argued that ROS are needed for proper NF-κB-driven cytokine production. Most of these studies have employed various antioxidants or NADPH oxidase inhibitors to abrogate ROS; in the presence of these agents NF-κB activation was blocked (Schreck et al., 1992; Yoshida et al., 2001; Matsunaga et al., 2003) and cytokine production diminished (Asehnoune et al., 2004; Park et al., 2004; Yu et al., 2005). In the presence of diphenyleneiodonium (DPI; a NADPH oxidase inhibitor), a marked decrease in
TNF-α production after stimulation was recorded (Fig. 2.1C). This was consistent with previously published data, but not with the hyperresponsive phenotype of CGD cells, indicating that this inhibitor did not replicate the conditions of CGD cells, at least with respect to cytokine production.

2.4.2 Enhanced proinflammatory cytokine responses of murine CGD spleen cells

As opposed to inbred strains of mice, the human population has substantial genetic variability, such that relatively high inter-individual variations can be anticipated. To ascertain whether the absence of a functional NADPH oxidase was the reason behind the hyperinflammatory phenotype seen for human CGD cells, we performed experiments using mononuclear spleen cells from gp91phox−/− mice and age- and gender-matched wild type (WT) mice of isogenic background (CB57BL/6J). As was the case for human CGD cells, the murine CGD cells responded to stimulation with significantly higher production of proinflammatory cytokines than WT cells (Fig. 2.2A, B). In general, LPS and PGN were very poor stimuli for cytokine production from murine cells, but IL-6 production from CGD cells was still enhanced compared to WT cells in response to these stimuli (data not shown). Interestingly, a particularly potent inflammatory stimulus for murine CGD cells was formalin-killed *S. aureus* (Fig. 2.2A, B), a bacterial pathogen to which CGD patients are particularly susceptible. As the mice used in these experiments were genetically identical, except for the gp91phox gene, the data strongly suggested that the hyperinflammatory phenotype was indeed due to the absence of a functional NADPH oxidase. We also monitored cellular survival in the presence or absence of stimulation and found no significant differences between CGD and WT cells (Fig. 2.2C), indicating that the hyperinflammatory phenotype of CGD cells was not due to increased cell survival in culture.

2.4.3 ROS production

To test if the stimuli used were able to induce ROS production in WT cells, we employed a luminol-enhanced chemiluminescence (CL) system (Dahlgren and Karlsson, 1999). Spleen cells
Figure 2.2  Hyperinflammatory phenotype and the absence of ROS production of murine gp91phox−/− spleen mononuclear cells.

Mononuclear cells from WT (open bars) or CGD mice (filled bars) were cultured in the presence of formalin-killed *S. aureus* (MOI 1:25), lipoteichoic acid (2 µg/ml) or CpG containing oligodeoxynucleotides (3.3 µM) for 24 hours after which cell free supernatants were analyzed for IL-6 (A) or IFN-γ (B) using CBA. (C) Viability of WT (open bars) and CGD (filled bars) cells in the presence or absence (control) of stimulation with formalin-killed *S. aureus* for 24 h was analyzed by flow cytometry and the cell-impermeable nuclear dye EthD-1 that only permeates dead cells. (D) Spleen cells from WT (black lines) or CGD (gray lines) were stimulated with buffer (background; dotted lines) or CpG (3.3 µM; solid lines) and the generation of ROS was followed by luminol-enhanced CL. Shown are representative experiments (including cells from four mice of each type) that were repeated independently at least four times (A and B), mean ± SD (n = 4; C), and a representative experiment repeated four times (D). Asterisks denote statistically significant differences between control and CGD cells (*P <0.05; ***P <0.0005).

from WT mice produced measurable levels of ROS when equilibrated (in the absence of stimulation) at 37°C; these levels were clearly enhanced upon stimulation with CpG (Fig. 2.2D). Cells from CGD mice on the other hand produced no ROS at all and the light emission
(reflecting superoxide production) from these cells was indistinguishable from light emission in the absence of cells (not shown). These data indicated that WT cells produced ROS in response to CpG and confirmed the need for gp91phox for ROS production. We also corroborated these findings with human PBMCs, comparing cells from one healthy control donor with cells from one CGD patient. In a similar manner, the control cells produced clearly detectable levels of ROS, whereas the CGD cells were completely devoid of ROS production regardless of stimulation (Appendix 5).

2.4.4 **Cytoplasmic phosphorylation and degradation of I-κBα.**

Next, we investigated how the NF-κB signaling pathway was activated in the absence of ROS and subjected PBMCs from CGD patients or healthy controls to LPS stimulation. After stimulation, cells were harvested at different time points and cytoplasmic and nuclear extracts were prepared. Cytoplasmic extracts from stimulated PBMCs were immunoblotted using a monoclonal antibody directed against the phosphorylated form of I-κBα; phosphorylation occurred as early as 20 minutes after LPS stimulation, peaked at 40 minutes and then declined (Fig. 2.3) as anticipated given that phosphorylation marks I-κBα for ubiquitination and proteasomal degradation (Henkel *et al.*, 1993). CGD cells tended to display weak phosphorylation of I-κBα even in the absence of stimulation, possibly indicative of enhanced resting levels of NF-κB activity, but in terms of magnitude and kinetics of the phosphorylation, CGD cells responded in a similar manner as the control cells. Both normal and CGD cells degraded cytosolic I-κBα upon LPS stimulation with a slightly more robust degradation displayed by CGD cells (Fig. 2.3). These data indicated that NADPH oxidase-derived ROS were not needed for phosphorylation and degradation of I-κBα in human PBMCs. Degradation of I-κBα and subsequent activation of NF-κB appeared to occur via the normal ubiquitination
proteasome pathway since production of both IL-6 and TNF-α from control and CGD cells was completely abrogated in the presence of proteasome inhibitor MG-132 (Appendix 6).

![Figure 2.3 Phosphorylation and degradation of cytoplasmic I-κBα in response to LPS.](image)

**Figure 2.3 Phosphorylation and degradation of cytoplasmic I-κBα in response to LPS.**

PBMCs from healthy controls and CGD patients were equilibrated at 37°C for 30 min, stimulated with LPS (100 ng/ml in the presence of 1% serum) for indicated periods of time and cytoplasmic- and nuclear fractions were prepared. The cytoplasmic fractions (14 µg/sample) were immunoblotted with antibody directed against the phosphorylated form of I-κBα (upper panel), followed by blotting with antibody against total I-κBα (middle panel) after stripping of the membranes. CGD cells displayed slightly elevated resting levels of I-κBα phosphorylation and responded to stimulation by a more pronounced degradation of this protein. Equal loading was ascertained by Coomassie Blue staining of the membranes (lower panel). Representative blots from four different experiments are shown.

### 2.4.5 Nuclear translocation of NF-κB binding proteins

After nuclear translocation, NF-κB subunits bind promoter elements of various proinflammatory genes containing the NF-κB consensus sequence (Natoli et al., 2005). Using EMSA, we assayed nuclear extracts from human PBMCs for NF-κB binding activity, both before and after LPS stimulation. In control cells, NF-κB binding activity was induced 20 minutes after LPS stimulation, and the binding continued to increase for up to 120 minutes (Fig. 4). In CGD cells, some NF-κB-binding activity was evident even in the absence of stimulation. This activity was, however, markedly increased upon LPS stimulation in a manner similar to that seen for control cells (Fig. 2.4). Specificity of the NF-κB binding was demonstrated by performing the reaction in the presence of excess unlabelled NF-κB probe (cold probe), which completely abolished the signals, both from control and CGD cell nuclear extracts.
Figure 2.4  Induction of NF-κB DNA binding by LPS.
Nuclear extracts (5 µg) from PBMCs stimulated with LPS for the indicated times were subjected to EMSA using a biotin-labeled NF-κB consensus sequence oligonucleotide as a probe. CGD extracts exhibited slightly higher NF-κB-binding activity than control extracts in the absence of stimulation and the activity was markedly increased upon stimulation. Addition of excess unlabeled NF-κB probe (cold probe) was used as a competitor (added to the LPS samples treated for 40 min) to determine the specificity of DNA binding. NF-κB-specific complexes, which were out competed by excess cold probe, are indicated by arrows; one representative experiment of four independent experiments is shown.

2.4.6  Nuclear translocation of NF-κB p50
The NF-κB species most heavily implicated in proinflammatory responses is the p50/p65 heterodimer. The EMSA showed specific binding of NF-κB to consensus sequences but did not distinguish between the various NF-κB subunits. To ascertain whether p50 was translocated into the nucleus upon LPS stimulation, we performed immunoblotting of nuclear extracts using an antibody directed to this subunit. Minor background levels of p50 were detected in the nuclei of resting cells, but the signal was robustly increased after LPS stimulation (Fig. 2.5A). We also subjected nuclear extracts to an NF-κB p50 ELISA in order to quantify p50 translocation and binding activity. Accumulation of nuclear p50 occurred in a very similar fashion in both control and CGD cells (Fig. 2.5B), indicating that both translocation and DNA binding of p50 was unimpeded in the absence of ROS.
2.4.7 Translocation of p65 is needed for production of proinflammatory cytokines

Only three out of the five NF-κB members, *i.e.* p65, c-Rel and RelB, contain transactivation domains needed to form transcriptionally active DNA complexes (Magnani *et al.*, 2000). Thus, p50 needs to partner with a transactivation domain-containing component in order to activate gene transcription. We employed a cell-permeable p65 inhibitor peptide (p65i), which has been shown to inhibit specifically NF-κB activation (Takada *et al.*, 2004). In the presence of p65i, LPS-induced production of both IL-6 (Fig. 2.6A) and TNF-α (Fig. 2.6B) was markedly decreased in control and CGD cells. At the concentration used (100 µM), the control peptide only modestly decreased cytokine production, indicating that the inhibitory action of p65i was not due to cytotoxic/unspecific effects and that regardless of the presence of ROS, LPS-induced production of IL-6 and TNF-α required phosphorylation and translocation of NF-κB p65.
Figure 2.5  Nuclear translocation of NF-κB p50 subunit.
PBMCs from healthy controls or CGD patients were stimulated with LPS for the indicated times and nuclear extracts (9 µg) were subjected to immunoblotting (A) with an antibody against the NF-κB p50 subunit (upper panel). The kinetics of NF-κB p50 translocation was similar for control and CGD cells. Equal loading was ascertained by Coomassie Blue staining of membranes after stripping (lower panel); one representative blot from four independent experiments is shown. Nuclear extracts from healthy controls (open bars) or CGD patients (filled bars) were subjected to ELISA to quantify the levels of NF-κB p50 present in the nucleus at the indicated time-points after LPS stimulation. Similar amounts of NF-κB p50 were present in nuclear extracts from control and CGD cells (B). The graph depicts mean values from two independent experiments.
Figure 2.6 Involvement of NF-κB p65 in the hyperinflammatory responses.

PBMCs from healthy controls (open bars) or CGD patients (filled bars) were preincubated for one hour in the presence of medium, p65i (100 µM) with a decoy phosphorylation site corresponding to Ser276 on NF-κB p65, or a control peptide (100 µM) that lacks the Ser276 site. The cells were then stimulated with LPS (100 ng/ml in the presence of 1% serum) for 24 hours in culture and the cells free supernatants were analyzed using the CBA for IL-6 (A) and TNF-α (B). In both control and CGD cells, p65i abolished cytokine production in response to LPS, whereas the control peptide only slightly decreased the responses. The graphs show mean + SEM of three independent experiments performed in triplicate.
2.5 Discussion

CGD is a very rare disorder caused by an inability of affected individuals’ leukocytes to produce ROS. This inability is due to genetic defects in genes encoding the NADPH oxidase; as a result, CGD patients frequently face problematic infections by a narrow range of fungal and bacterial pathogens (Winkelstein et al., 2000). Regardless of the exact mechanism behind ROS-mediated microbial killing, the lack of ROS production from CGD phagocytes confers defective phagocytic killing (Curnutte, 1993), resulting in an increased susceptibility to infection. In addition to predisposition to life-threatening infection, CGD patients suffer from an array of inflammatory complications, most prominently granuloma formation in hollow viscera, inflammatory bowel disease, progressive sterile pulmonary inflammatory disease, and lupus-like syndromes in patients and their obligate heterozygous mothers (Segal et al., 2000). To date, a convincing explanation for the exaggerated inflammatory responses seen in CGD patients is lacking, and the notion that the inflammatory state stems from subclinical infections (due to the decreased antimicrobial action) can not explain, for example, why granulomas are typically sterile (Gallin and Buescher, 1983; Segal et al., 2000). Functionally, CGD cells have been shown to display prolonged intracellular calcium transients upon stimulation, leading to exaggerated cellular responses (Tintinger et al., 2001). ROS have also been implicated in numerous processes of potential importance for the inflammatory conditions displayed by CGD patients: neutrophil cell death (both apoptosis (Kasahara et al., 1997) and necrosis (Bylund et al., 2005b)), secondary clearance of apoptotic cells (Hampton et al., 2002; Brown et al., 2003), and functional downregulation of immunocompetent lymphocytes (Asea et al., 1996). In addition, rats with decreased capacity to produce ROS display increased susceptibility to autoimmune arthritis (Olofsson et al., 2003). Very recent data showing that ROS may dampen the reactivity of neighbouring T cells suggests an intriguing mechanism whereby ROS production could quench autoimmune responses by decreasing the number of reduced thiol groups on T cell
surfaces (Gelderman et al., 2006). Future studies will hopefully determine how these ideas relate to the hyperinflammatory phenotype of human CGD.

Several inflammatory pathways converge on the transcription factor NF-κB, e.g. those triggered by recognition of conserved microbial signatures by genome-encoded TLR (Beutler et al., 2003). As ROS are able to react with, and alter, most types of biomolecules, they have been implicated in a variety of cell-signaling processes including NF-κB activation (Kabe et al., 2005). The role of ROS in NF-κB activation has been studied using a variety of techniques, e.g. a murine model for CGD (Sadikot et al., 2004), or cells in the presence of various antioxidants/NADPH oxidase inhibitors (Shrivastava and Aggarwal, 1999; Fan et al., 2003; Asehnoune et al., 2004; Park et al., 2004). These reports suggest that a lack of ROS production leads to diminished NF-κB activation and thus decreased inflammatory responses, i.e. a fundamentally different scenario from that seen in CGD patients. Here, we show that leukocytes from CGD patients are hyperresponsive to stimulation and produce more proinflammatory cytokines than cells with a functional NADPH oxidase. We also show DPI, an inhibitor of the NADPH oxidase, failed to confer the hyperinflammatory phenotype of CGD cells and is thus a poor tool for studying the role of ROS in inflammatory signaling. This is in line with data describing ROS-independent effects of antioxidants and NADPH oxidase inhibitors (Bowie and O'Neill, 2000; Hayakawa et al., 2003) and points to the importance of interpreting data generated with such substances with great caution. Because CGD is such a rare condition and CGD patients are not readily available, many investigators employ CGD mice to study the role of NADPH oxidase derived ROS. A critical role for ROS in the activation of NF-κB was recently proposed, whereby p47phox -/- CGD mice were unable to activate NF-κB to the same extent as mice with a functional NADPH oxidase upon intratracheal infection with Pseudomonas aeruginosa (Sadikot et al., 2004). Furthermore, the CGD mice had markedly lower levels of
TNF-α in their lungs after infection and were impaired in bacterial clearance. Our contrasting data, showing dramatically enhanced production of proinflammatory cytokines from murine CGD mononuclear spleen cells, were generated using standardized in vitro conditions whereas the previous study employed a complex in vivo model of infection, making direct comparisons difficult. It should also be noted that the gp91<sup>phox</sup>-/- murine model used here differs from the p47<sup>phox</sup> deficient mouse employed by Sadikot <em>et al.</em> (<em>Sadikot et al.</em>, 2004). These different mouse strains represent the two most common genetic deficiencies among human CGD patients (gp91<sup>phox</sup> being the X-linked form of CGD and p47<sup>phox</sup> representing an autosomal recessive form), and differences in severity of symptoms between these patient groups have been reported (<em>Winkelstein et al.</em>, 2000). It should be noted that the murine spleen cell preparations used in our study were not identical to the human PBMC preparations in terms of cellular composition. Murine spleen cell preparations represent a well-established mixed leukocyte system and in both the murine and human systems, lymphocytes were the dominating cell type; no significant differences in cell composition were found between WT/control and CGD preparations. Our data also show that cells from the gp91<sup>phox</sup>-deficient mice were completely devoid of ROS production, regardless of stimulation, whereas WT cells produced ROS both spontaneously and in response to stimulation.

Relatively few studies have been published on inflammatory responses of human CGD cells, a fact probably relating to the scarcity of patients. The available reports invariably describe increased inflammatory reactions in CGD cells/patients (<em>Gallin</em> and <em>Buescher</em>, 1983; <em>Warris et al.</em>, 2003; <em>Hatanaka et al.</em>, 2004; <em>Lekstrom-Himes et al.</em>, 2005), and a recent microarray study described upregulation of various proinflammatory genes in unstimulated CGD cells compared to controls (<em>Kobayashi et al.</em>, 2004). Thus, the data presented in our work are consistent with these published studies and suggest that ROS exhibit a dampening effect on inflammatory reactions, which fits very well with clinical data on exuberant inflammatory
responses in CGD patients (Segal et al., 2000; Schappi et al., 2003; Levine et al., 2005). The level at which this dampening occurs and the mechanistic details behind the phenomenon remain to be elucidated.

The hyperinflammatory phenotype of CGD leukocytes was evident regardless of the nature of the stimulus, indicating general dysregulation rather than an abnormality in a specific activation pathway and/or a specific cell type. A plausible explanation for the increased cytokine production displayed by CGD cells could be that these cells survived better than WT cells in culture. In contrast, CGD cells were slightly less viable than WT cells after 24 h in culture, although the difference was not statistically significant. These data clearly indicated that the exaggerated cytokine production displayed by CGD cells in vitro was not due to increased survival in culture. Although we could not detect any profound differences in the abilities of CGD cells to execute the various steps involved in NF-κB activation, other ROS dependent cell signaling systems might be involved in the hyperresponsiveness of CGD cells. One such system is the pathway that involves activator protein-1 (AP-1), a dimeric transcription factor that is implicated in inflammatory signaling and has recently been connected to inflammatory complications such as psoriasis and arthritis (Zenz et al., 2005). Evidence suggests that AP-1 is redox sensitive (Liu et al., 2005) and can be activated by ROS (Iles et al., 2002). Whether AP-1 activation is somehow defective or abnormal in CGD cells, has to our knowledge, never been investigated.

Our data clearly show that CGD cells are competent to activate the NF-κB pathway in the absence of ROS and present with a general hyperinflammatory phenotype in response to multiple different stimuli, e.g. TLR stimulation. Although at present we can only speculate on the underlying reason for the hyperinflammatory phenotype of CGD cells, it seems obvious that the lack of ROS as microbicidal effectors is only part of the explanation for CGD pathology.
2.6 References


3. *Burkholderia cenocepacia* but not *Burkholderia multivorans* disrupts maturation and induces necrosis in human dendritic cells

3.1 Summary

*Burkholderia cepacia* complex (BCC) bacteria cause pulmonary infections which can evolve into fatal overwhelming septicemia in chronic granulomatous disease (CGD) or cystic fibrosis (CF) patients. *B. cenocepacia* and *B. multivorans* are responsible for the majority of BCC infections in CF patients, but *B. cenocepacia* is generally associated with a poorer prognosis than *B. multivorans*. The present study investigated whether these pathogens could modulate the normal functions of primary human monocyte-derived dendritic cells (DCs), important phagocytic cells that act as critical orchestrators of the immune response. Effects of the bacteria on maturation of DCs were determined using flow cytometry. DCs co-incubated for 24 hours with *B. cenocepacia*, but not *B. multivorans*, had reduced expression of co-stimulatory molecules when compared with standard BCC lipopolysaccharide-matured DCs. *B. cenocepacia*, but not *B. multivorans*, also induced necrosis in DCs after 24 h, as determined by annexin V and propidium iodide staining. DC necrosis only occurred after phagocytosis of live *B. cenocepacia*; DCs exposed to heat-killed bacteria, bacterial supernatant, or those pre-treated with cytochalasin D then exposed to live bacteria remained viable. The ability of *B. cenocepacia* to interfere with normal DC maturation and induce necrosis may contribute to its pathogenicity in susceptible hosts.

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3.2 Introduction

*B. cepacia* complex (BCC) is a group of opportunistic bacterial pathogens that cause serious infections in patients with chronic granulomatous disease (CGD) or cystic fibrosis (CF) (Isles et al., 1984; Corey and Farewell, 1996; Hutchison and Govan, 1999; Winkelstein et al., 2000; Johnston, 2001; Speert, 2002). CGD is a rare inherited disorder of phagocytic cells caused by mutations in components of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which is responsible for generating the respiratory burst (Segal et al., 2000; Johnston, 2001). Despite the profound defect in phagocytic cells and severe inflammatory complications associated with this disease, CGD patients are susceptible to only a narrow spectrum of pathogens, with BCC being the most virulent Gram negative bacterium (Winkelstein et al., 2000; Speert, 2002). BCC bacteria predominantly cause lung infections in CGD that can develop into bacteremia, a rare occurrence with other CGD pathogens (Speert, 2002). CF, the most common fatal autosomal recessive disease among Caucasians, is caused by a defect in the CF transmembrane conductance regulator (Rommens et al., 1989). CF patients are susceptible to chronic bacterial pulmonary infections with a characteristic array of pathogens. Though *Pseudomonas aeruginosa* is the most prevalent CF pathogen, infections with BCC are associated with a worse prognosis (Corey and Farewell, 1996; Hutchison and Govan, 1999; Chaparro et al., 2001). BCC may cause “cepacia syndrome”, characterized by rapid pulmonary deterioration, septicaemia, and death in about 20% of infected CF patients (Isles et al., 1984; Hutchison and Govan, 1999). This type of severe complication is virtually never observed with *P. aeruginosa*. Adding to the complexity of this clinical picture, different species within the complex display discrete pathogenicity in CF patients. *B. multivorans* and *B. cenocepacia* are the two most clinically important members of the BCC. Prior to adoption of stringent infection control policies, *B. cenocepacia* comprised 80% of BCC clinical CF isolates in Canada and was associated with a higher mortality rate than the less prevalent *B. multivorans*, and, unlike most
strains of *B. multivorans*, may be readily transmitted among patients (Chaparro *et al.*, 2001; Speert *et al.*, 2002).

The virulence of *B. multivorans* and *B. cenocepacia* in CF and CGD suggests an interplay between bacterial and host determinants, but clues about this complex interaction are just beginning to emerge (Mahenthiralingam *et al.*, 2005). Abrogation of normal phagocytic cell function may play an important role in these processes. BCC bacteria are impervious to non-oxidative phagocytic killing by neutrophils (Speert *et al.*, 1994) and avoid oxidative killing actively by producing superoxide scavengers (Zughaier *et al.*, 1999b; Lefebre and Valvano, 2001; Smalley *et al.*, 2001; Bylund *et al.*, 2005b). BCC bacteria also thrive in environments where oxidative killing is compromised such as CGD lung, which is inherently devoid of superoxide, and the CF lung, where an oxidant/antioxidant imbalance has been reported (Speert, 2002; Bylund *et al.*, 2005b). These organisms also survive within macrophages (Saini *et al.*, 1999; Martin and Mohr, 2000; Chu *et al.*, 2004) perhaps by delaying phagosomal acidification, as has been recently demonstrated in cultured macrophages infected with *B. cenocepacia* (Lamothe *et al.*, 2007). BCC lipopolysaccharide (LPS) is reportedly extremely pro-inflammatory (Shaw *et al.*, 1995; Zughaier *et al.*, 1999a; Hutchison *et al.*, 2000; Shimomura *et al.*, 2001; Gronow *et al.*, 2003), yet some BCC species persist within mice in the absence of overt inflammation (Speert *et al.*, 1999; Chu *et al.*, 2002; Chu *et al.*, 2004), which also suggests that these organisms may be occupying a privileged intracellular niche. Indeed, in a murine pulmonary infection model, *B. multivorans* persists within mononuclear phagocytes in the absence of inflammation, while *B. cenocepacia* induces greater inflammation and toxicity (Chu *et al.*, 2004). Secreted bacterial products from this group of pathogens also induce apoptosis or necrosis in neutrophils and macrophages (Hutchison *et al.*, 1998; Melnikov *et al.*, 2000; Punj *et al.*, 2003).
Dendritic cells (DCs) also reside in the lung, act as crucial mediators between innate and adaptive immunity, and may therefore play a role in the pathogenesis of BCC. Immature DCs act as sentinels in the tissue; they are very efficient in antigen capture through macropinocytosis, receptor-mediated endocytosis, and phagocytosis (Banchereau et al., 2000). A broad repertoire of opsonic and non-opsonic phagocytic receptors enable DCs to bind and internalize microbes (Banchereau et al., 2000; Cutler et al., 2001). Upon capture of soluble antigen or bacteria, immature dendritic cells migrate to secondary lymphoid organs while undergoing phenotypic and functional changes that transform them from antigen capturing cells to mature antigen processing and presenting cells. The latter have the critical capacity to activate naïve T cells, inducing the appropriate immune response to deal with the invading microbe (Banchereau and Steinman, 1998; Banchereau et al., 2000; Kapsenberg, 2003). The importance of DCs to immunity is highlighted by the fact that microbial pathogens have evolved diverse strategies to avoid or subvert DCs. Microbial pathogens can thwart DC differentiation, survival, maturation, antigen processing and presentation, and the induction of T cell immunity (Palucka and Banchereau, 2002; Moll, 2003; Steinman and Banchereau, 2007; Ueno et al., 2007). Because dendritic cells migrate from the sites of infection to secondary lymph nodes to present antigen, they may also serve as systemic vectors (“trojan horses”) for persistent intracellular bacteria. This concept is tantalizing in light of the proclivity of BCC organisms to cause fatal necrotizing pneumonia and sepsis in susceptible individuals (Hutchison and Govan, 1999).

We studied the interaction between B. multivorans/B. cenocepacia and primary human monocyte-derived DCs to gain new insights into the pathogenesis of BCC. We hypothesized that B. cepacia complex subvert normal antibacterial mechanisms of DCs. The ability of B. multivorans and B. cenocepacia to modulate DC functions was investigated by examining DC maturation, cytokine production, and cell death processes. B. cenocepacia, unlike B.
multivorans, impaired DC maturation and stimulated DC necrosis, despite inducing similar amounts of IL-6, IL-12, TNF-α, and IL-10.

3.3 Materials and methods

3.3.1 Bacterial strains, media, and growth conditions

B. multivorans C5568 and B. cenocepacia C6433 are isolates from patients with CF that had been stored in the Canadian B. cepacia complex Research and Referral Repository. Eight additional clinical isolates from CF and CGD patients were selected from the BCC strain panel (B. multivorans C5393, C1576, and JTC and B. cenocepacia J2315 and K56-2) (Mahenthiralingam et al., 2000a) and from the Canadian B. cepacia complex Research and Referral Repository (B. multivorans D0999 and B. cenocepacia C8963 and Cep1067). Bacteria were stored at -70°C in Mueller Hinton broth with 8% (v/v) dimethyl sulfoxide. Routine culturing was performed on blood agar plates (PML Microbiologicals, Richmond, British Columbia, Canada) or Luria-Bertani (LB) agar. Bacteria were grown in 5 ml of LB broth at 37°C with agitation at 250 r.p.m. for 16.5 h to stationary phase, pelleted at 4500 g for 10 min at room temperature, then adjusted to the correct optical density (OD_{610nm} of 0.600). Further dilutions were performed in R10 cell culture medium (RPMI 1640 supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine and 1 mM sodium pyruvate) to a concentration of 6 × 10^5 cfu/ml. For some experiments, bacteria were heat-killed by incubating 1 ml of culture in a 56°C water bath for 15 min. To prepare bacterial cell-free supernatants, cultures were grown for 20 h in R10 at 37°C, 5% CO₂ and filtered through 0.22 μm cellulose acetate filters (Costar, Corning, NY). The concentration of viable bacteria and the efficiency of heat-killing and filtration was confirmed by viable plate counting.
3.3.2 Preparation of lipopolysaccharide (LPS)

Purified LPS from *B. multivorans* C5568 and *B. cenocepacia* C6433 was kindly provided by R.K. Ernst (Univ. of Washington). LPS had been isolated using a hot water-phenol extraction method (Westphal and Jann, 1965), and then treated with RNase A, DNase I and proteinase K to remove contaminating nucleic acids and proteins (Fischer *et al.*, 1983). Subsequent purifications had been performed to eradicate contaminating phospholipids (Folch *et al.*, 1957) and proteins which signal through toll-like receptor 2 (Hirschfeld *et al.*, 2000).

3.3.3 Monocyte isolation and culture into DCs

Monocyte-derived DCs were generated using standard protocols (Sallusto and Lanzavecchia, 1994; Davidson *et al.*, 2004). Briefly, human venous blood was collected from normal adult volunteers, according to the University of British Columbia Clinical Research Ethics Board protocol C04-0193. Heparanized blood was diluted 1:1 in R10 medium, then overlayed onto Ficoll-Paque plus (GE Healthcare, Uppsala, Sweden). Following centrifugation at 450 g for 20 min at 20°C, the peripheral blood mononuclear cells were collected from the interface and washed with phosphate buffered saline (PBS). T cells were depleted by incubation with neuraminidase-treated sheep erythrocytes and an additional Ficoll-Paque purification. Remaining mononuclear cells were collected from the interface, washed with PBS, resuspended in R10, and cultured in six-well plates at a concentration of $4 \times 10^6$ cells per well for 1 h at 37°C, 5% CO$_2$. After washing with PBS to remove non-adherent cells, the adherent monocytes were cultured in R10 containing 100 ng/ml granulocyte-macrophage colony stimulating factor (GM-CSF) and 100 ng/ml interleukin-4 (IL-4) (Fitzgerald, Concord, MA) for seven days at 37°C, 5% CO$_2$ to generate immature dendritic cells.

After seven days, DCs were harvested from six-well plates, washed with PBS, and resuspended in R10. Enumeration and confirmation of viability was performed using Trypan blue dye exclusion as assessed in a haemocytometer. Flow cytometry was used to confirm the
purity and immature phenotype of the resultant cells, as previously described (Davidson et al., 2004). Briefly, an aliquot of DCs was resuspended in FACS buffer (PBS + 10 % (v/v) FCS, 0.01% (v/v) sodium azide, and 0.01% (v/v) human AB serum (Sigma)) at a concentration of \(1 \times 10^6\) DCs/ml. One hundred microlitres of DCs/tube were stained for 1 h at 4°C with FITC-conjugated monoclonal antibodies specific for CD1a (Serotec, Raleigh, NC), CD14, CD86 (BD Biosciences, Mississauga, Ontario, Canada), CD83, CD19, IgG2a or IgG1 isotype controls (eBioscience, San Diego, CA) and with PE-conjugated antibodies raised against CD206 (BD Biosciences), CD80, TCR alpha/beta, and an IgG1 isotype control (eBioscience). After two washes in PBS, DCs were resuspended in 200 µl of PBS + 2% (v/v) formaldehyde for flow cytometry.

### 3.3.4 DC infection assays

DCs were seeded into 96-well or 24-well plates at a concentration of \(5 \times 10^5\) cells/ml and challenged with live *B. multivorans* or *B. cenocepacia* at a multiplicity of infection (MOI) of 0.3:1. Untreated DCs in R10 served as a control. Plates were incubated at 37°C in 5% CO\(_2\) for 2 h, after which 8 µg/ml of meropenem (one to two times the minimum inhibitory concentration for each organism), was added to each well. DCs were then re-incubated up to 24 h. This experimental set-up was based on preliminary experiments of BCC bacteria growing in cell culture media in the absence of DCs. BCC bacteria multiplied two to three log-fold over 24 h in R10 alone. When 8 µg/ml of meropenem was added, the cfu/ml of BCC bacteria decreased by four to five log-fold after 24 h, from a starting inoculum of \(1.25 \times 10^5\) to 5-30 cfu/ml. Therefore, after 2 hours of DC:BCC co-incubation, meropenem was added to control extracellular growth of the bacteria. At the appropriate time points, 20 mM EDTA was added to each well, the plate was incubated at 37°C for ten minutes, and then DCs were harvested from the appropriate wells.
3.3.5 Association assays and viable plate counts
DCs were co-incubated with *B. multivorans, B. cenocepacia*, or R10 alone as a control, and then harvested at 2, 6, 12, 18 and 24 h. Small aliquots from each well were diluted in Hank’s balanced salt solution containing 10% gelatin and 1% triton X-100 to lyse DCs and release any associated bacteria. The resulting lysates were plated on LB agar, and incubated at 37°C for enumeration. The remaining cells were washed to remove non-adherent bacteria then cytocspun onto glass slides. The slides were stained with Diff-Quik (Dade Behring, Newark, DE) and permounted. The number of bacteria associated with each DC was determined by light microscopy for 100 DCs per slide.

3.3.6 DC maturation and cytokine enzyme-linked immunosorbent assays
DCs were seeded into Teflon inserts in 24 well plates at a concentration of $5 \times 10^5$ cells/ml and then exposed to R10 only, 200 µg/ml LPS, or heat-killed or live bacteria at an MOI of 0.3:1. After 24 h of incubation at 37°C, 5% CO2, supernatants were collected for cytokine enzyme-linked immunosorbent assays (ELISA) and stored at -70°C. DCs were then harvested, washed, resuspended in FACS buffer and $1 \times 10^5$ DCs/tube were stained with FITC CD83, FITC CD86 and PE CD80, or the appropriate isotype controls for 1 h at 4°C, washed twice with PBS, and fixed in 200 µl of PBS + 2% (v/v) formaldehyde for flow cytometry. Cytokine ELISAs were performed using commercial ELISA kits for IL-6, TNF-α, IL-10 and IL-12p70 (BD Biosciences), according to the manufacturer’s instructions. Plates were read using a Model 3550 Microplate reader (Bio-Rad Laboratories, Mississauga, Ontario, Canada).

3.3.7 Apoptosis necrosis assays
Induction of cell death was examined by co-incubating $5 \times 10^5$ DCs /ml in 96-well plates with *B. multivorans, B. cenocepacia*, or R10 (negative control) for 2, 6, 12, 18 and 24 h. For some experiments, DCs were exposed to heat-killed bacteria, bacterial cell-free supernatant, or
pre-treated for ten minutes with 10 µg/ml of cytochalasin D prior to addition of live bacteria. Positive controls for apoptosis and necrosis were used to validate these assays at each time point. DCs were exposed to 1 µg/ml of actinomycin D (Calbiochem) to induce apoptosis (Colino and Snapper, 2003). Untreated DCs were exposed to 0.05 % (wt/vol) of the detergent saponin (Sigma) prior to harvesting to cause complete cell necrosis (Colino and Snapper, 2003). DCs were next stained with Annexin V (BD Biosciences) and propidium iodide (BD Biosciences) in Annexin V binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂). Cells were analyzed by flow cytometry within 1 h. Based on differential staining with Annexin V and propidium iodide, this assay distinguishes among viable (Annexin V-negative, propidium iodide-negative), apoptotic (Annexin V-positive, propidium iodide-negative), and necrotic (Annexin V-positive, propidium iodide-positive) cells.

3.3.8 Flow cytometry.
A minimum of 1×10⁴ gated events were collected for each condition using a FACSCalibur system and CellQuest, version 3.1, software (BD Biosciences). Data were analyzed using Flowjo 7.2.2 flow cytometry software (Tree Star, Ashland, OR).

3.3.9 Statistical analyses
Data are expressed as mean ± SEM. Paired Student’s t tests were used to evaluate the statistical differences between B. multivorans- and B. cenocepacia- treated DCs. Comparisons between a control and several experimental groups were performed using one-way analyses of variance with a Dunnett's multiple comparison test. Multiple group comparisons were performed using one-way analyses of variance with a Tukey post-test. A value of \( P < 0.05 \) was taken as a statistically significant difference.
3.4 Results

3.4.1 DC phenotype

Monocytes cultured for seven days with GM-CSF and IL-4 displayed the expected immature DC phenotype; staining strongly for the DC markers CD1a and CD206 (mean fluorescent intensities (MFI) of 134.14 ± 16.77 and 104.66 ± 26.33, respectively) and weakly for the monocyte/macrophage surface molecule CD14 (4.27 ± 0.56), the lymphocyte markers TCR alpha/beta and CD19 (0.93 ± 0.32 and 0.14 ± 0.06, respectively) and 3 markers of mature DCs: CD83, CD80 and CD86 (1.01 ± 0.59, 2.96 ± 1.63, 6.87± 1.27, respectively). Cell viability was 93.42% ± 1.02%, as determined by Trypan blue dye exclusion. Subsequent long-term incubations in meropenem had no impact on DC viability or cell surface marker expression (data not shown).

3.4.2 Bacterial association with DCs

During the first 6 h of co-incubation, similar numbers of *B. multivorans* and *B. cenocepacia* associated with DCs, with approximately 40% of DCs binding at least one BCC bacterium. A considerable proportion of this early association was due to phagocytic uptake of the bacteria into DCs rather than simple binding of the bacteria to the external surface of cells, as pre-exposure to cytochalasin D, which paralyses the cytoskeleton and prevents uptake, caused a substantial reduction in the numbers of BCC associating with DCs. At 2 h, there were 50.52% ± 10.70% and 73.42% ± 1.83% fewer *B. multivorans* and *B. cenocepacia* bacteria respectively, per cytochalasin D-treated DC than per normal phagocytic DC. From 2 to 24h, the mean number of bacteria per DC increased from 1.40 ± 0.50 and 0.52 ± 0.11 to 10.92 ± 2.38 and 19.81 ± 4.55 for *B. multivorans* and *B. cenocepacia*, respectively (Fig. 3.1A). At 24 h, DCs associated with significantly more *B. cenocepacia* than *B. multivorans* (Fig. 3.1A, *P* < 0.05). This corresponded to an increase in the percentage of DCs associating with more than ten *B. cenocepacia* when
compared to the percentage of DCs associating with more than ten *B. multivorans* (47.20% ± 4.00% vs. 21.20% ± 9.49%). At later time points, DCs exposed to *B. cenocepacia* had noticeably more peripheral cell debris and more bacterial cells associated with this debris than on slides of DCs treated with *B. multivorans*. The numbers of live *B. multivorans* and *B. cenocepacia* remained constant for the first 6 h of interaction with DCs and slowly increased from 6 to 24 h, with doubling times of 5.32 h and 2.73 h over the final 18 h of the incubation (Fig. 3.1B). In comparison, *B. multivorans* and *B. cenocepacia* had doubling times of 2.12 h and 1.98 h, respectively, during exponential growth in R10 alone, in the absence of DCs and meropenem. These results contrast with the deleterious effect of R10 with meropenem on BCC

![Graph A](image1.png)

![Graph B](image2.png)

**Figure 3.1** BCC association with DCs and viability over time.

(A) Mean number of *B. multivorans* and *B. cenocepacia* associated with DCs over time, as assessed by light microscopic enumeration of cyto-spun and Diff-Quik-stained DCs (average of 3, 4, 3, 2, 5 donors per time point ± SEM). (*significant difference between the mean number of *B. multivorans* and *B. cenocepacia* associating with DCs at indicated time points, *P* < 0.05). (B) Cfu/ml of *B. multivorans* and *B. cenocepacia* over 24 h of interaction with DCs, as assessed by viable plate counting of DC lysates (average of 4, 3, 3, 2, 4 donors per time point ± SEM).
growth in the absence of DCs: a 4 to 5 log-fold decrease in the colony-forming unit (cfu)/ml of
*B. multivorans* and *B. cenocepacia* over 24 h. There were no significant differences in viable *B. multivorans* and *B. cenocepacia* when co-incubated with DCs and meropenem at each time point (Fig. 3.1B).

### 3.4.3 Influence of BCC on DC cytokine production and maturation

DCs co-incubated for 24 h with live *B. multivorans* and *B. cenocepacia* bacteria produced significantly more IL-6, TNF-α, IL-10 and IL-12 than control DCs or DCs stimulated with heat-killed BCC (Fig. 3.2, *P* < 0.001). Live BCC bacteria were also a more potent stimulus for cytokine release than BCC LPS. IL-6, TNF-α, and IL-10 were produced in significantly higher quantities after stimulation with live *B. multivorans* vs. *B. multivorans* LPS (*P*<0.01, *P*<0.001, *P*<0.05, respectively), while IL-6, TNF-α and IL-12 concentrations were significantly higher after stimulation with live *B. cenocepacia* vs. *B. cenocepacia* LPS (*P*<0.001, *P*<0.001, *P*<0.01, respectively). Although DCs stimulated with BCC LPS released more cytokine than unstimulated DCs, these differences were only significant for IL-6 (*P* < 0.01 and *P* < 0.05 for *B. multivorans* LPS versus control and *B. cenocepacia* LPS versus control, respectively) and IL-10 (*P*<0.05 and *P*<0.01 for *B. multivorans* LPS versus control and *B. cenocepacia* LPS versus control, respectively). When the cytokine-inducing ability of each species was directly compared, not only were amounts of cytokines elicited by *B. multivorans* and *B. cenocepacia* LPS similar, but also the levels of IL-6, TNF-α, IL-10 and IL-12 were uniformly high and not significantly different whether DCs were stimulated with live *B. multivorans* or *B. cenocepacia* (Fig. 3.2).
Figure 3.2  24 h cytokine production.
Production of (A) IL-6, (B) TNF-α, (C) IL-10, and (D) IL-12 by DCs after 24h exposure to B. multivorans or B. cenocepacia LPS, heat killed or live bacteria as determined by ELISA (average ng/ml of six donors ± SEM).  *P < 0.05, **P < 0.01, ***P < 0.001, significantly different than control, as calculated by a one-way analysis of variance using Tukey’s multiple comparisons test).

As expected, DCs exposed to B. multivorans and B. cenocepacia LPS upregulated CD83, CD80 and CD86 and over 70 % of cells stained positively for both costimulatory molecules CD80 and CD86 (Table 3.1).  All surface markers measured were significantly upregulated after stimulation with BCC LPS (P < 0.05).  Heat-killed BCC failed to induce phenotypic maturation of DCs, as neither the mean fluorescent intensities of the three cell surface markers nor the percentage of cells positive for both CD80 and CD86 were significantly different from unstimulated DCs (Table 3.1).  CD83 expression on DCs exposed to live BCC was approximately half that of DCs exposed to BCC LPS, and was not significantly greater than CD83 expression on control DCs (Table 3.1).  However, DCs co-incubated with B. multivorans or B. cenocepacia displayed distinct patterns of costimulatory molecule expression.  B. multivorans-exposed DCs, as compared to control DCs, had two fold greater expression of
CD86, significantly greater expression of CD80 ($P < 0.05$), and a significantly greater proportion of cells that were doubly positive for the two co-stimulatory molecules ($P < 0.01$) (Table 1). In contrast, DCs infected with live *B. cenocepacia* failed to upregulate CD80 and CD86, and showed similar patterns of expression as control cells for all markers examined (Table 3.1). In addition, when *B. cenocepacia* LPS and live bacteria were simultaneously co-incubated with DCs for 24h, there was no upregulation of co-stimulatory molecules (data not shown). In the course of the maturation experiments, it was also observed that 24 h of exposure to live *B. cenocepacia*, but not to live *B. multivorans*, heat-killed BCC, or BCC LPS, resulted in a decrease in the total number of viable cells as assessed by Trypan blue staining, and there was a dramatic increase in the number of cells that appeared dead (i.e. cells with low forward scatter and intermediate side scatter on flow cytometry plots).

**Table 3.1** Surface marker expression of DCs after exposure to *B. multivorans* and *B. cenocepacia*.

Surface marker expression of DCs after 24 h exposure to *B. multivorans* or *B. cenocepacia* LPS, heat-killed or live bacteria, displayed as mean fluorescent intensity of the maturation markers CD83, CD80, and CD86 and the percent of DCs positive for both CD80 and CD86 (n=5).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean fluorescent intensity (± SEM)</th>
<th>Percent Positive (± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD83 (± SEM)</td>
<td>CD80 (± SEM)</td>
</tr>
<tr>
<td>Control</td>
<td>1.48 (0.37)</td>
<td>13.23 (3.60)</td>
</tr>
<tr>
<td><em>B. multivorans</em> C5568 LPS</td>
<td>7.80 (2.33)*</td>
<td>34.69 (5.51)**</td>
</tr>
<tr>
<td><em>B. cenocepacia</em> C6433 LPS</td>
<td>8.65 (2.73)**</td>
<td>32.26 (5.01)*</td>
</tr>
<tr>
<td><em>B. multivorans</em> C5568 Heat-killed</td>
<td>1.63 (0.18)</td>
<td>17.12 (4.81)</td>
</tr>
<tr>
<td><em>B. cenocepacia</em> C6433 Heat-killed</td>
<td>2.08 (0.37)</td>
<td>18.45 (3.80)</td>
</tr>
<tr>
<td><em>B. multivorans</em> C5568</td>
<td>3.28 (0.55)</td>
<td>33.40 (5.78)*</td>
</tr>
<tr>
<td><em>B. cenocepacia</em> C6433</td>
<td>4.71 (1.34)</td>
<td>18.31 (5.23)</td>
</tr>
</tbody>
</table>

*P* < 0.05, **P* < 0.01, significantly different from unstimulated control as calculated by a one-way analysis of variance with Dunnett’s multiple comparisons test
3.4.4 Induction of DC necrosis by *B. cenocepacia*

DCs exposed to *B. cenocepacia* remained viable, i.e. the vast majority of cells were negative for both annexin V and propidium iodide, for the first 6 h, then sequentially lost viability from 12 to 24h, while the viability of *B. multivorans*-treated DCs and control DCs remained consistently high over 24 h (Fig. 3.3A). Significantly fewer DCs infected with *B. cenocepacia* were viable than control DCs at 18 (46.70% versus 86.74%, *P* < 0.01) and 24 h (27.60% versus 79.30%, *P* < 0.01). This drop in viability did not elicit a corresponding rise in apoptosis over time for DCs treated with *B. cenocepacia*. Indeed, similarly low background levels of apoptosis ranging from 5.75% to 15.36% were observed in untreated DCs and those co-incubated with *B. cenocepacia* or *B. multivorans* at all time points, though DCs given the apoptosis inducer actinomycin D displayed a time dependant increase in apoptosis, peaking at 63.39% after 12 h of co-incubation (Fig. 3.3B). Instead of inducing apoptosis, exposure to *B. cenocepacia* began to induce necrosis in DCs after 12 h of co-incubation; the proportion of DCs staining positively for annexin V and propidium iodide increased from 18.82% at 12 h to 57.63% at 24 h (Fig. 3.3C). DCs exposed to *B. cenocepacia* demonstrated a higher percentage of necrotic cells than control DCs at 12, 18 and 24h (*P* < 0.01). Untreated DCs and those co-incubated with *B. multivorans* displayed only background levels of necrosis, with at most 5.24% of control and 13.44% of *B. multivorans*-treated cells staining positively for both dyes, unlike DCs treated with the detergent saponin, of which 95.34-99.00% were dead (Fig. 3.3C).

Unlike DCs exposed to live *B. cenocepacia*, DCs treated with LPS, heat-killed bacteria or overnight cell-free supernatant for 24 h did not display significantly more necrosis than the background level of necrosis in control cells (Fig. 3.4). At the standard MOI of 0.3:1, DCs did bind fewer heat-killed than live *B. cenocepacia*, at earlier time points. Association of heat-killed and live bacteria with DCs was therefore equilibrated by increasing the MOI of heat-killed *B. cenocepacia* by 100 fold to 30:1 (the MOI of live *B. cenocepacia* remained at 0.3:1) and by
Figure 3.3  Induction of DC necrosis by *B. cenocepacia* over time.
Annexin V and propidium iodide staining profile of control DCs and DCs exposed to *B. multivorans* or *B. cenocepacia*. DCs treated with actinomycin D and saponin were controls for apoptosis and necrosis, respectively. (A) Viability of cells was measured by the percentage of annexin V−, propidium iodide− DCs, (B) Apoptosis of cells was measured by the percentage of annexin V+, propidium iodide− DCs, (C) Necrosis of cells was measured by the percentage of annexin V+, propidium iodide+ DCs. (average of 3, 3, 3, 2, 4 donors per time point ± SEM, *P* < 0.05, **P* < 0.01, significantly different than control at particular time points, as measured by a one-way analysis of variance with Dunnett’s multiple comparison test).
Figure 3.4  Induction of DC necrosis by live *B. cenocepacia*.

Percentage of necrotic (annexin V+, propidium iodide+) DCs after 24 h exposure to *B. multivorans* or *B. cenocepacia* LPS, heat killed or live bacteria, bacterial cell-free supernatant, or live bacteria following a cytochalasin D pre-treatment. Untreated and saponin-exposed DCs served as negative and positive controls for necrosis, respectively (average of 3 donors ± SEM, **significantly different than untreated control, *P* < 0.01, as measured by a one-way analysis of variance with Dunnett’s multiple comparisons test).

Centrifuging the bacteria onto the DCs. Even at this high MOI, heat-killed *B. cenocepacia* had no effect on DC viability, unlike live *B. cenocepacia* (Appendix 7). DCs also failed to become necrotic when pre-treated with cytochalasin D and then exposed to live *B. cenocepacia*.

Cell death assays were also performed for DCs exposed to four other non-clonal clinical isolates each of *B. multivorans* and *B. cenocepacia* as well as the original pair of *B. multivorans* C5568 and *B. cenocepacia* C6433. None of the five clinical isolates of *B. multivorans* induced significant necrosis after 24 h, while four out of five *B. cenocepacia* isolates caused necrotic cell death in DCs (*P*<0.01, Fig. 3.5). Only *B. cenocepacia* Cep1067 failed to induce significant necrosis in DCs.
Figure 3.5  Four out of five *B. cenocepacia* isolates induced necrosis in DCs.
Percentage of necrotic (annexin V+, propidium iodide+) DCs after 24 h exposure to five clinical isolates of *B. multivorans* and five clinical isolates of *B. cenocepacia*. Unstimulated DCs and saponin-treated DCs were used as controls (average of 4 donors ± SEM, ** significantly different than control, *P* < 0.01, as measured by a one-way analysis of variance with Dunnett’s multiple comparisons test).

3.5 Discussion

This study is the first report of the interaction between human DCs and two important opportunistic pathogens of CF and CGD patients, *B. multivorans* and *B. cenocepacia*. We analyzed the ability of these bacteria to associate with DCs, to induce maturation, to stimulate cytokine production, and to affect cell death processes. We demonstrated that DCs bind and internalize *B. multivorans* and *B. cenocepacia* at a very low MOI and in the absence of opsonins and mechanical manipulations, i.e. no centrifugation of bacteria onto cells. The number of bacteria associated with DCs and viable bacterial counts both increased over time. The increase in cfu/ml was quite gradual then levelled off for *B. multivorans* between 6 and 24 h, with a doubling time of 5.32 h, in contrast to the relatively fast *B. multivorans* doubling time of 2.12 h in R10 alone. However, the doubling time of *B. cenocepacia* after 6-24h in the presence of DCs and meropenem was 2.73 h, only slightly slower than 1.98 h, the doubling time of *B.
B. *cenocepacia* in R10 media alone. This raises the question of whether BCC replicate inside DCs, which, if true, would be in contrast to previous studies of BCC and murine macrophage cell lines which show uptake but no intracellular growth of BCC (Saini et al., 1999; Lamothe et al., 2007). However, we could not conclusively demonstrate intracellular *B. cenocepacia* replication, due to the concomitant induction of necrosis. *B. cenocepacia* were associated with live, dying, and dead cells at 12, 18 and 24 h, some or all of which may have been permissive to bacterial growth and subsequent increased association. Bacteria may also have escaped from dead or dying cells and bound to other DCs. Indeed, we observed many *B. cenocepacia* among cell debris at 24 h: this likely accounted for the significantly increased cellular association of *B. cenocepacia* when compared with *B. multivorans* at this late time point. In addition, meropenem, though effective at reducing viable bacterial counts to near zero in cell culture media, may not have completely eliminated all of the extracellular bacteria; for instance, it may not have killed bacteria in close association, but uningested by DCs. Meropenem is also not as completely cell-impermeable as aminoglycosides, though it does not accumulate inside cells (Lemaire et al., 2005). The activity of meropenem may have been compromised at later time points, overwhelmed by the release of *B. cenocepacia* from many necrotic DCs. Therefore, more tools are needed to draw conclusions about intracellular BCC replication in DCs. We are currently developing such tools.

Once the ability of BCC to associate with DCs had been established, it was important to examine whether BCC induced DCs to produce cytokines and to undergo maturation. Significant amounts of IL-6, TNF-α, IL-10 and IL-12 were produced by DCs exposed to BCC LPS and live bacteria. DCs secreted similar amounts of IL-6, TNF-α, IL-10 and IL-12 upon exposure to either *B. multivorans* or *B. cenocepacia*, the only significant differences in cytokine induction were observed between live BCC bacteria and BCC LPS. The pro-inflammatory nature of LPS from clinical BCC strains has been well described in studies of human whole blood, peripheral blood mononuclear cells, monocyte-derived macrophages, macrophage cell
lines, and murine macrophages (Shaw et al., 1995; Zughai er et al., 1999a; Hutchison et al., 2000; Shimomura et al., 2001; Gronow et al., 2003; De Soyza et al., 2004; Bamford et al., 2007; Silipo et al., 2007). Human DCs can now be added to this list of responsive cells. Some variation in the potency of LPS has been described within and between clinical isolates of B. multivorans or B. cenocepacia (De Soyza et al., 2004; Silipo et al., 2007), which was not observed with our two clinical isolates.

In general, live BCC bacteria were more potent stimuli of cytokine production than BCC LPS. This result is in contrast to a previous study using a macrophage cell line and a higher concentration of less highly purified LPS (Zughai er et al., 1999a), which may account for the discrepancy observed, as unpurified LPS can stimulate cells via multiple mechanisms besides ligation of CD14/TLR4. Our data are not unexpected, as live bacteria interact more dynamically with host cells than a simple TLR ligand, both at the cell surface and in the phagosome, and can actively modulate an array of DC processes (Palucka and Banchereau, 2002; Steinman and Banchereau, 2007; Ueno et al., 2007). Indeed, it is interesting to note that heat-killed bacteria, which are often used as surrogates for live bacteria, failed to induce DCs to secrete cytokines, upregulate surface markers or undergo necrosis. This may be due to lack of forced contact between immotile, dead bacteria and DCs. However, necrosis was still not induced by heat-killed B. cenocepacia even when the MOI was increased and the bacteria were spun onto the cells to equilibrate early association levels (data not shown). The present study may suggest a role for one or more heat labile bacterial components in these diverse DC processes. It has been recently demonstrated that live but not heat-killed B. cenocepacia cause a delay in phagosomal maturation of murine macrophages (Saini et al., 1999; Lamothe et al., 2007). Distinct phenotypes of DCs exposed to live and heat-killed BCC may be related to potential phagosomal maturation alterations, but further investigation is required. Though some questions remain, it
was clear that, in this system, live bacteria were required to engage DCs effectively and generated more cytokine release than heat-killed bacteria or even purified LPS.

The maturation experiment demonstrated that DCs challenged with live *B. cenocepacia* failed to upregulate the CD80 and CD86 costimulatory molecules significantly. As effective co-stimulation is required to activate T cells (Kapsenberg, 2003; Steinman and Banchereau, 2007; Ueno et al., 2007), this could have profound effects on the ability of DCs to drive an immune response directed against this serious pathogen. This is not to say that adaptive immunity is completely compromised in patients infected with this organism. Indeed, it has been reported that *B. cepacia* complex-specific antibodies are produced by infected CF patients (Hendry et al., 2000b; Hendry et al., 2000a), likely by interaction with other antigen presenting cells such as macrophages. However, the presence of antibodies does not play an obvious salutary role in chronic disease, as is expected in a group of organisms that appear to adopt an intracellular lifestyle. A robust Th1 response is required to combat intracellular pathogens (Kapsenberg, 2003; Steinman and Banchereau, 2007; Ueno et al., 2007). DCs produced a large amount of IL-12 in response to BCC, but failed to upregulate co-stimulatory molecules, which likely impeded their capacity to activate T cells.

This study also demonstrated that *B. cenocepacia* but not *B. multivorans* caused necrosis of human DCs. Necrosis occurred following exposure to live *B. cenocepacia*, not heat-killed BCC or BCC supernatant. DCs pre-treated with cytochalasin D prior to exposure to live *B. cenocepacia* also did not become necrotic. These results suggest that uptake of live *B. cenocepacia* was required to induce necrosis in DCs. We also determined the scope and relevance of this observation by examining a panel of *B. multivorans* and *B. cenocepacia* clinical isolates. None of the five *B. multivorans* isolates induced significant DC necrosis after 24 h, including C1576, a highly virulent and transmissible strain of *B. multivorans* responsible for significant paediatric mortality during an outbreak in Glasgow, Scotland (Whiteford et al., 2000).
In contrast, four out of five *B. cenocepacia* isolates induced DC cell death. Only *B. cenocepacia* Cep1067, a member of RecA group III-B, a distinct phylogenetic cluster within *B. cenocepacia* (Mahenthiralingam *et al.*, 2000b), failed to induce significant necrosis.

Other researchers have reported that BCC, and specifically *B. cenocepacia*, can induce death in non-phagocytic and phagocytic cells. *B. cenocepacia* cable (cbl) pili have recently been implicated in lung epithelial cell death (Cheung *et al.*, 2007). The cbl pili are mainly produced by *B. cenocepacia* strains belonging to the epidemic ET12 lineage (Mahenthiralingam *et al.*, 2000a) which are members of phylogenetic cluster III-A. Since equivalent necrosis is induced by *B. cenocepacia* J2315 and K56-2, cblA positive ET12 isolates, and *B. cenocepacia* C6433 and 8963 which are not part of the ET12 lineage and are cblA negative (Mahenthiralingam *et al.*, 2000a), another mechanism must be involved in DC death induced by this organism, perhaps involving other types of BCC pili or non-fimbrial adhesins (Chiu *et al.*, 2001; Mohr *et al.*, 2001).

Both apoptotic and necrotic cell death have been described in murine macrophages and human neutrophils following exposure to purified secreted BCC enzymes such as hemolysin, ATP-utilizing enzymes, azurin and cytochrome c551 (Hutchison *et al.*, 1998; Melnikov *et al.*, 2000; Punj *et al.*, 2003). In rodent models, extracellular proteases are associated with tissue damage, and the production of at least one zinc metalloprotease is required for virulence (McKevitt *et al.*, 1989; Kooi *et al.*, 2006). Of particular interest, azurin and ZmpB are secreted by *B. cenocepacia* but not by *B. multivorans* (Punj *et al.*, 2003; Kooi *et al.*, 2006). The relevance of those studies to the present one is unclear, because, in our investigations, DCs remained viable when exposed to BCC supernatants. As the clinical *B. multivorans* and *B. cenocepacia* strains used in this study likely secrete their respective full arsenal of enzymes, it may be that DCs are insensitive to these enzymes, or that these enzymes are not present in sufficient quantity in the 20 h BCC supernatant to cause necrosis. As uptake of live *B. cenocepacia* appears to be required for DC necrosis; the enzymes specifically secreted by *B.
Cenocepacia may contribute to the process if they are produced in sufficient quantities in the microenvironment of the phagosome, but this has not been determined.

Studies from our lab have recently demonstrated that human neutrophils are killed by live *B. cenocepacia* (Bylund *et al.*, 2005a). Neutrophils are short-lived cells that undergo spontaneous apoptosis after 24 h (Savill *et al.*, 1989). Exposure to live *B. cenocepacia*, but not killed bacteria or LPS, enhances apoptosis as compared to untreated cells. Upon challenge with *B. cenocepacia*, CGD neutrophils have slightly higher levels of apoptosis than control, but also undergo necrosis more readily than normal neutrophils. This suggests that *B. cenocepacia* can induce neutrophil necrosis when reactive oxygen species production is compromised, as is found in CGD (Bylund *et al.*, 2005a).

It is difficult to draw parallels between the effect of *B. cenocepacia* on neutrophils and DCs. Though both are phagocytes, DCs, like macrophages, are longer lived cells and do not undergo spontaneous apoptosis after 24 h. DCs are also resistant to many common stimulators of apoptosis (Ashany *et al.*, 1999; Leverkus *et al.*, 2000), consistent with their complimentary role in phagocytosing and eliminating apoptotic cells. Apoptosis represents the normal termination of DC maturation, as DCs encounter microbes or microbial components, mature into antigen-presenting cells, migrate to the lymph node, present to T cells, and then eventually undergo apoptosis; but this entire process takes days (Hou and Van Parijs, 2004; De Trez *et al.*, 2005) rather than hours as is the case with neutrophils. Indeed, apoptosis was not observed over the 24 h study period even in response to LPS, the prototypic DC maturation stimulus.

It is interesting to note that live *B. cenocepacia* induced necrosis in both CGD neutrophils and normal DCs. Bylund et al. (Bylund *et al.*, 2005a) demonstrated that reactive oxygen species are critical for eliminating ingested bacteria and abrogate necrosis of the ingesting neutrophil. Previous work has shown that immature DCs, even from normal donors, produce a lower amount of reactive oxygen species than neutrophils, macrophages and
monocytes (Vulcano et al., 2004). However, DC maturation following LPS exposure stimulates increased ROS production in response to soluble agonists (Vulcano et al., 2004), which has also been confirmed using BCC LPS-matured DCs (K.L. MacDonald, L.A. Burgess, and D.P. Speert, unpublished data). The fact that DCs challenged with *B. cenocepacia* failed to mature suggests that the bacteria remained in an immature ROS-deficient phagocyte that may be incapable of producing much ROS. This may contribute to the toxicity of *B. cenocepacia* toward immature DCs, but remains to be investigated.

In this study, we have demonstrated that, although both BCC species induced significant cytokine release from DCs, *B. cenocepacia*, unlike *B. multivorans*, effectively interfered with the normal functioning of DCs by inhibiting upregulation of costimulatory molecules and inducing necrosis. It was striking that the more clinically virulent pathogen, *B. cenocepacia*, had such a deleterious effect on DCs, cells which represent a vital component of the innate immune response in the lung and a critical coordinator of an effective adaptive immune response. We believe that the ability of *B. cenocepacia* to subvert DC function and target these critical host defense cells for destruction may be of central relevance to the complicated pathogenesis of BCC in CGD and CF patients. The differences between the two species which we observed may provide valuable new insights into both understanding and treating this serious complex of opportunistic pathogens.
3.6 References


4. Subcellular localization of *Burkholderia cenocepacia* and *Burkholderia multivorans* in human dendritic cells

4.1 Summary

*B. cenocepacia* and *B. multivorans* are serious opportunistic pathogens infecting cystic fibrosis (CF) and chronic granulomatous disease (CGD) patients, and recently these bacteria have been shown to invade primary human monocyte-derived dendritic cells (DCs). In the present study, transmission electron microscopy was utilized to examine the intracellular lifestyle of these bacteria within DCs. Ultrathin sections of DCs exposed to *B. cenocepacia* and *B. multivorans* for 6 h revealed that approximately one in every 100 DCs contained intracellular bacteria. The intracellular niche of each organism was clearly distinct; *B. cenocepacia* was seen in phagosomes and *B. multivorans* was free in the cytoplasm of DCs. Although more details remain to be obtained, the unique intracellular location of *B. cenocepacia* and *B. multivorans* in DCs may be an important feature of differential pathogenesis between these species of the *Burkholderia cepacia* complex (BCC).

4.2 Introduction

*Burkholderia cepacia* complex (BCC) bacteria are important opportunistic pathogens of Cystic Fibrosis (CF) and Chronic Granulomatous Disease (CGD) patients (Isles *et al.*, 1984; Corey and Farewell, 1996; Hutchison and Govan, 1999; Winkelstein *et al.*, 2000; Johnston, 2001; Speert, 2002). CF, the most common potentially fatal autosomal recessive disease among Caucasians, is caused by mutations in the CF transmembrane conductance regulator (CFTR) (Rommens *et al.*, 1989). CGD is a rare primary immunodeficiency characterized by the failure of phagocytic cells to generate an oxidative burst, as a result of defects in the nicotinamide

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adenine dinucleotide phosphate (NADPH) oxidase (Segal et al., 2000; Johnston, 2001). In both CGD and CF patients, BCC manifests as pulmonary infections which can escalate into fulminant septicemia (Isles et al., 1984; Hutchison and Govan, 1999; Speert, 2002). *B. cenocepacia* and *B. multivorans* are the two most clinically significant members of the BCC in CF patients. In general, *B. cenocepacia* is more transmissible among patients, more prevalent - especially prior to adoption of strict infection control measures - and is associated with a higher mortality rate than *B. multivorans* (Chaparro et al., 2001; Speert, 2002).

There is increasing evidence that BCC bacteria are intracellular pathogens, with the ability to invade and survive *in vitro* within amoebae (Marolda et al., 1999; Landers et al., 2000), human primary and cultured epithelial cells (Martin and Mohr, 2000; Cieri et al., 2002; Schwab et al., 2002; Duff et al., 2006; Cheung et al., 2007; Mullen et al., 2007; Moura et al., 2008; Pirone et al., 2008; Sajjan et al., 2008), and human and murine cultured macrophages (Saini et al., 1999; Martin and Mohr, 2000; Lamothe et al., 2007; Lamothe and Valvano, 2008; Sajjan et al., 2008). *In vivo*, BCC bacteria are found associated with bronchiolar epithelium, between adjacent epithelial cells, and within alveolar septae and macrophages in CF lung explants (Sajjan et al., 2008). In the murine host, *B. cenocepacia* invades respiratory epithelial cells and pulmonary macrophages and induces inflammation (Chiu et al., 2001; Chu et al., 2002) while *B. multivorans* persists in the murine lung within alveolar macrophages (Chu et al., 2002; Chu et al., 2004) and in the spleen (Speert et al., 1999) without causing overt inflammation and pathology, which suggests that this organism may be occupying a privileged intracellular niche.

We have previously begun to characterize the interaction of *B. cenocepacia* and *B. multivorans* with human monocyte-derived dendritic cells (DCs) (MacDonald and Speert, 2008). DCs are one of the resident lung phagocytic cells and serve as intermediaries between innate and adaptive immunity, with a vital role in the generation of an immune response (Banchereau and
Steinman, 1998; Banchereau et al., 2000; Kapsenberg, 2003). Microbial pathogens can interfere with all aspects of the DC life cycle, including differentiation from monocytic precursors, survival, maturation from immature antigen capturing cells to mature antigen presenting cells, migration from tissue to lymph nodes, antigen processing and presentation, and the induction of T cell immunity (Palucka and Banchereau, 2002; Moll, 2003; Steinman and Banchereau, 2007; Ueno et al., 2007).

We have recently demonstrated that BCC can also modulate normal DC functions. Although both B. cenocepacia and B. multivorans associate with DCs and induce inflammation, B. cenocepacia also interferes with DC maturation and gradually induces necrosis, beginning 12 h after co-incubation (MacDonald and Speert, 2008). Experiments using cytochalasin D, which paralyses the cytoskeleton and prevents phagocytosis, clearly demonstrate that both organisms are taken up into DCs (MacDonald and Speert, 2008), but the details of this intracellular dynamic remain to be established. In the present study, we utilize transmission electron microscopy to examine the intracellular lifestyle of B. cenocepacia and B. multivorans in human DCs, early in the infection process, before any induction of necrosis.

4.3 Materials and methods

4.3.1 Bacterial strains, media, and growth conditions

B. multivorans C5568 and B. cenocepacia C6433 are CF patient isolates from the Canadian B. cepacia complex Research and Referral Repository. Bacteria were stored at -70°C in Mueller Hinton broth with 8% (v/v) dimethyl sulfoxide. Routine culturing was performed on blood agar plates (PML Microbiologicals, Richmond, British Columbia, Canada) or Luria-Bertani (LB) agar. Bacteria were grown in 5 ml of LB broth at 37°C with agitation at 250 r.p.m. for 16.5 h to stationary phase, pelleted at 4500 g for 10 min at room temperature, then adjusted to the correct optical density (OD_{610nm} of 0.600). Further dilutions were performed in R10 cell
culture medium (RPMI 1640 supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine and 1 mM sodium pyruvate) to a concentration of $6 \times 10^5$ cfu/ml. The concentration of viable bacteria was confirmed by viable plate counting.

4.3.2 Monocyte isolation and culture into DCs

Monocyte-derived DCs were generated using standard protocols (Sallusto and Lanzavecchia, 1994; Davidson et al., 2004). Briefly, human venous blood was collected from normal adult volunteers, according to the University of British Columbia Clinical Research Ethics Board protocol C04-0193. Heparanized blood was diluted 1:1 in R10 medium, then overlayed onto Ficoll-Paque plus (GE Healthcare, Uppsala, Sweden). Following centrifugation at 450 g for 20 min at 20°C, the peripheral blood mononuclear cells were collected from the interface and washed with phosphate buffered saline (PBS). T cells were depleted by incubation with neuraminidase-treated sheep erythrocytes and an additional Ficoll-Paque purification. Remaining mononuclear cells were collected from the interface, washed with PBS, resuspended in R10, and cultured in six-well plates at a concentration of $4 \times 10^6$ cells per well for 1 h at 37°C, 5% CO₂. After washing with PBS to remove non-adherent cells, the adherent monocytes were cultured in R10 containing 100 ng/ml granulocyte-macrophage colony stimulating factor (GM-CSF) and 100 ng/ml interleukin-4 (IL-4) (Fitzgerald, Concord, MA) for seven days at 37°C, 5% CO₂ to generate immature dendritic cells.

After seven days, DCs were harvested from six-well plates, washed with PBS, and resuspended in R10. Enumeration and confirmation of viability was performed using Trypan blue dye exclusion as assessed in a haemocytometer. Flow cytometry was used to confirm the purity and immature phenotype of the resultant cells, as previously described (Davidson et al., 2004). Briefly, an aliquot of DCs was resuspended in FACS buffer (PBS + 10 % (v/v) FCS, 0.01% (v/v) sodium azide, and 0.01% (v/v) human AB serum (Sigma)) at a concentration of
1×10^6 DCs/ml. One hundred microlitres of DCs/tube were stained for 1 h at 4°C with FITC-conjugated monoclonal antibodies specific for CD1a (Serotec, Raleigh, NC), CD14, CD86 (BD Biosciences, Mississauga, Ontario, Canada), CD83, CD19, IgG2a or IgG1 isotype controls (eBioscience, San Diego, CA) and with PE-conjugated antibodies raised against CD206 (BD Biosciences), CD80, TCR alpha/beta, and an IgG1 isotype control (eBioscience). After two washes in PBS, DCs were resuspended in 200 µl of PBS + 2% (v/v) formaldehyde for flow cytometry.

4.3.3 DC infection assays

DCs were seeded into 24-well plates at a concentration of 5 × 10^5 cells/ml and challenged with live *B. multivorans* or *B. cenocepacia* at a multiplicity of infection (MOI) of 0.3:1. Untreated DCs in R10 served as a control. Plates were incubated at 37°C in 5% CO2 for 2 h, after which 8 µg/ml of meropenem (one to two times the minimum inhibitory concentration for each organism), was added to each well. DCs were then re-incubated until 6 h. This time point was chosen because at 6 h, there is roughly equal association of *B. multivorans* and *B. cenocepacia*, with approximately 40% of DCs binding at least one BCC bacterium, and there is no evidence of induction of cell death (MacDonald and Speert, 2008).

4.3.4 Transmission electron microscopy

For the initial two experiments, samples were subjected to high-pressure freezing and freeze substitution, prior to embedding and sectioning, as outlined in Appendix 8. This method was chosen based on its requirement for less biological material and its potential to provide exceptional preservation of membrane structure. However, this approach did not yield samples of sufficient quality or quantity, so conventional processing was used for the final experiment. DCs were harvested, washed with warm PBS, and fixed with pre-warmed 2.5% (v/v) glutaraldehyde (Canemco, Canton de Gore, QC) in 0.1 M phosphate buffer (pH 7.4) at 37°C for
1 h. Following two washes in 0.1 M phosphate buffer, the fixed cells were pelleted and enrobed in 2% (w/v) low melting point agarose in 0.1 M phosphate buffer. The enrobed samples were allowed to solidify, overlayed with 0.1 M phosphate buffer, and stored at 4°C overnight. The enrobed pellet was dissected into 1 mm squares, washed in 0.1 M sodium cacodylate buffer (pH 7.4), and post-fixed in 1% (v/v) osmium tetroxide (Canemco) in 0.1 M sodium cacodylate buffer for four cycles of 2 min on power level 2 under vacuum, 2 min off in a Pelco laboratory microwave (Ted Pella, Redding, CA). The samples were washed in distilled water, then dehydrated through a graded ethanol series, and infiltrated with increasing concentrations of Epon-Spur’s resin (Canemco) under vacuum at power level 3 using the microwave. The samples were finally polymerized in 100% resin in a 60°C oven overnight. Ultrathin sections were mounted in 100-mesh Formvar-coated copper grids, stained with 2% (w/v) uranyl acetate and 2% (w/v) lead citrate, and viewed on a Hitachi H7600 transmission electron microscope (Hitachi, Toronto, ON) operating at 80 kV.

4.4 Results

4.4.1 DC phenotype
The surface marker expression and cell morphology of monocyte-derived DCs was consistent with the immature DC phenotype, as previously reported (MacDonald and Speert, 2008). DC viability was greater than 90%, as determined by Trypan blue dye exclusion.

4.4.2 Transmission electron microscopy
Transmission electron micrographs of DCs co-incubated for 6 h with B. multivorans and B. cenocepacia and control DCs are shown (Figure 4.1). Control cells exhibited the typical immature DC morphology, with extensive dendrites projecting from the cell surface, heterochromatin clustered around the inner nuclear membrane, and abundant mitochondria, Golgi Bodies, endoplasmic reticulum, and electron-dense granules in the cytoplasm (Fig. 4.1A,
B). In general, DCs co-incubated with BCC were a heterogeneous population of smaller, organelle- and granule-replete cells resembling control DCs along with larger, highly vacuolated, seemingly activated cells (Fig. 4.1C, D). Of the three to six hundred DCs examined for each condition, we observed four DCs infected with intracellular *B. cenocepacia* and seven DCs harbouring intracellular *B. multivorans*. Therefore, approximately one in every hundred DCs contained intracellular bacteria. This is close to the expected probability, as, in each 100 μm section, there is a one in twenty chance of observing a 1 μm bacterium in a 20 μm cell and it was previously determined that approximately four out of every ten DCs are positive for at least one BCC bacterium (MacDonald and Speert, 2008). Interestingly, *B. cenocepacia* was only observed in the phagosome of DCs (Fig. 4.1C, E) while *B. multivorans* was only observed in the cytoplasm of DCs (Fig. 4.1D, F). All individual DCs harbouring *B. cenocepacia*-occupied phagosomes resembled resting cells. *B. multivorans* resided in the cytoplasm of both morphologically resting (smaller, organelle- and granule-replete cells) and activated DCs (larger, highly vacuolated cells). Infected DCs contained between one and four BCC bacterial cells. No evidence of bacterial cell degradation was observed, in fact some cytosolic *B. multivorans* cells appeared to be dividing (Fig. 4.1D). Control and BCC-exposed DCs all appeared healthy at this 6 h time point, with intact nuclear and cytoplasmic membranes and no nuclear condensation.
Figure 4.1  Transmission electron micrographs of control DCs and DCs with intracellular BCC.

Representative transmission electron micrographs of control DCs (A, B), and DCs co-incubated for 6 h with *B. cenocepacia* (C, E) and *B. multivorans* (D, F, bacteria are indicated with an asterisk in panels C and D).  Higher magnification images show *B. cenocepacia* within DC phagosomes (E, arrowheads indicate the phagosomal membrane), and *B. multivorans* within the cytoplasm (F, arrow indicates the dividing bacterial septum).  Scale bars are indicated.
4.5 Discussion

The intracellular niches occupied by *B. cenocepacia* and *B. multivorans* were identified in human dendritic cells using transmission electron microscopy. Six hours post-infection, *B. cenocepacia* was found within the phagosome while *B. multivorans* was cytosolic.

The presence of *B. cenocepacia* in the DC phagosome is consistent with previous publications showing *B. cenocepacia* within membrane-bound vacuoles in human and murine cultured macrophages (Martin and Mohr, 2000; Lamothe et al., 2007; Lamothe and Valvano, 2008; Sajjan et al., 2008), primary murine macrophages and epithelial cells (Chiu et al., 2001), primary and cultured human epithelial cells (Schwab et al., 2002; Cheung et al., 2007; Sajjan et al., 2008) and amoebae (Marolda et al., 1999). One of these studies does report occasional *B. cenocepacia* bacteria in the cytoplasm of primary human epithelial cells (Sajjan et al., 2008), but this was not observed in DCs.

The presence of *B. cenocepacia* in the DC phagosome naturally prompts speculation about phagosomal trafficking of *B. cenocepacia* in DCs. It has been previously found that *B. cenocepacia* delays phagosomal maturation in murine macrophages by impairing phagosome acidification, the acquisition of the late endosomal marker lysosome-associated membrane protein-1, and phagosome-lysosome fusion (Lamothe et al., 2007). This phagosomal maturation defect is even more enhanced when *B. cenocepacia* infects macrophages derived from CF mice (Lamothe and Valvano, 2008). Based on recent observations of human cultured macrophages infected with *B. cenocepacia*, it has also been suggested that *B. cenocepacia*-occupied vacuoles are not simply delayed in maturation, but may divert to an alternative pathway which involves the endoplasmic reticulum (Sajjan et al., 2008). Researchers have also shown that typical DC phagosomal maturation differs from that of macrophages, with reduced NADPH oxidase activity and less lysosomal recruitment, resulting in reduced proteolysis (Banchereau and Steinman,
1998; Trombetta et al., 2003; Savina and Amigorena, 2007). Motivated by the potential of \( B. cenocepacia \) to further aggravate this process, we are currently investigating the kinetics of phagosomal maturation and the constitution of the \( B. cenocepacia \)-occupied vacuole in DCs using confocal microscopy.

Very few studies have examined the intracellular life cycle of \( B. multivorans \), but this is the first report of \( B. multivorans \) in the cytoplasm of phagocytic cells. A previous study noted the presence of an environmental isolate of \( B. multivorans \) within cultured human macrophages, but no supporting micrographs were shown and the subcellular location of the bacteria was not reported (Martin and Mohr, 2000). \( B. multivorans \) was previously localized to the phagosome of murine alveolar macrophages (Chu et al., 2004). The apparent disparity between these reports may represent inherent differences between murine and human cells, between macrophages and DCs, or simply kinetic differences, as it is likely that \( B. multivorans \) is first taken up into a phagosome and then escapes into the cytoplasm, although this is yet to be conclusively shown.

BCC bacteria are closely related to the primary human intracellular pathogen \( B. pseudomallei \), which, like \( Listeria monocytogenes \), can lyse phagosomal membranes, escape into the cytoplasm, and form polar actin tails (Harley et al., 1998; Kespichayawattana et al., 2000; Stevens and Galyov, 2004). \( B. pseudomallei \) is clearly adept at intracellular replication and cell-to-cell spread (Jones et al., 1996; Kespichayawattana et al., 2000; Kespichayawattana et al., 2004). Although \( B. multivorans \) is less virulent than \( B. cenocepacia \) clinically in CF patients (Chaparro et al., 2001; Speert, 2002) and in vitro in DCs (MacDonald and Speert, 2008), nevertheless, it shares one potentially key pathogenic factor with its dangerous relative \( B. pseudomallei \) that \( B. cenocepacia \) apparently does not, a rarefied niche in the cytoplasm of infected phagocytes. It may be that \( B. multivorans \) has acquired the ability to escape from the
phagosome, but there is no evidence that it can co-opt host actin machinery or spread to adjacent cells.

Interestingly, cytosolic *L. monocytogenes*, but not a mutant which remains in the phagosome, induces upregulation of costimulatory molecules in DCs, leading to optimal priming of T cells (Brzoza et al., 2004). This is similar to our observations, as cytosolic *B. multivorans* but not phagosome-bound *B. cenocepacia* was previously shown to up-regulate DC co-stimulatory molecules (MacDonald and Speert, 2008). However, other intracellular pathogens that reside in the cytoplasm, such as *Mycobacterium tuberculosis*, induce normal maturation of DCs (Giacomini et al., 2001; Tailleux et al., 2003). The significance of intracellular niche to induction of DC maturation warrants further investigation.

The replicative ability of *B. multivorans* within DCs is another interesting unknown. We did observe dividing *B. multivorans* bacteria in the cytoplasm of DCs; however, the cytoplasm of each infected cell contained no more than four and typically one or two bacterial cells. In addition, we have previously demonstrated that *B. multivorans* does not undergo profound replication in DCs, as there was only a slight increase in viable plate counts over 24 h, corresponding to a doubling time of 5.32 h over the final 18 h of incubation (MacDonald and Speert, 2008). *B. multivorans* does not replicate uncontrollably and adversely affect DC functions, as DCs infected with *B. multivorans* undergo normal DC maturation and do not undergo cell death, even after 24 h (MacDonald and Speert, 2008). Clearly, more research is needed into the intracellular lifestyle of *B. multivorans*, an important pathogen within the BCC which is sometimes overlooked in favour of the more virulent *B. cenocepacia*.

In the present study, DCs were exposed to *B. cenocepacia* and *B. multivorans* for 6 h and the subcellular location of the intracellular bacteria was characterized. *B. cenocepacia* was located within membrane-bound phagosomes, while *B. multivorans* was found in the cytoplasm.
of DCs. Research into the dynamics of DC phagosome maturation and the precise details of the
disparate BCC intracellular survival strategies are ongoing and may lead to further insight into
the intracellular pathogenicity of these serious opportunistic human pathogens.
4.6 References


5. General Discussion

5.1 Introduction

This thesis has explored the diverse topics of inflammation and infection by examining inflammation in peripheral blood mononuclear cells (PBMCs) from chronic granulomatous disease (CGD) patients and the interaction of *B. multivorans* and *B. cenocepacia* with human monocyte-derived dendritic cells (DCs). The purpose of this concluding chapter is to summarize the major findings of the thesis, especially in light of the most current research. I will also critically analyze this work and suggest future directions of study in this field. Finally, overarching conclusions will be drawn and the implications of this research for understanding the pathology of CGD and pathogenesis of *B. cepacia* complex (BCC) bacteria will be addressed.

5.2 Inflammation in CGD

5.2.1 Major findings

The goal of the first part of the thesis was to clarify the issue of inflammation in CGD, in light of two competing sets of observations. One is the clinical observation that CGD patients, who lack the ability to produce reactive oxygen species (ROS), experience hyperinflammatory complications, often in the absence of an obvious and sustained infectious trigger (Segal et al., 2000). However, there are reports that ROS directly activates nuclear factor-κB (NF-κB), a key transcription factor involved in inflammatory signaling, and that deficient ROS production leads to a resulting decrease in inflammation (Flohe et al., 1997; Fan et al., 2003; Asehnoune et al., 2004; Sadikot et al., 2004). The hypothesis that lack of ROS does not decrease activation of NF-κB and, in fact, enhances production of pro-inflammatory cytokines was therefore tested.

Pro-inflammatory cytokine production and NF-κB signaling by PBMCs from four X-linked, gp91phox-deficient CGD patients and from healthy controls was examined following
stimulation with a panel of toll-like receptor ligands. Upon stimulation, PBMCs from CGD patients secreted significantly more TNF-α and IL-6 than PBMCs from healthy controls. This hyperinflammatory phenotype was also found in mononuclear cells from gp91phox(−/−) CGD mice but not in control human PBMCs treated with the NADPH oxidase inhibitor diphenyleneiodonium (DPI), suggesting that this commonly used chemical likely has NADPH oxidase-independent effects. NF-κB signaling in PBMCs from CGD patients and healthy controls upon lipopolysaccharide (LPS) stimulation was also examined. The principal steps in activation of NF-κB, namely phosphorylation and degradation of I-κBα, DNA binding of NF-κB and translocation of NF-κB p50 were all normal in CGD cells. Therefore, ROS was not essential for NF-κB activation and may in fact attenuate inflammation in human PBMCs.

5.2.2 Critique

One of the major strengths of this research is the use of primary cells from CGD patients to demonstrate the hyperinflammatory phenotype. CGD is a rare disease and we were fortunate to have access to four patients for our initial series of experiments. These observations were confirmed using splenic cells from a murine model of CGD, which was not identical in composition to human PBMCs, but did generate comparable results. Some of the later follow-up experiments were only possible with cells from one patient, due to limited patient availability and decline in the health of the patients. All of our experiments were done in vitro using isolated human PBMCs or murine splenic cells, often stimulated with highly purified TLR ligands, which is an artificial system. Some of the intricacies of in vivo inflammation were undoubtedly missed using this simplified approach.

5.2.3 Recent advances in the literature and future directions

The observation of the hyperinflammatory phenotype in CGD PBMCs was further explored by microarray analysis of monocytes from CGD patients and healthy control performed
in collaboration with our laboratory (Brown et al., 2008). Resting and LPS- and peptidoglycan-(PGN-) activated monocytes from CGD patients have significantly higher expression levels of inflammatory genes than control cells; in fact, gene expression in resting CGD cells resembled that of LPS-stimulated control cells. Enhanced gene expression of inflammatory mediators correlates with elevated NF-κB expression and is dependent on signaling through the extracellular response kinase- (ERK-) 1/2, a mitogen activated protein (MAP) kinase. There was no evidence of acute illness, infection, or detectable levels of circulating endotoxin in all four CGD patients, suggesting that this phenotype is not driven by underlying infection.

Excessive inflammation upon stimulation was also found in mononuclear cells from gp91phox(-/-) CGD mice, a finding that is consistent with previous studies of CGD murine infection models (Jackson et al., 1995; Pollock et al., 1995; Morgenstern et al., 1997), including a recent paper which examines the response of gp91phox(-/-) and p47phox(-/-) CGD and control mice to injected LPS (Zhang et al., 2009). As was noted in the chapter 2 discussion, this contradicts the findings of Sadikot et al., who found diminished cytokine production and NF-κB activation in p47phox(-/-) CGD mice inoculated intratracheally with Pseudomonas aeruginosa (Sadikot et al., 2004).

Some inconsistencies in the literature surrounding the role of ROS in inflammation may be attributable to the use of chemical inhibitors of the NADPH oxidase or antioxidants as surrogates for human or murine CGD cells (Shrivastava and Aggarwal, 1999; Fan et al., 2003; Asehnoune et al., 2004; Park et al., 2004). Our finding that PBMCs treated with DPI, the NADPH oxidase inhibitor, do not adopt a hyperinflammatory phenotype is in line with other reports suggesting that such chemical inhibitors and antioxidants exert unexpected, pleiotrophic effects on cells (Bowie and O'Neill, 2000; Hayakawa et al., 2003).
Despite some contradictory reports, the body of evidence now points to a role for ROS in dampening or controlling inflammation. Recently, researchers have begun elucidating the underlying inherent anti-inflammatory mechanisms of ROS. The major focuses have been on examining the ability of ROS to directly affect gene expression and the bystander effects of phagocyte ROS on controlling inflammation in adjacent cells (Björkman et al., 2008). Clues about the mechanism of ROS control of inflammation comes from literature examining the effect of cellular redox states on key proteins. The oxidant-antioxidant balance influences immune cell function (Knight, 2000). Most transcription factors and RNA-stabilizing proteins have zinc-finger domains involved in DNA and RNA binding that are redox-sensitive (Liu et al., 2005), so an oxidant-antioxidant imbalance in CGD may enhance transcription or affect mRNA stability, leading to continuous inflammation. In addition, cellular redox states can change the activity of phospho-tyrosine kinases (Knight, 2000; Liu et al., 2005), which may influence downstream signaling. Indeed, Brown et al. found upregulation of genes encoding NF-κB subunits and proinflammatory mediators, which was dependent on the ERK1/2 MAP kinase, another protein partly activated by redox-sensitive phosphorylation of tyrosine kinases (Brown et al., 2008).

It has also been suggested that the absence of ROS may not simply cause enhanced proinflammatory gene expression, but also may result in the failure to produce anti-inflammatory mediators to return the system to homeostasis following an infectious insult or injury. Consistent with this hypothesis, a decrease in the gene expression of several anti-inflammatory mediators in CGD monocytes has been described (Brown et al., 2008).

In vivo, the picture becomes more complicated as ROS produced by phagocytes may also affect other adjacent cells, leading to uncontrolled inflammation. As discussed in chapter 2, rodents with \( p47^{phox} \) polymorphisms are vulnerable to autoimmune arthritis through a complicated mechanism involving loss of the normal thiol regulatory function of phagocyte-
derived ROS on neighbouring lymphocytes, resulting in activation of the normally controlled arthritogenic T cells. Recently, another ROS-driven mechanism has been proposed to explain acute inflammation upon *Aspergillus fumigatus* challenge of p47phox(-/-) mice (Romani *et al.*, 2008). Lack of ROS adversely affects phagocyte tryptophan metabolism, which alters phagocyte-mediated T cell skewing, leading to uncontrolled expansion of inflammatory Th17 cells and decreased activity of anti-inflammatory, tolerogenic T regulatory cells (Romani *et al.*, 2008). The indirect effects of ROS on T cells may help explain other phenomena, such as the recently observed increased susceptibility of gp91phox(-/-) CGD mice to collagen-induced arthritis (George-Chandy *et al.*, 2008) and a higher clinical incidence of discoid lupus in maternal CGD carriers (Winkelstein *et al.*, 2000).

Future studies should focus on carefully elucidating the precise details and downstream effects of redox-sensitive signal transduction and further unraveling the complex mechanisms by which ROS produced by the phagocyte NADPH oxidase regulate inflammation *in vitro* and *in vivo*. The present study represents an important contribution to this emerging field. It is hoped that a greater understanding of inflammation in CGD may lead to the development of new therapeutics to help these vulnerable patients.

### 5.3 The interaction of BCC bacteria with DCs

#### 5.3.1 Major findings

The second half of this thesis explored the role of DCs in the pathogenesis of BCC. BCC bacteria can exert a range of effects on phagocytes such as neutrophils and macrophages, including avoidance of phagocytic cell killing, adoption of an intracellular lifestyle, and stimulation of inflammation and cell death (MacDonald and Speert, 2007). The crucial role of DCs in orchestrating the immune response is well established and the concurrent ability of bacterial pathogens to subvert DC functions is increasingly recognized (Cutler *et al.*, 2001;
Palucka and Banchereau, 2002). We therefore investigated the hypothesis that *B. multivorans* and *B. cenocepacia* modulate the normal functions of primary human monocyte-derived DCs, to subvert antibacterial mechanisms and persist intracellularly.

*B. multivorans* and *B. cenocepacia* were co-incubated with DCs for up to 24 h and bacterial association and viable plate counts, DC maturation, cytokine production, and induction of apoptosis or necrosis were assessed. These bacteria bound and were taken up into DCs. The numbers of live *B. multivorans* and *B. cenocepacia* remained constant for the first 6 h of co-incubation, and then slowly increased from 6 to 24 h. Although both BCC species induced significant IL-6, TNF-α, IL-10 and IL-12 cytokine release from DCs, *B. cenocepacia*, unlike *B. multivorans*, also effectively interfered with the normal DC maturation by inhibiting upregulation of costimulatory molecules after 24 h of co-incubation. *B. cenocepacia*, but not *B. multivorans*, also induced necrosis in DCs after 24 h, as determined by annexin V and propidium iodide staining. DC necrosis only occurred after phagocytosis of live *B. cenocepacia*; DCs exposed to heat-killed bacteria, bacterial supernatant, or those pre-treated with cytochalasin D then exposed to live bacteria remained viable. In addition, when a panel of BCC clinical isolates was examined, none of the five clinical isolates of *B. multivorans* induced significant necrosis after 24 h, while four out of five *B. cenocepacia* isolates caused necrotic cell death in DCs. The ability of *B. cenocepacia* to interfere with normal DC functions may contribute to its pathogenicity in susceptible hosts.

The subcellular location of *B. cenocepacia* and *B. multivorans* within DCs was also determined by transmission electron microscopy following 6 h of co-incubation. *B. cenocepacia* resided in the phagosome of DCs while *B. multivorans* was found in the cytoplasm. Although more details remain to be obtained, the unique intracellular niches of *B. cenocepacia* and *B. multivorans* in DCs may be significant to BCC pathogenesis.
5.3.2 Critique

The major strength of this work is the in depth comparison of the ability of two important CF and CGD pathogens, *B. multivorans* and *B. cenocepacia*, to modulate phagocytic cell functions, using a novel model for investigating BCC pathogenesis: primary human DCs. The use of primary human cells was a challenge, these cells are time-consuming to isolate and limited in number, but human monocyte-derived DCs are the best *in vitro* model available for human DCs. However, there may be phenotypic differences between DCs differentiated *in vitro* from human monocytes and the *in vivo* human pulmonary DCs they are intended to represent. One of the frequent criticisms of using primary cells is the inter-donor variability, which may make it difficult to obtain statistically significant data, unlike inbred murine cells or immortalized cells, which are more homogeneous. However, in this study, we were able to see clear, statistically significant differences in the data using DCs from 3 to 5 human donors.

Other potential criticisms of the present study involve the use of control DCs from healthy subjects instead of patient cells and the focus on an *in vitro* investigation of many DC functions that BCC bacteria may affect. We investigated opportunistic pathogens of CF and CGD patients, yet we only employed DCs from healthy controls, assuming that *in vitro* observations in control DCs may be applicable to DCs from CF and CGD patients. The validity of this approach remains to be determined, but it was important to optimize procedures and baseline responses in cells from healthy controls, which are more readily available than patient cells. *In vitro* experiments allowed a detailed, comprehensive examination of processes like DC maturation, cytokine production, and necrosis induction that set the stage for further explorations of the interaction between BCC and DCs from CF and CGD patients *in vitro* and the *in vivo* role of DCs in BCC pathogenesis. However, the decision to focus on the diverse processes involved in the interaction of DCs and BCC bacteria meant that specific *B. cenocepacia* virulence factors, which contribute to necrosis induction and maturation impairment, were not identified. Finally,
the results illustrating the unique intracellular habitats of *B. multivorans* and *B. cenocepacia* are intriguing, but must be confirmed with confocal microscopy and further kinetic analyses.

### 5.3.3 Recent advances in the literature and future directions

Chapter 3 and 4 represent recently published or due to be published data, therefore most of the relevant literature concerning the interaction between BCC bacteria and phagocytes has already been discussed. It is worth commenting on some general trends in the literature to highlight where our current research fits and illuminate some future avenues of investigation. Research into BCC pathogenesis has broadened beyond a few immortalized macrophage and epithelial cell lines to include primary human differentiated epithelial cells from CF and non-CF patients (Sajjan *et al.*, 2008), macrophages from murine models of CF and their healthy littermates (Lamothe and Valvano, 2008), neutrophils (Bylund *et al.*, 2005b; Bylund *et al.*, 2005a) and now primary human dendritic cells. This allows us to gain a more complete picture of the *in vitro* interactions between BCC bacteria and all the relevant cells present in the lung, and provides impetus to move into more sophisticated *in vivo* murine models of CF and CGD and to examine human CF or CGD lung explants, bronchiolar lavage, or sputum samples. It would be particularly useful to examine maturation and cell death induction in pulmonary DCs from mice or patients infected with BCC bacteria and to examine trafficking of BCC-infected DCs from the murine lung to the lymph node, to determine if BCC bacteria hijack DCs to escape the lung and become systemic.

Knowledge of BCC pathogenesis is rapidly expanding. Indeed, a very recent paper describes delayed association of membrane and cytosolic components of the NADPH oxidase with the *B. cenocepacia*-occupied phagosome of a murine macrophage cell line (Keith *et al.*, 2009), which follows a previous paper demonstrating delayed phagosome acidification and acquisition of lysosome-associated membrane protein-1 (Lamothe *et al.*, 2007). Phagosomal
maturation is further delayed when these cells are treated with an inhibitor of CFTR (Lamothe and Valvano, 2008; Keith et al., 2009) or when macrophages derived from CF mice are examined (Lamothe and Valvano, 2008). As previously discussed, normal DC phagosomal maturation differs from that of macrophages, with reduced NADPH oxidase activity and lysosomal recruitment, resulting in reduced proteolysis (Banchereau and Steinman, 1998; Trombetta et al., 2003; Savina and Amigorena, 2007). Interestingly, bone-marrow derived DCs have recently been found to express CFTR (Xu et al., 2009). These CFTR-defective DCs have alterations in lipid metabolism, including caveolin-1, a lipid raft protein involved in endocytosis (Xu et al., 2009). However, CFTR has not been identified in human DCs. It is interesting to speculate that the maturation delay induced by *B. cenocepacia* in macrophages may be even more enhanced in DCs, especially in DCs from CF patients. However, this remains to be investigated.

One of the gaps in the literature is the identification of the specific receptors involved in uptake of BCC bacteria in DCs and macrophages. Targeting bacteria to certain receptors can have dramatic downstream effects, such as impacting the ability of DCs to process and present *Salmonella typhimurium* antigens due to poor phagosome-lysosome fusion (Tobar et al., 2004), making the search for BCC receptors a worthwhile line of future investigation.

The recent publication of the *B. cenocepacia* genome (Holden et al., 2009) and the development of the *B. cenocepacia* microarray (Drevinek et al., 2008) really point the way forward. This will facilitate identification of putative virulence factors. It should also be possible to compare gene expression of BCC bacteria infecting macrophages and DCs or CF, CGD and normal cells. Once sequencing and annotation of the *B. multivorans* genome is performed, the genomes of *B. multivorans* and *B. cenocepacia* can be compared to identify shared and divergent genes which may influence common and distinct mechanisms of
pathogenesis. Presently, only a handful of genetically tractable strains have been identified (Mahenthiralingam et al., 2000), therefore new genetic tools must also be developed to allow manipulation of a wider variety of clinically relevant isolates of BCC. This will facilitate the exploration of the virulence factors involved in BCC pathogenesis.

The present study contributes to the emerging field of BCC pathogenesis, in particular the interaction of bacteria with phagocytic cells. By increasing our comprehension of these basic mechanisms, we come closer to grasping how BCC bacteria infect CF and CGD patients and to developing new treatment strategies for this serious opportunistic pathogen.

5.4 Conclusions

In this thesis, profound inflammatory responses in PBMCs from CGD patients coupled with normal activation of NF-κB were demonstrated. We were therefore able to conclude that superoxide was not required for activation of NF-B and actually may be involved in controlling inflammation.

Through the investigation of DCs and BCC bacteria, we found that B. cenocepacia was able to subvert normal DC functions by impairing maturation and inducing necrosis, unlike DCs exposed to B. multivorans which matured normally and remained viable. Within DCs, B. cenocepacia occupied the phagosome while B. multivorans resided in the cytoplasm. The contribution of these different intracellular niches to overall pathogenesis of BCC remains to be established. However, it is clear that these two BCC species interact with DCs in dramatically distinct ways, which mirrors their contrasting clinical phenotypes. B. cenocepacia is therefore a more dangerous pathogen than B. multivorans both clinically for CF patients and in vitro for DCs, as it is appears to be more adept at modulating DC functions.
Infection and inflammation are integral components of many human diseases, including CF and CGD. To combat these diseases effectively, we must first understand these complex and multi-factorial processes. This thesis has made solid contributions to this great endeavor.
References


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Appendices

Appendix 1. Human ethics approval certificate.

ETHICS CERTIFICATE OF EXPEDITED APPROVAL: RENEWAL

PRINCIPAL INVESTIGATOR: David P. Speert
DEPARTMENT: 
UBC CREB NUMBER: H04-70193

INSTITUTION(S) WHERE RESEARCH WILL BE CARRIED OUT:
Children's and Women's Health Centre of BC (incl. Sunny Hill) Children's and Women's Health Centre of BC (incl. Sunny Hill)
Other locations where the research will be conducted: N/A

CO-INVESTIGATOR(S):
Kelly MacDonald
Stuart Turvey
Trevor Hind
Christopher Blomke
Billie Valastino
Aaron Hirschfeld
James E A Zdanit

SPONSORING AGENCIES:
- British Columbia Lung Association - "Use of Primary Epithelial Cells to Identify Novel Anti-Inflammatory Targets for Cystic Fibrosis Lung Disease"
- Canadian Cystic Fibrosis Foundation - "The role of mucoidity in the pathogenesis of Burkholderia cepacia complex"
- Canadian Cystic Fibrosis Foundation - "Toll-Like Receptor 5: A Novel Anti-Inflammatory Target for Cystic Fibrosis"
- Canadian Cystic Fibrosis Foundation - "Virulence of Burkholderia Cepacia Bacteria in Cystic Fibrosis: The Role of Microbial Surface Determinants"
- Genome Canada - "The Pathogenomics of Innate Immunity in Defense Against Bacterial Infection in Humans"
- Genome Canada - "The Pathogenomics of Innate Immunity (PI2)"

PROJECT TITLE:
The Role of In innate Phagocytic Immunity in Defense Against Bacterial Infection in Humans

EXPIRY DATE OF THIS APPROVAL: February 10, 2010

APPROVAL DATE: February 10, 2009

CERTIFICATION:
In respect of clinical trials:
1. The membership of this Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Division 5 of the Food and Drug Regulations.
2. The Research Ethics Board carries out its functions in a manner consistent with Good Clinical Practices.
3. This Research Ethics Board has reviewed and approved the clinical trial protocol and informed consent form for the trial which is to be conducted by the qualified investigator named above at the specified clinical trial site. This approval and the views of this Research Ethics Board have been documented in writing.

The Chair of the UBC Clinical Research Ethics Board has reviewed the documentation for the above named project. The research study, as presented in the documentation, was found to be acceptable on ethical grounds for research involving human subjects and was approved for renewal by the UBC Clinical Research Ethics Board.

Approval of the Clinical Research Ethics Board by:

Dr. Stephen Hofton Cann, Associate Chair
Appendix 2. Biohazard approval certificate.

The University of British Columbia

Biohazard Approval Certificate

PROTOCOL NUMBER: B06.0051
INVESTIGATOR OR COURSE DIRECTOR: David P. Speert
DEPARTMENT: Infectious & Immune Disease - Paeds
PROJECT OR COURSE TITLE: The Pathogenomics of Innate Immunity
APPROVAL DATE: March 14, 2006
START DATE: March 22, 2006
APPROVED CONTAINMENT LEVEL: 2
FUNDING TITLE: Innate Immune Response in Premature Neonates at risk of Bronchopulmonary Dysplasia
FUNDING AGENCY: British Columbia Lung Association
FUNDING TITLE: Virulence of Burkholderia cepacia bacteria in cystic fibrosis: the role of microbial surface determinants
FUNDING AGENCY: Canadian Cystic Fibrosis Foundation
FUNDING TITLE: The Pathogenomics of Innate Immunity
FUNDING AGENCY: Genome Canada
FUNDING TITLE: Virulence of Burkholderia cepacia bacteria in cystic fibrosis: the role of microbial surface determinants
FUNDING AGENCY: Canadian Cystic Fibrosis Foundation
FUNDING TITLE: Immune Function and Biodefence in Children, Elderly and Immuno-compromised Populations
FUNDING AGENCY: National Institutes of Health
FUNDING TITLE: The role of innate phagocytic immunity in defense against bacterial infections in humans
FUNDING AGENCY: Canadian Institutes of Health Research (CIHR)

UNFUNDED TITLE: The Pathogenomics of Innate Immunity

The Principal Investigator/Course Director is responsible for ensuring that all research or course work involving biological hazards is conducted in accordance with the University of British Columbia Policies and Procedures, Biosafety Practices and Public Health Agency of Canada guidelines.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there are no changes. Annual review is required.

A copy of this certificate must be displayed in your facility.

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Appendix 3. Published manuscript #1.
Appendix 3 has been removed because of copyright restrictions. Full text of the published manuscript can be accessed by following this link:
http://www3.interscience.wiley.com/journal/114131834/abstract?CRETRY=1&SRETRY=0

Appendix 4. Published manuscript #2.
Differential modulation of innate immune cell functions by the *Burkholderia cepacia* complex: *Burkholderia cenocepacia* but not *Burkholderia multivorans* disrupts maturation and induces necrosis in human dendritic cells.

Differential modulation of innate immune cell functions by the *Burkholderia cepacia* complex: *Burkholderia cenocepacia* but not *Burkholderia multivorans* disrupts maturation and induces necrosis in human dendritic cells

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Summary

*Burkholderia cepacia* complex (BCC) bacteria cause pulmonary infections that can evolve into fatal overwhelming septicemia in chronic granulomatous disease or cystic fibrosis patients. *Burkholderia cenocepacia* and *Burkholderia multivorans* are responsible for the majority of BCC infections in cystic fibrosis patients, but *B. cenocepacia* is generally associated with a poorer prognosis than *B. multivorans*. The present study investigated whether these pathogens could modulate the normal functions of primary human monocyte-derived dendritic cells (DCs), important phagocytic cells that act as critical orchestrators of the immune response. Effects of the bacteria on maturation of DCs were determined using flow cytometry. DCs co-incubated for 24 h with *B. cenocepacia*, but not *B. multivorans*, had reduced expression of costimulatory molecules when compared with standard BCC lipopolysaccharide-matured DCs. *B. cenocepacia*, but not *B. multivorans*, also induced necrosis in DCs after 24 h, as determined by annexin V and propidium iodide staining. DC necrosis only occurred after phagocytosis of live *B. cenocepacia*; DCs exposed to heat-killed bacteria, bacterial supernatant or those pre-treated with cytochalasin D then exposed to live bacteria remained viable. The ability of *B. cenocepacia* to interfere with normal DC maturation and induce necrosis may contribute to its pathogenicity in susceptible hosts.

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less-prevalent *B. multivorans* and, unlike most strains of *B. multivorans*, may be readily transmitted among patients (Chaparro et al., 2001; Speert et al., 2002).

The virulence of *B. multivorans* and *B. cenocepacia* in CF and CGD suggest an interplay between bacterial and host determinants, but clues about this complex interaction are just beginning to emerge (Mahenthiralingam et al., 2005). Abrogation of normal phagocytic cell function may play an important role in these processes. BCC bacteria are impervious to non-oxidative phagocytic killing by neutrophils (Speert et al., 1994) and avoid oxidative killing actively by producing superoxide scavengers (Zughaier et al., 1999a, Lefebre and Valseco, 2001; Smalley et al., 2001; Bylund et al., 2005a). BCC bacteria also thrive in environments where oxidative killing is compromised, such as the CGD lung, which is inherently devoid of superoxide, and the CF lung, where an oxidant/antioxidant imbalance has been reported (Speert, 2002; Bylund et al., 2005a). These organisms also survive within macrophages (Saint et al., 1999; Martin and Mohr, 2000; Chu et al., 2004) perhaps by delaying phagosomal acidification, as has been recently demonstrated in cultured macrophages infected with *B. cenocepacia* (Lamothé et al., 2007). BCC lipopolysaccharide (LPS) is reportedly extremely pro-inflammatory (Snow et al., 1995; Zughaier et al., 1999b; Hutchison et al., 2000; Shimomura et al., 2001; Gronow et al., 2003), yet some BCC species persist within macrophages in the absence of overt inflammation (Speert et al., 1999; Chu et al., 2002, 2004), which also suggests that these organisms may be occupying a privileged intracellular niche. Indeed, in a murine pulmonary infection model, *B. multivorans* persists within monocytic phagocytes in the absence of inflammation, while *B. cenocepacia* induces greater inflammation and toxicity (Chu et al., 2004). Secreted bacterial products from this group of pathogens also induce apoptosis or necrosis in neutrophils and macrophages (Hutchison et al., 1998; Malhotra et al., 2000; Pung et al., 2003).

Dendritic cells (DCs) also reside in the lung, act as crucial mediators between innate and adaptive immunity, and may therefore play a role in the pathogenesis of BCC. Immature DCs act as sentinels in the tissue, they are very efficient in antigen capture through macrophagy, receptor-mediated endocytosis and phagocytosis (Banchereau et al., 2000). A broad repertoire of opsonic and non-opsonic phagocytic receptors enable DCs to bend and internalize microbes (Banchereau et al., 2000; Culler et al., 2001). Upon capture of soluble antigen or bacteria, immature DCs migrate to secondary lymphoid organs while undergoing phenotypic and functional changes that transform them from antigen-capturing cells to mature antigen-processing and -presenting cells. The latter have the critical capacity to activate naive T cells, inducing the appropriate immune response to deal with the invading microbe (Banchereau and Steinman, 1998; Banchereau et al., 2000; Kapsenberg, 2003). The importance of DCs to immunity is highlighted by the fact that microbial pathogens have evolved diverse strategies to avoid or subvert DCs. Microbial pathogens can thwart DC differentiation, survival, maturation, antigen processing and presentation and the induction of T cell immunity (Palucka and Banchereau, 2002; Moll, 2003; Steinman and Banchereau, 2007; Ueno et al., 2007). Because DCs migrate from the sites of infection to secondary lymph nodes to present antigen, they may also serve as systemic vectors (‘Trojan horses’) for persistent intracellular bacteria. This concept is tantalizing in light of the proximity of BCC organisms to cause fatal necrotizing pneumonitis and sepsis in susceptible individuals (Hutchison and Govan, 1999).

We studied the interaction between *B. multivorans* and *B. cenocepacia* in primary human monocytic-derived DCs to gain new insights into the pathogenesis of BCC. We hypothesized that *B. cepacia* complex subvert normal antibacterial mechanisms of DCs. The ability of *B. multivorans* and *B. cenocepacia* to modulate DC functions was investigated by examining DC maturation, cytokine production and cell death processes. *B. cenocepacia*, unlike *B. multivorans*, impaired DC maturation and stimulated DC necrosis, despite inducing similar amounts of IL-6, IL-12, TNF-α and IL-10.

**Results**

**DC phenotype**

Monocytes cultured for 7 days with GM-CSF and IL-4 displayed the expected immature DC phenotype, staining strongly for the DC markers CD1a and CD309 (mean fluorescent intensities of 134.14 ± 16.77 and 164.66 ± 26.33 respectively) and weakly for the monocyte/macrophage surface molecule CD14 (4.27 ± 0.56), the lymphocyte markers TCR alpha/beta and CD19 (0.93 ± 0.32 and 0.14 ± 0.06 respectively) and three markers of mature DCs: CD83, CD86 and CD86 (1.01 ± 0.59, 2.96 ± 1.63, 6.87 ± 1.27 respectively). Cell viability was 93.42% ± 1.02%, as determined by Trypan blue dye exclusion. Subsequent long-term incubations in monoparametric had no impact on DC viability or cell surface marker expression (data not shown).

**Bacterial association with DCs**

During the first 6 h of co-incubation, similar numbers of *B. multivorans* and *B. cenocepacia* associated with DCs, with approximately 40% of DCs binding at least one BCC bacterium. A considerable proportion of this early association was due to phagocytic uptake of the bacteria into...
DCs rather than simple binding of the bacteria to the external surface of cells, as pre-exposure to cytochalasin D, which paralyses the cytoskeleton and prevents uptake, caused a substantial reduction in the numbers of BCC-associated with DCs. At 2 h, there were 50.92% ± 10.70% and 73.42% ± 1.93% fewer B. multivorans and B. cenocepacia bacteria respectively, per cytochalasin D-treated DC than per normal phagocytic DC. From 2 to 24 h, the mean number of bacteria per DC increased from 1.40 ± 0.50 to 5.25 ± 0.11 to 10.92 ± 2.38 and 19.81 ± 4.55 for B. multivorans and B. cenocepacia respectively (Fig. 1A). At 24 h, DCs associated with significantly more B. cenocepacia than B. multivorans (Fig. 1A, P < 0.05). This corresponded to an increase in the percentage of DCs associated with more than 10 B. multivorans (47.20% ± 4.04% versus 21.20% ± 4.04%). At later time points, DCs exposed to B. cenocepacia had noticeably more peripheral cell debris and more bacterial cells associated with this debris than on slides of DCs treated with B. multivorans. The numbers of live B. multivorans and B. cenocepacia remained constant for the first 6 h of inter-

action with DCs and slowly increased from 6 to 24 h, with doubling times of 5.32 h and 2.73 h over the final 18 h of the incubation (Fig. 1B). In comparison, B. multivorans and B. cenocepacia had doubling times of 2.12 h and 1.98 h respectively, during exponential growth in R10 alone, in the absence of DCs and murine pneumonitis. These results contrast with the deleterious effect of R10 with murine pneumonitis BCG growth in the absence of DCs. A 4–6 log-fold decrease in the colony-forming unit (cfu) ml⁻¹ of B. multivorans and B. cenocepacia over 24 h. There were no significant differences in viable B. multivorans and B. cenocepacia when co-incubated with DCs and murine pneumonitis at each time point (Fig. 1B).

Influence of BCC on DC cytokine production and maturation

Dendritic cells co-incubated for 24 h with live B. multivorans and B. cenocepacia bacteria produced significantly more IL-6, TNF-α, IL-10 and IL-12 than control DCs or DCs stimulated with heat-killed BCC (Fig. 2, P < 0.001). Live BCC bacteria were also a more potent stimulus for cytokine release than BCC LPS. IL-6, TNF-α and IL-10 were produced in significantly higher quantities after stimulation with live B. multivorans versus B. multivorans LPS (P < 0.01, P < 0.001, P < 0.05 respectively), while IL-6, TNF-α and IL-12 concentrations were significantly higher after stimulation with live B. cenocepacia versus B. cenocepacia LPS (P < 0.001, P < 0.01, P < 0.01 respectively). Although DCs stimulated with BCC LPS released more cytokine than unstimulated DCs, these differences were only significant for IL-6 (P < 0.01 and P < 0.05 for B. multivorans LPS versus control and B. cenocepacia LPS versus control respectively) and IL-10 (P < 0.05 and P < 0.01 for B. multivorans LPS versus control and B. cenocepacia LPS versus control respectively). When cytokine-inducing ability of each species was directly compared, not only were amounts of cytokines elicited by B. multivorans and B. cenocepacia LPS similar, but the levels of IL-6, TNF-α, IL-10 and IL-12 were also uniformly high and not significantly different whether DCs were stimulated with live B. multivorans or B. cenocepacia (Fig. 2).

As expected, DCs exposed to B. multivorans and B. cenocepacia LPS upregulated CD83, CD86 and CD86 over 70% of cells stained positively for both co-stimulatory molecules CD80 and CD86 (Table 1). All surface markers measured were significantly upregulated after stimulation with BCC LPS (P < 0.05). Heat-killed BCC failed to induce phenotypic maturation of DCs, as neither the mean fluorescent intensities of the three cell surface markers nor the percentage of cells positive for both CD80 and CD86 were significantly different from unstimulated DCs (Table 1). CD83 expression on DCs exposed to
live BCG was approximately half of that of DCs exposed to BCC LPS, and was not significantly greater than CD83 expression on control DCs (Table 1). However, DCs co-incubated with *B. multivorans* or *B. cenocepacia* displayed distinct patterns of co-stimulatory molecule expression. *B. multivorans*-exposed DCs, as compared with control DCs, had twofold greater expression of CD86, significantly greater expression of CD80 (*P* < 0.05) and a significantly greater proportion of cells that were doubly positive for the two co-stimulatory molecules (*P* < 0.01) (Table 1). In contrast, DCs infected with live *B. cenocepacia* failed to upregulate CD80 and CD86, and showed similar patterns of expression as control cells for all markers examined (Table 1). In addition, when *B. cenocepacia* LPS and live bacteria were simultaneously co-incubated with DCs for 24 h, there was no upregulation of co-stimulatory molecules (data not shown).

In the course of the maturation experiments, it was also observed that 24 h of exposure to live *B. cenocepacia*, but not to live *B. multivorans*, heat-killed BCC, or BCC LPS,

**Table 1.** Surface marker expression of DCs after 24 h exposure to *B. multivorans* or *B. cenocepacia* LPS, heat-killed or live bacteria, displayed as mean fluorescent intensity of the maturation markers CD80, CD86 and CD93 and the per cent of DCs positive for both CD80 and CD86 (*n* = 5).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CD80</th>
<th>CD86</th>
<th>CD80 and CD86</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.48 (±0.37)</td>
<td>13.23 (±3.60)</td>
<td>35.90 (±7.35)</td>
</tr>
<tr>
<td><em>B. multivorans</em> C5668 LPS</td>
<td>7.80 (±2.39)*</td>
<td>34.69 (±5.61)**</td>
<td>106.34 (±16.62)**</td>
</tr>
<tr>
<td><em>B. cenocepacia</em> C6433 LPS</td>
<td>8.65 (±5.73)**</td>
<td>32.26 (±5.01)*</td>
<td>89.49 (±10.07)*</td>
</tr>
<tr>
<td><em>B. multivorans</em> C6433 heat-killed</td>
<td>1.63 (±0.18)</td>
<td>17.12 (±4.81)</td>
<td>33.80 (±5.98)</td>
</tr>
<tr>
<td><em>B. cenocepacia</em> C6433 heat-killed</td>
<td>2.06 (±0.37)</td>
<td>18.46 (±3.80)</td>
<td>32.65 (±5.87)</td>
</tr>
<tr>
<td><em>B. multivorans</em> C6686</td>
<td>3.28 (±0.65)</td>
<td>33.40 (±5.78)*</td>
<td>76.53 (±10.01)</td>
</tr>
<tr>
<td><em>B. cenocepacia</em> C6433</td>
<td>4.71 (±1.34)</td>
<td>18.31 (±2.23)</td>
<td>30.65 (±7.47)</td>
</tr>
</tbody>
</table>

* *P* < 0.05, ** *P* < 0.01, significantly different from unstimulated control as calculated by a one-way analysis of variance with Dunnett's multiple comparisons test.
resulted in a decrease in the total number of viable cells as assessed by Trypan blue staining, and there was a dramatic increase in the number of cells that appeared dead (i.e. cells with low forward scatter and intermediate side scatter on flow cytometry plots).

**Induction of DC necrosis by *B. cenocepacia***

Dendritic cells exposed to *B. cenocepacia* remained viable, i.e. the vast majority of cells were negative for both annexin V and propidium iodide, for the first 6 h, then sequentially lost viability from 12 to 24 h, while the viability of *B. multivorans*-treated DCs and control DCs remained consistently high over 24 h (Fig. 3A). DCs infected with *B. cenocepacia* were significantly less viable than control DCs at 18 (46.70% versus 86.74%, *P < 0.01) and 24 h (27.60% versus 79.30%, *P < 0.01). This drop in viability did not elicit a corresponding rise in apoptosis over time for DCs treated with *B. cenocepacia*. Indeed, similarly low background levels of apoptosis ranging from 5.75% to 15.36% were observed in untreated DCs and those co-incubated with *B. cenocepacia* or *B. multivorans* at all time points, although DCs given the apoptosis inducer actinomycin D displayed a time-dependent increase in apoptosis, peaking at 63.30% after 12 h of co-incubation (Fig. 3B). Instead of inducing apoptosis, exposure to *B. cenocepacia* began to induce necrosis in DCs after 12 h of co-incubation; the proportion of DCs staining positively for annexin V and propidium iodide increased from 18.82% at 12 h to 57.63% at 24 h (Fig. 3C). DCs exposed to *B. cenocepacia* demonstrated a higher percentage of necrotic cells than control DCs at 12, 18 and 24 h (*P < 0.01). Untreated DCs and those co-incubated with *B. multivorans* displayed only background levels of necrosis, with at most 5.24% of control and 13.44% of *B. multivorans*-treated cells staining positively for both dyes, unlike DCs treated with the detergent saponin, of which 95.34–99.66% were dead (Fig. 3C).

Unlike DCs exposed to live *B. cenocepacia*, DCs treated with LPS, heat-killed bacteria or overnight cell-free supernatant for 24 h did not display significantly more necrosis than the background level of necrosis in control cells (Fig. 4). At the standard multiplicity of infection (MOI) of 0.3:1, DCs did bind fewer heat-killed than live *B. cenocepacia* at earlier time points. Association of heat-killed and live bacteria with DCs was therefore equilibrated by increasing the MOI of heat-killed *B. cenocepacia* by 100-fold to 30:1 (the MOI of live *B. cenocepacia* remained at 0.3:1) and by centrifuging the bacteria onto the DCs. Even at this high MOI, heat-killed *B. cenocepacia* had no effect on DC viability, unlike live *B. cenocepacia* (data not shown). DCs also failed to become necrotic when pre-treated with cytochalasin D and then exposed to live *B. cenocepacia*.

**Fig. 3.** Annexin V and propidium iodide staining profile of control DCs and DCs exposed to *B. multivorans* or *B. cenocepacia* DCs treated with actinomycin D and saponin were controls for apoptosis and necrosis respectively.

A. Viability of cells was measured by the percentage of annexin V–propidium iodide–DCs. B. Apoptosis of cells was measured by the percentage of annexin V+, propidium iodide–DCs. C. Necrosis of cells was measured by the percentage of annexin V–, propidium iodide+DCs (average of 9, 3, 3, 2, 4 donors per time point ± SEM; *P < 0.05, **P < 0.01, significantly different from control at particular time points, as measured by one-way analysis of variance with Dunnett`s multiple comparisons test).

Cell death assays were also performed for DCs exposed to four other clinical isolates each of *B. multivorans* and *B. cenocepacia* as well as the original pair of *B. cenocepacia* C5568 and *B. cenocepacia* C6433. None of the five clinical isolates of *B. multivorans* induced significant necrosis after 24 h, while four out of five *B. cenocepacia* isolates caused necrotic cell death in DCs.
Fig. 4. Percentage of necrotic (annexin V+ propidium iodide+).
DCs after 24 h exposure to B. multivorans or B. cenocepacia LPS, heat-killed or live bacteria, bacterial cell-free supernatant or live bacteria following a cytochalasin D pretreatment. Untreated and saponin-exposed DCs served as negative and positive controls for necrosis respectively (average of 3 donors ± SEM; ***significantly different from untreated control, \( P < 0.01 \), as measured by a one-way analysis of variance with Dunnett's multiple comparisons test).

\( P < 0.01 \), Fig. 5). Only B. cenocepacia Cep1067 failed to induce significant necrosis in DCs.

Discussion

This study is the first report of the interaction between human DCs and two important opportunistic pathogens of CF and CGD patients, B. multivorans and B. cenocepacia. We analysed the ability of these bacteria to associate with DCs, to induce maturation, to stimulate cytokine production and to affect cell death processes. We demonstrated that DCs bind and internalize B. multivorans and B. cenocepacia at a very low MOI and in the absence of opsonins and mechanical manipulations, i.e. no contribution of bacteria onto cells. The number of bacteria associated with DCs and viable bacterial counts both increased over time. The increase in cfu ml\(^{-1}\) was quite gradual than levelled off for B. multivorans between 6 and 24 h, with a doubling time of 5.32 h, in contrast to the relatively fast B. multivorans doubling time of 2.12 h in \( \text{F}10 \) alone. However, the doubling time of B. cenocepacia after 6–24 h in the presence of DCs and meropenem was 2.73 h, only slightly slower than 1.90 h, the doubling time of B. cenocepacia in \( \text{F}10 \) media alone. This raises the question of whether BCC replicate inside DCs, which, if true, would be in contrast to previous studies of BCC and murine macrophage cell lines that show uptake but no intracellular growth of BCC (Sains et al., 1999, Lamto et al., 2007). However, we could not conclusively demonstrate intracellular B. cenocepacia replication, due to the concomitant induction of necrosis. B. cenocepacia were associated with live, dying and dead cells at 12, 18 and 24 h, some or all of which may have been permissive to bacterial growth and subsequent increased association. Bacteria may also have escaped from dead or dying cells and bound to other DCs. Indeed, we observed many B. cenocepacia among cell debris at 24 h, this likely accounted for the significantly increased cellular association of B. cenocepacia when compared with B. multivorans at this late time point. In addition, meropenem, although effective at reducing viable bacterial counts to near zero in cell culture media, may not have completely eliminated all of the extracellular bacteria, for instance, it may not have killed bacteria in close association, but untargeted by DCs. Meropenem is also not as completely cell-impermeable as aminoglycosides, although it does not accumulate inside cells (Lemaire et al., 2005). The activity of meropenem may have been compromised at later time points, overwhelmed by the release of B. cenocepacia from many necrotic DCs. Therefore, more tools are needed to draw conclusions about intracellular BCC replication in DCs. We are currently developing such tools.

Once the ability of BCC to associate with DCs had been established, it was important to examine whether BCC induced DCs to produce cytokines and to undergo maturation. Significant amounts of IL-6, TNF-\( \alpha \), IL-10 and IL-12 were produced by DCs exposed to BCC LPS and live bacteria. DCs secreted similar amounts of IL-6, TNF-\( \alpha \), IL-10 and IL-12 upon exposure to either B. multivorans or B. cenocepacia, and the only significant differences in cytokine induction were observed between live BCC bacteria and BCC LPS. The pro-inflammatory
nature of LPS from clinical BCC strains has been well
described in studies of human whole blood, peripheral
blood mononuclear cells, monocyte-derived macro-
phages, macrophage cell lines and murine macrophages
(Shew et al., 1995; Zughbi et al., 1999b; Hutchinson
et al., 2000; Shimomura et al., 2001; Groenew et al.,
2003; De Soya et al., 2004; Barnford et al., 2007; Silipo et al.,
2007). Human DCs can now be added to this list of
responsive cells. Some variation in the potency of LPS
has been described within and between clinical isolates of
B. multiroractis or B. cenoacapsa (De Soya et al., 2004; Silipo et al.,
2007), which was not observed with our two
clinical isolates.

In general, live BCC bacteria were more potent stimuli
of cytokine production than BCC LPS. This result is in
contrast to a previous study using a macrophage cell
line and a higher concentration of less highly purified
LPS (Zughbi et al., 1999b), which may account for the
discrepancy observed, as unpurified LPS can stimulate
cells via multiple mechanisms besides ligation of CD14-
TLR4. Our data are not unexpected, as live bacteria
interact more dynamically with host cells than a simple
TLR ligand, both at the cell surface and in the phago-
some, and can actively modulate an array of DC pro-
cessors (Palucka and Banchereau, 2002; Steinman and
Banchereau, 2007; Ueno et al., 2007). Indeed, it is inter-
esting to note that heat-killed bacteria, which are often
used as a surrogate for live bacteria, failed to induce
DCs to secrete cytokines, upregulate surface markers or
undergo necrosis. This may be due to lack of forced
contact between immotile, dead bacteria and DCs.

However, necrosis was still not induced by heat-killed
B. cenoacapsa even when the MOI was increased and
the bacteria were spun onto the cells to equilibrate early
association levels (data not shown). The present study
may suggest a role for one or more heat-labile bacterial
components in these diverse DC processes. It has been
recently demonstrated that live but not heat-killed
B. cenoacapsa cause a delay in phagosomal maturation
of murine macrophages (Lamothe et al., 2007). Distinct
phenotypes of DCs exposed to live and heat-killed BCC
may be related to potential phagosomal maturation alter-
ations, but further investigation is required. Although
some questions remain, it was clear that in this system
live bacteria were required to engage DCs effectively
and generated more cytokine release than heat-killed
bacteria or even purified LPS.

The maturation experiment demonstrated that DCs
challenged with live B. cenoacapsa failed to upregulate
the CD80 and CD86 costimulatory molecules signifi-
cantly. As effective costimulation is required to activate T cells
(Kapsenberg, 2003; Steinman and Banchereau, 2007;Ueno et al., 2007),
this could have profound effects on the ability of DCs to drive an immune response directed
against this serious pathogen. This is not to say that
adaptive immunity is completely compromised in patients
infected with this organism. Indeed, it has been reported
that B. cenoacapsa complex-specific antibodies are produced
by infected CF patients (Hendry et al., 2006a,b), likely by
interaction with other antigen-presenting cells such as
macrophages. However, the presence of antibodies does
not play an obvious salutary role in chronic disease, as is
expected in a group of organisms that appear to adopt an
intracellular lifestyle. A robust Th1 response is required
to control intracellular pathogens (Kapsenberg, 2003; Stein-
man and Banchereau, 2007; Ueno et al., 2007). DCs pro-
duced a large amount of IL-12 in response to BCC, but
failed to upregulate costimulatory molecules, which likely
impaired their capacity to activate T cells.

This study also demonstrated that B. cenoacapsa
but not B. multiroractis caused necrosis of human DCs.
Necrosis occurred following exposure to live B. cenoac-
apsa, not heat-killed BCC or BCC supernatant. DCs
pre-treated with the cytochrome D prior to exposure to
live B. cenoacapsa also did not become necrotic. These
results suggest that uptake of live B. cenoacapsa was
required to induce necrosis in DCs. We also determined
the scope and relevance of this observation by examining
a panel of B. multiroractis and B. cenoacapsa clinical
isolates. None of the five B. multiroractis isolates induced
significant DC necrosis after 24 h, whereas four out of five
B. cenoacapsa isolates induced DC cell death. Only B.
cenoacapsa Cep1067, a member of RcsA group III B,
a distinct phylogenetic cluster within B. cenoacapsa
(Mahenthiralingam et al., 2008a), failed to induce signifi-
cant necrosis.

Other researchers have reported that BCC, and specifi-
cally B. cenoacapsa, can induce death in non-phagocytic
and phagocytic cells. B. cenoacapsa cable (cb) pil have
recently been implicated in lung epithelial cell death
(Cheung et al., 2007). The cbA pil are mainly produced by
B. cenoacapsa strains belonging to the epidemic
ET12 lineage (Mahenthiralingam et al., 2008b), which are
members of phylogenetic cluster III A. As equivalent
necrosis is induced by B. cenoacapsa J2315 and K56-2,
ubcA-positive ET12 isolates, and B. cenoacapsa G6433
and 8963 that are not part of the ET12 lineage and are
ubcA-negative (Mahenthiralingam et al., 2008b), another
mechanism must be involved in DC death induced by this
organism, perhaps involving other types of BCC pil or
non-fimbrial adhesins (Chiu et al., 2001; Mohr et al.,
2001).

Both apoptotic and necrotic cell death have been
described in murine macrophages and human neutrophils
following exposure to purified secreted BCC enzymes
such as hemolysin, ATP-utilizing enzymes, azurin and
cytochrome c551 (Hutchison et al., 1998; Mehnke et al.,
2000, Punj et al., 2003). In rodent models, extracellular
proteases are associated with tissue damage, and the production of at least one zinc metalloprotease is required for virulence (McKeever et al., 1989; Kooi et al., 2006). Of particular interest, azurin and ZmpB are secreted by B. cenocepacia but not by B. multivorans (Punj et al., 2003; Kooi et al., 2006). The relevance of these studies to the present one is unclear because, in our investigations, DCs remained viable when exposed to BCC supernatants. As the clinical B. multivorans and B. cenocepacia strains used in this study likely secrete their respective full arsenal of enzymes, it may be that DCs are insensitive to those enzymes, or that these enzymes are not present in sufficient quantity in the 20 h BCC supernatant to cause necrosis. As uptake of live B. cenocepacia appears to be required for DC necrosis, the enzymes specifically secreted by B. cenocepacia may contribute to the process if they are produced in sufficient quantities in the microenvironment of the phagosome, but this has not been determined.

Studies from our lab have recently demonstrated that human neutrophils are killed by live B. cenocepacia (Bylund et al., 2005b). Neutrophils are short-lived cells that undergo spontaneous apoptosis after 24 h (Szejtli et al., 1989). Exposure to live B. cenocepacia, but not killed bacteria or LPS, enhances apoptosis as compared with untreated control cells. Upon challenge with B. cenocepacia, CGD neutrophils have slightly heightened levels of apoptosis than control, but also undergo necrosis more readily than normal neutrophils. This suggests that B. cenocepacia can induce neutrophil necrosis when reactive oxygen species production is compromised, as is found in CGD (Bylund et al., 2005b).

It is difficult to draw parallels between the effect of B. cenocepacia on neutrophils and DCs. Although both are phagocytes, DCs, like macrophages, are longer-lived cells and do not undergo spontaneous apoptosis after 24 h. DCs are also resistant to many common stimulators of apoptosis (Ashary et al., 1996; Levittus et al., 2009) consistent with their complimentary role in phagocytosing and eliminating apoptotic cells. Apoptosis represents the normal termination of DC maturation, as DCS encounter microbes or microbial components, mature into antigen-presenting cells, migrate to the lymph node, present to T cells and then eventually undergo apoptosis. This entire process takes days (Hou and Van Putten, 2004; De Trous et al., 2005) rather than hours as is the case with neutrophils. Indeed, apoptosis was not observed over the 24 h study period even in response to LPS, the prototypic DC maturation stimulus.

It is interesting to note that live B. cenocepacia induced necrosis in both CGD neutrophils and normal DCs. Bylund et al. (2005b) demonstrated that reactive oxygen species are critical for eliminating ingested bacteria and abrogate necrosis of the ingesting neutrophil. Previous work has shown that immature DCs, even from normal donors, produce a lower amount of reactive oxygen species than neutrophils, macrophages and monocytes (Vulcano et al., 2004). However, DC maturation following LPS exposure stimulates increased reactive oxygen species (ROS) production in response to soluble agonists (Vulcano et al., 2004), which has also been confirmed using BCC LPS-matured DCs (K.L. MacDonald, L.A. Burgess, and D.P. Speert, unpubl. data). The fact that DCs challenged with B. cenocepacia failed to mature suggests that the bacteria remained in an immature ROS-deficient phagocyte that may be incapable of producing much ROS. This may contribute to the toxicity of B. cenocepacia towards immature DCs, but remains to be investigated.

In this study, we have demonstrated that although both BCC species induced significant cytokine release from DCs, B. cenocepacia, unlike B. multivorans, effectively interfered with the normal functioning of DCs by inhibiting upregulation of costimulatory molecules and inducing necrosis. It was striking that the more clinically relevant pathogen, B. cenocepacia, had such a deleterious effect on DCs, cells that represent a vital component of the innate immune response in the lung and a critical co-ordinator of an effective adaptive immune response. We believe that the ability of B. cenocepacia to subvert DC function and target these critical host defence cells for destruction may be of central relevance to the complicated pathogenesis of BCC in CGD and CF patients. The differences between the two species which we observed may provide valuable new insights into both understanding and treating this serious complex of opportunistic pathogens.

Experimental procedures

Bacterial strains, media and growth conditions

All isolates of B. multivorans C6568 and B. cenocepacia C6493 are isolates from patients with CF that had been stored in the Canadian B. cepacia complex Research and Reference Repository. Eight additional clinical isolates from CF and CGD patients were selected from the BCC strain panel (B. multivorans C6593, C1576 and JTC, and B. cenocepacia J2316 and K56-2) (Maharajrungsluck et al., 2000b), and from the Canadian B. cepacia complex Research and Reference Repository (B. multivorans D0599 and B. cenocepacia C8963 and Cep15027). Bacteria were stored at −70°C in Mueller Hinton broth with 10% (v/v) dimethyl sulfoxide. Routine culturing was performed on blood agar plates (PM, Microbiologicals, Richmond, British Columbia, Canada) or Luria–Bertani (LB) agar. Bacteria were grown in 5 ml of LB broth at 37°C with agitation at 200 r.p.m. for 16 h to stationary phase, pelleted at 4500 g for 10 min at room temperature, then adjusted to the correct optical density (OD600 of 0.600). Further dilutions were performed in R10 cell culture medium (RPMI 1640 supplemented with 10% [v/v] fetal calf serum, 2 mM L-glutamine and 1 μM sodium selenite) to a concentration of 6 × 10^5 cfu/ml. For some experiments, bacteria
were heat-killed by incubating 1 ml of culture in a 56°C water bath for 15 min. To prepare bacterial cell-free supernatants, cultures were grown for 20 h in R10 at 37°C. 5% CO₂ and filtered through 0.22 μm cellulose acetate filters (Costar, Corning, NY). The concentration of viable bacteria and the efficiency of heat-killing and filtration were confirmed by viable plate counting.

Preparation of LPS
Purified LPS from B. multivorans C6559 and B. cepacia C6439 was kindly provided by R.K. Ernst (University of Washington). LPS had been isolated using a hot water-phenol extraction method (Westphal and Jann, 1965), and then treated with RNase A, DNase I, and protease K to remove contaminating nucleic acids and proteins (Fischer et al., 1993). Subsequent purifications had been performed to eradicate contaminating phospholipids (Follih et al., 1997), and proteins that signal through toll-like receptor 2 (Hirschfield et al., 2000).

Monocyte isolation and culture into DCs
Monocyte-derived DCs were generated using standard protocols (Salusto and Lanzavecchia, 1994; Davidson et al., 2004). Briefly, human venous blood was collected from normal adult volunteers, according to the University of British Columbia Clinical Research Ethics Board protocol C04-0193. Separated blood was diluted 1:1 in R10 medium, then overlayed onto Ficol-Paque plus (GE Healthcare, Waukesha, WI, USA). Following centrifugation at 450 g for 20 min at room temperature, the peripheral blood mononuclear cells were collected from the interface and washed with phosphate-buffered saline (PBS). T cells were depleted by incubation with sheep anti-human platelet antiserum and an additional Ficol-Paque purification. Remaining mononuclear cells were collected from the interface, washed with PBS, re-suspended in R10 and cultured in six-well plates at a concentration of 4 × 10⁶ cells per well for 7 days at 37°C, 5% CO₂. After washing with PBS to remove non-adherent cells, the monocytes were cultured in R10 containing 100 ng/ml granulocyte-macrophage colony-stimulating factor and 100 ng/ml interleukin-4 (FITC, Concorde, MA) for 7 days at 37°C, 5% CO₂ to generate immature DCs.

After 7 days, DCs were harvested from six-well plates, washed with PBS and re-suspended in PBS. Enumeration and confirmation of viability was performed using Trypan blue dye exclusion as assessed in a hemocytometer. Flow cytometry was used to confirm the purity and immature phenotype of the resultant cells, as previously described (Davidson et al., 2004). Briefly, an aliquot of DCs was re-suspended in FACS buffer (PBS + 1% (w/v) FCS, 0.01% (w/v) sodium azide and 0.01% (w/v) human AB serum (Sigma)) at a concentration of 1 × 10⁶ DCs ml⁻¹. One hundred microliters of DCs were stained for 1 h at 4°C with FITC-conjugated monoclonal antibodies specific for CD1a (Serotec, Raleigh, NC), CD14, CD16 (BD Biosciences, Mississauga, Ontario, Canada), CD83, CD19, IgG2a or IgG1 isotype controls (eBioscience, San Diego, CA) and with PE-conjugated antibodies raised against CD80 (BD Biosciences), CD86, TCR alpha/beta and an IgG1 isotype control (eBioscience). After two washes in PBS, DCs were re-suspended in 200 μl of PBS + 2% (w/v) formaldehyde for flow cytometry.

Association assays and viable plate counts
Dendritic cells were co-inoculated with B. multivorans, B. cepacia or R10 alone as a control, and then harvested at 2, 4, 6, 12, 18 and 24 h. Small aliquots from each well were diluted in Hank’s balanced salt solution containing 10% gelatin and 1% triton X-100 to lyse DCs and release any associated bacteria. The resulting lysates were plated on LB agar, and incubated at 37°C for enumeration. The remaining cells were washed to remove non-adherent bacteria then opto-spin on glass slides. The slides were stained with Diff-Quik (Dade Behring, Newark, DE) and permounted. The number of bacteria associated with each DC was determined by light microscopy for 100 DCs per slide.

DC maturation and cytokine enzyme-linked immunosorbent assays
Dendritic cells were seeded into Teflon inserts in 24-well plates at a concentration of 5 × 10⁵ cells ml⁻¹ and exposed to R10 at 37°C, 5% CO₂ for 1 h at 4°C with FITC-conjugated monoclonal antibodies specific for CD1a (Serotec, Raleigh, NC), CD14, CD16 (BD Biosciences, Mississauga, Ontario, Canada), CD83, CD19, IgG2a or IgG1 isotype controls (eBioscience, San Diego, CA) and with PE-conjugated antibodies raised against CD80 (BD Biosciences), CD86, TCR alpha/beta and an IgG1 isotype control (eBioscience). After two washes in PBS, DCs were re-suspended in 200 μl of PBS + 2% (w/v) formaldehyde for flow cytometry.

Apoptosis necrosis assays
Induction of cell death was examined by co-incubating 5 × 10⁵ DCs ml⁻¹ in 96-well plates with B. multivorans, B. cepacia or R10 (negative control) for 2, 6, 12, 18 and 24 h. For some
Flow cytometry

A minimum of 1×10^6 gated events were collected for each condition using a FACSCalibur system and CellQuest, version 3.1, software (BD Biosciences). Data were analyzed using FlowJo 7.2.2 flow cytometry software (Tree Star, Ashland, OR).

Statistical analyses

Data are expressed as mean ± SEM. Paired Student’s t-tests were used to evaluate the statistical differences between B. multivorans- and B. cenocepacia-treated DCs. Comparisons between a control and several experimental groups were performed using one-way analyses of variance with a Dunnett’s multiple comparison test. Multiple group comparisons were performed using one-way analyses of variance with a Tukey post-test. A value of P<0.05 was taken as a statistically significant difference.

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Burkholderia cenocepacia subspecies human dendritic cells


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Appendix 5. ROS production by control and CGD PBMCs following TLR stimulation.

PBMCs were stimulated with buffer (Ctrl, dotted black line; CGD, dotted gray line), LPS (100 ng/ml in the presence of 1% serum, Ctrl, red line; CGD, green line), or PGN (10 µg/ml, Ctrl, blue line; CGD, yellow line) and ROS production was followed by luminol-enhanced CL. Shown is one matched experiment with CGD and Ctrl, though this experiment was repeated two other times with control PBMCs only.
Appendix 6. Effect of MG-132 on the hyperinflammatory phenotype of CGD cells. PBMCs from a healthy control (open bars) and a CGD patient (filled bars) were preincubated in the absence or presence of MG-132 (5 μM) for 30 min before stimulation with medium (unstimulated), LPS (100 ng/ml in the presence of 1% serum), PGN (10 μg/ml), IL-1β (250 ng/ml) or PHA (10 μg/ml). Pre-treatment with the DMSO vector followed by stimulation with medium or LPS + serum were also included as controls. Following a 20 hour incubation, the cell-free supernatants were analyzed by ELISA for the production of IL-6 (A) and TNF-α (B). MG-132 inhibited cytokine production from both CGD and control cells.
Appendix 7. **Analysis of necrosis in live and heat-killed *B. cenocepacia* C6433 upon equilibration of early association.**

(A) Mean number of live and heat-killed *B. cenocepacia* associated with DCs and (B) percentage of DCs associating with at least one bacterium after 6 h of co-incubation, as assessed by microscopic enumeration of cyto-spun and Diff-Quik-stained DCs (C) Percentage of necrotic (AnnV+, PI+) DCs after 24h exposure to live *B. cenocepacia* C6433 at an MOI of 0.3:1 and heat-killed *B. cenocepacia* C6433 at an MOI of 30:1. Unstimulated DCs and saponin-treated DCs were used as controls (average of 3 donors ± SEM, ***significantly different than control, p < 0.001 as measured by a one-way analysis of variance with Tukey’s multiple comparisons test).
Appendix 8. High-pressure freezing and freeze substitution methods used for transmission electron microscopy.

High pressure freezing was initially chosen to process DCs for TEM. It required less material so up to nine samples could be processed per experiment. There are also fewer artifacts and better resolution of the membranes with this technique, as samples are instantly frozen and then the vitreous water is slowly replaced by the osmium tetroxide fixative in acetone over 6 days, instead of the normal harsher room temperature dehydration of conventional processing.

Control DCs and DCs exposed to *B. cenocepacia* and *B. multivorans* for 2, 6 and 24 h were fixed with 2.5 % (v/v) glutaraldehyde (Canemco, Canton de Gore, QC) and 2 % (v/v) formaldehyde (Canemco) in 0.1 M phosphate buffer (pH 7.4) glutaraldehyde for thirty minutes at 37°C, washed twice in 0.1 M phosphate buffer, and resuspended in 0.1 M phosphate buffer. Samples were then sealed in dialysis tubing, high pressure frozen in planchets, and transferred to liquid nitrogen pre-cooled cryovials containing 1 % (v/v) osmium tetroxide (Canemco) in 20 % methanol/80% acetone (v/v). All planchets were then transferred to plastic capsules (Leica Microsystems, Richmond Hill, ON) under liquid nitrogen using pre-cooled forceps. The capsules were then transferred to the pre-cooled automatic freeze substitution chamber (Leica) and left for 6 days. The fixed and freeze-substituted samples were then infiltrated with increasing concentrations of Spurr’s resin (Canemco) in acetone under vacuum at power level 3 using the Pelco laboratory microwave (Ted Pella, Redding, CA). The samples were finally polymerized in 100% resin in a 60°C oven overnight. Ultrathin sections were mounted in 100-mesh Formvar-coated copper grids, stained with 2% (w/v) uranyl acetate and 2% (w/v) lead citrate, and viewed on a Hitachi H7600 transmission electron microscope (Hitachi, Toronto, ON) operating at 80 kV. However, the DCs were not properly taken up by the dialysis tubing or the dialysis tubing collapsed during processing, so all the dialysis tubes sectioned were empty.
In the second high pressure freezing attempt, DC samples were combined with the cryoprotectant dextran (25% (w/v) in 0.1 M phosphate buffer), and at least five batches of samples were subjected to high pressure freezing, freeze substitution, and resin infiltration and polymerization. One batch of samples was processed as before, but these samples failed to substitute properly and broke apart during sectioning. The freeze substitution time was extended and other strategies were attempted for the remaining batches of samples, but useable sections were not obtained.