Biometals (2011) 24:1–17
DOI 10.1007/s10534-010-9376-3

Chronic beryllium disease: an updated model interaction between innate and acquired immunity

Richard T. Sawyer · Lisa A. Maier

Received: 16 August 2010 / Accepted: 30 August 2010 / Published online: 28 October 2010

Abstract During the last decade, there have been concerted efforts to reduce beryllium (Be) exposure in the workplace and thereby reduce potential cases of this occupational lung disorder. Despite these efforts, it is estimated that there are at least one million Be-exposed individuals in the U.S. who are potentially at risk for developing chronic beryllium disease (CBD). Previously, we reviewed the current CBD literature and proposed that CBD represents a model interaction between innate and acquired immunity (Sawyer et al., Int Immunopharmacol 2:249–261, 2002). We closed this review with a section on "future directions" that identified key gaps in our understanding of the pathogenesis of CBD. In the intervening period, progress has been made to fill in some of these gaps, and the current review will provide an update on that progress. Based on recent findings, we provide a new hypothesis to explain how Be drives sustained chronic inflammation and granuloma formation in CBD leading to progressive compromised lung function in CBD patients. This paradigm has direct implications for our understanding of the development of an immune response to Be, but is also likely applicable to other immune-mediated lung diseases of known and unknown etiology.

Keywords Beryllium · Chronic beryllium disease · Granuloma · Innate immunity · Acquired immunity

R. T. Sawyer
Asthma, Allergy and Inflammation Branch, Division of Allergy, Immunology and Transplantation, National Institute of Allergy and Infectious Disease, National Institutes of Health, 6610 Rockledge Drive, Bethesda, MD, USA

L. A. Maier (✉)
Robert H. Hollis Laboratory of Environmental and Occupational Health Sciences, Division of Environmental and Occupational Health Science, Department of Medicine, National Jewish Health, Denver, CO, USA
e-mail: maierl@njc.org

L. A. Maier
Division of Pulmonary Sciences and Critical Care Medicine, Department of Medicine, School of Medicine, University of Colorado Denver, Denver, CO, USA

L. A. Maier
Department of Environmental & Occupational Health, Colorado School of Public Health, University of Colorado Denver, Denver, CO, USA

Introduction

Chronic beryllium disease (CBD) is a disorder mediated by cellular and molecular components of the innate and acquired immune systems (Sawyer et al. 2002; Fontenot and Kotzin 2003; Samuel and Maier 2008), occurring principally in Be-exposed industry workers (Henneberger et al. 2004; Infante and Newman 2004). In a subset of those exposed, Be may result in the generation of Be-specific $CD4^+$ T cells in the peripheral circulation, or beryllium sensitization

(BeS). Over time, these "sensitized" T cells may accumulate within the lungs and eventually form granulomas. Exposure to Be triggers T cell proliferation in the lung with the accompanied production and release of pro-inflammatory T helper 1 (Th1) type cytokines, and this response to Be mediates a state of chronic lung inflammation that lasts for decades. This Be-specific lung inflammation is slowly progressive but may result in debilitating and potentially fatal pulmonary fibrosis. The increasing world-wide industrial use of Be and consumption of Be products may be contributing to a new epidemic of this chronic lung disorder (Infante and Newman 2004).

Beryllium

Beryllium (Be) is familiar to all in its gemstone forms, aquamarine, emerald, beryl and chrysoberyl. Although a rare element, Be is found naturally, and its ore (beryl ore and/or bertrandite) is currently mined and processed into various forms, including pure metal, alloys and ceramics, in the U.S. (Henneberger et al. 2004). Purified elemental Be occurs as a fine gray powder with a metallic luster (Mckeehan 1922). With atomic number 4, Be heads the Group IIA alkaline earth series of metals in the periodic table of elements. The Be ion, and the metals in the Group IIA series including magnesium, calcium, strontium, barium and radium, all have a bi-positive charge (Be_2^+). However, with an atomic weight of 9.013 and a charge number, $z = 2$, as compared to the other alkaline earth metals, Be has the smallest ionic radius, $r = 0.31$ nm. The ratio of the charge number to the ionic radius is large, $z/r = 6.45$, and the chemical properties of Be are due to this high density charge (Everest 1964). Beryllium accepts two electrons and forms tetrahedral structures, principally with oxygen, but also due to this high z/r ratio Be forms complex compounds with biologically important macromolecules, for example ferritin (Price and Joshi 1983). It's extremely light weight, stiffness six times that of steel, very low density 1.85 times that of water, and high melting point of 1,285°C, make Be a strategically important metal finding application in many high-technology industries.

Once mined, Be ore is converted to a variety of chemical compounds used in various industrial applications including Be metal, Be oxide (BeO) and Be alloys with copper and aluminum (Day et al. 2007; Taiwo et al. 2008; Nilsen et al. 2010). Be-exposure in the workplace is a growing public health concern. It is estimated that over one million Be-exposed individuals are at risk to develop CBD. Current studies show that specific job titles and tasks, such as work as a Be machinists, are associated with greater risk for progression to CBD with significantly worse lung disease (Mroz et al. 2009). CBD is not limited to individuals who work directly with Be but also occurs in workers with relatively low levels of exposure, including those with bystander exposure and dust disturbers, such as decontamination and decommission workers, and construction trade workers at U.S. Department of Energy nuclear sites (Welch et al. 2004). Recent work has raised the important concern that Be inhalation may not be the only route of workplace exposure leading to BeS and CBD. Industry reduction in inhalation exposure to Be has not resulted in a reduction in BeS and it is hypothesized that workplace skin exposure may also lead to BeS, which then may progress to CBD in those with inhalational exposure (Day et al. 2006; Tinkle et al. 2003). This hypothesis is supported by studies showing that fine particles of Be metal are present in Be workplace dust, and that Be is found on the skin of Be workers (Day et al. 2007; Rouleau et al. 2005). Early studies, that used skin patch testing showed that exposure to Be salts in normal control subjects resulted in immune sensitization to Be and potentially impaired lung function (Rossman 2001), demonstrating that Be skin exposure can result in the induction of a Be-specific immune response.

The U.S. is the world's beryllium industry leader and consumer. However, world-wide use of Be is increasing and occupational Be-exposure continues to occur in the aerospace, nuclear, automotive, electronics and telecommunications industries (Henneberger et al. 2004). New industries that recycle electronics, computers and scrap alloys to recover copper and aluminum have resulted in a new group of occupationally exposed workers (Balkissoon and Newman 1999; Hasejima et al. 1995; Taiwo et al. 2010). The current U.S. Occupational Safety and Health Administration permissible exposure limit (PEL) of 2 μg per cubic meter of air as an 8-h time weighted average has failed to protect workers from Be health effects including BeS and CBD (Infante and Newman 2004; McCleskey et al. 2009). OSHA is currently reevaluating this standard and may provide a new standard

for workers in the US, although the level for the PEL and other aspects of the standard are not currently known.

The spectrum of beryllium disease

Acute pulmonary and dermal reactions were observed in workers exposed to high Be levels in the 1940s. Acute beryllium disease is an acute pneumonitis characterized by a predominantly lymphocytic inflammation of the upper and lower respiratory tract, nasopharyngitis, tracheobronchitis and symptoms indicating acute inhalation injury of a chemical irritant. It is widely believed that the mandated reduction in workplace levels of Be made this disease infrequent. However, increasing world-wide use of Be in countries that do not adhere to U.S. standards may result in new cases of acute beryllium disease (Cummings et al. 2009).

Workplace exposure can result in the generation of an acquired immune response to Be as demonstrated by the proliferation of Be-specific $CD4^+$ T cells using the beryllium lymphocyte proliferation test (BeLPT) (Mroz et al. 2009). This test used extensively in workplace surveillance over the past decade to define Be-related health effects in the beryllium industry (Bailey et al. 2010; Sackett et al. 2004; Taiwo et al. 2008; Welch et al. 2004). The use of the BeLPT in medical surveillance has identified subjects who are BeS with an abnormal BeLPT but without evidence of CBD. Although the majority of Be-exposed workers remain symptom free, and demonstrate a negative BeLPT for their life-time, between 2 and 20% of workers progress to BeS (Mroz et al. 1991, 2009). These subjects are asymptomatic and have normal chest radiography, pulmonary physiology and lung biopsies (Pappas and Newman 1993). BeS subjects have a 6–8% risk of progressing to CBD and thus require on-going medical monitoring for progression to CBD (Newman et al. 2005).

Chronic beryllium disease is a debilitating and potentially fatal occupational lung disorder. Progression to CBD is characterized by the presence of interstitial mononuclear cell infiltrates and/or non-caseating granulomas on lung biopsy, usually obtained via bronchoscopy with transbronchial biopsy. Progression from BeS to CBD can occur within months to decades from the time of the initial exposure and the period of the initial Be-exposure can be very brief (Rossman 2001). While individuals with CBD diagnosed due to determination of BeS during medical surveillance using the BeLPT may be asymptomatic, others may experience increasing pulmonary symptoms accompanied by abnormal pulmonary function and chest radiography. A recent study showed that individuals with CBD diagnosed through medical surveillance with the BeLPT demonstrated a greater decline in lung function than surveillance identified BeS subjects, indicating that the individuals with surveillance identified CBD are at risk of developing progressive lung disease (Mroz et al. 2009).

There is no cure for CBD. Patients with BeS are followed with ongoing clinical evaluations to determine if they are progressing to CBD. Treatment focuses on control of inflammation and immune reactions although studies show that Be can persist in CBD granuloma for decades after the last Be exposure (Sawyer et al. 2005a). Prednisone is administered when there is evidence of pulmonary impairment based on symptoms, pulmonary function tests and exercise testing. Corticosteroids are required life-long with other supportive treatment such as oxygen therapy for those with hypoxemia and/or pulmonary hypertension. Methotrexate may be used in patients that do not respond to steroids. Steroid treatment has been associated with improvement in lung function in CBD patients, suppressing lung granuloma formation and inhibiting progression to pulmonary fibrosis in some patients (Marchand-Adam et al. 2008).

Peripheral blood mononuclear cells (PBMCs) from CBD and BeS subjects contain Be-specific $CD4^+$ T cells that proliferate in response to Be-stimulation in the BeLPT. The bronchoalveolar lavage (BAL) mixed cell population from BeS subjects displays normal numbers of leukocyte classes, dominated by alveolar macrophages, whereas CBD BAL cells display a lymphocytic alveolitis due to the presence of pathogenic Be-specific $CD4^+$ T cells in the lung. In culture, CBD BAL T cells proliferate and produce Th1-type cytokines in response to Be-stimulation (Fontenot et al. 2005; Mroz et al. 1991; Rossman et al. 1988; Sawyer et al. 2004a; Tinkle and Newman 1997; Tinkle et al. 1997). In a previous review (Sawyer et al. 2002), we discussed the growing body of studies showing that in addition to the induction of

an acquired, T cell mediated, immune response, Be also engages cellular and molecular components of the innate immune system. We advanced the notion that initial Be exposure activates the innate immune system to generate an acquired immune response. Chronic Be exposure stimulates the acquired immune response to release mediators of chronic inflammation in the lung involving cellular and molecular components of innate immunity and it is this vicious cycle driven by Be that results in progressive impairment of lung function, granuloma formation and progression to lung fibrosis. At that time there were major gaps in our understanding of how Be might drive this cycle in the lung. Those gaps included:

(1) The complete chemical composition of Be-antigen was unknown.
(2) The role of Be-antigen processing and presentation was poorly studied.
(3) The role of Be-binding molecules, such as ferritin in the movement of Be within the host and host cells was poorly characterized.
(4) The effects of Be on reactive oxygen species (ROS) and oxidant lung injury were unknown.
(5) We did not know how Be-antigen activated $CD4^+$ T cells arise in CBD.
(6) The role of IL12, IL15 and IL18 and other cytokine mediator molecules had not been evaluated.
(7) Be stimulated macrophage apoptosis was new, and poorly understood.
(8) The role for Be persistence in the lung was unknown.
(9) Genetic components were just being identified, but were limited and worker population based studies were needed.
(10) There were only a few preliminary studies of the role of Be in pulmonary fibrosis.

Filling the gaps

Some of the gaps outlined above have been addressed either partially or more completely while others have not. The remainder of this review will focus on the progression in research studies to fill these gaps and our current understanding of the importance of the innate immune system in CBD and other similar granulomatous lung diseases. In addition, we will identify those areas that are still unresolved and where additional study is needed.

The complete chemical composition of Be-antigen was unknown

The activation of Be-specific $CD4^+$ T cells for proliferation and cytokine production depends on the presentation of Be-antigen by major histocompatibility class II (MHCII) molecules on antigen presenting cells (APCs) to T cell antigen receptors (TCR) (Fontenot et al. 2001). Studies have identified a strong association between CBD and MHC class II human leukocyte antigen (HLA)-DP alleles that contain glutamic acid at amino acid position 69 of the β-chain (βGlu69) (Maier et al. 2003b; McCanlies et al. 2004; Rossman et al. 2002). Several recent studies have attempted to model possible Be-antigen-MHCII structures and interactions. In those individuals without a βGlu69, studies suggest that a Glu at amino acid position 71 in HLA DRB1 β-chain may function in antigen presentation in CBD (Maier et al. 2003b; Bill et al. 2005).

The formation of MHCII-peptide complexes occurs in the endoplasmic reticulum of antigen presenting cells where the peptide binding pocket of MHCII is occupied by the class-II associated invariant chain peptide (CLIP) that blocks unwanted peptide binding at this point. The CLIP peptide is enzymatically removed allowing antigenic peptides to enter the binding pocket. The MHCII-peptide is transported to the APCs surface where it is now able to present the peptide to an antigen specific TCR on T cells. Amicosante and his coworkers (Amicosante et al. 2001, 2009; Berretta et al. 2003) found that Be can outcompete high affinity CLIP peptide for binding to soluble HLA-DP molecules. The data show the importance of the βGlu69 amino acid moiety present in HLA-DP, and possibly βGlu71 in HLA DRB1, and support the idea that selected peptides with high affinity for HLA-DP mediate Be binding and therefore Be presentation to T cells. Additional modeling suggested that interactions between Be and carboxylate groups in the HLA-DP binding pocket could result in formation of "Be clusters" in association with the bound peptide (Scott et al. 2003). Support for this idea comes from a study by Keizer et al. (2005) showing that at physiologic

pH, Be forms stable ring clusters with oxygen. The HLA-DP peptide binding pocket contains six carboxylate groups and Be may therefore form stable complexes bound via one or more of these carboxylates (Scott et al. 2003; Snyder et al. 2003; Keizer et al. 2005).

Fontenot and his coworkers (Dai et al. 2010) have now described the crystal structure of HLA-DPB1 0201 at 3.25 Å resolution. The solved structure has interesting features that suggest how Be could bind to HLA-DP. The peptide binding grove is exceptionally wide and bound peptide rides rather high in the groove. This feature leaves the acidic pocket on the floor of the peptide binging groove, formed by the carboxylates present on the βGlu69, βGlu26 and βGlu88 residues, free to react with and bind to Be. The model suggests that the Be-antigen presentation complex consists of MHCII HLA-DP with Be bound to carboxylate groups in the acidic pocket that is formed by a select group of peptides that give Be an appropriate amount of room to enter the acidic pocket. Mutation analysis of the acidic amino acids in the binding grove show that the βGlu69 position is necessary and sufficient to bind Be in a manner that allows T cell activation. Crystallization of the HLA-DP molecule significantly advances our understanding the chemical structure and composition of Be-antigen. Whether Be binds to MHCII molecules directly in vivo, or to peptides that then bind in the MHCII peptide binding groove is unresolved. The data suggest that Be likely binds directly to HLA-DP within the acidic pocket and this interaction is favored by specific permissive peptides. The model does not yet tell us how Be-antigen is then recognized by the corresponding TCR, or the composition of potentially permissive peptide(s) in vivo. Nevertheless, this study impacts directly on the issues related to our next gap, that of understanding how Be-antigen is processed and presented by APCs.

The role of Be-antigen processing and presentation was poorly studied

Antigen processing and presentation is a function shared by many classes of cells, most of which are found in the human airway, lung circulatory system and lung parenchymal tissues, including basophils (Perrigoue et al. 2009; Sokol et al. 2009; Yoshimoto et al. 2009), eosinophils (Wang et al. 2007), macrophages and DCs (Batista and Harwood 2009; Hume 2008), B cells (Chen and Jensen 2008; Reichardt et al. 2007) and human endothelial cells (Kreisel et al. 2002; Mestas and Hughes 2001). The potential role of these various APCs in CBD has not been fully explored. Two important concepts have begun to emerge from recent studies on the role of APCs in CBD. APCs may play an important role in establishing the initial state of immunologic sensitization and in responses to Be upon re-exposure and Be may directly activate Be-specific T cells in lung granulomas in the absence of a need for dedicated APCs.

Workers come into contact with Be by inhalation of dust and fumes and/or by direct skin contact. Both the skin and the human lung are inhabited by large populations of APCs, principally cells of the mononuclear phagocyte system (Hume 2008). The human lung has a dense network of dendritic cells extending from the nose, through the upper and lower airways into the terminal bronchioles and down to the alveoli, while large numbers of resident alveolar macrophages occupy the alveolar lumen (Holt et al. 1994; Schon-Hegrad et al. 1991). The skin is populated by specialized APCs (Shortman and Liu 2002; Shortman and Naik 2007). Together, these mononuclear phagocytes rapidly remove materials from inhaled air, or substances that overcome the skin barrier. APCs appear to remove Be by endocytic processes (Burgdorf et al. 2007; Dudziak et al. 2007), although there may exist receptor-mediated pathways of Be uptake by APCs (Sawyer et al. 2004b). Antigen processing and presentation is complex, and there are recent helpful reviews of the regulation and assembly of CD1 and major histocompatibility (MHC) class I (MHCI) and class II (MHCII) molecules (Gelin et al. 2009; Wearsch and Cresswell 2008). Endocytic uptake of antigenic substances results in their intracellular breakdown and the generating antigenic fragments that are cycled into molecular complexes with CD1 (lipids and glycolipids), or MHCI and MHCII molecules in association with peptides derived from the breakdown of glycoproteins. These complex molecules, for example MHCII-peptide (MHCIIp) complexes, are then cycled to the surface of the APC where the MHCIIp antigen complexes are presented to TCR. In concert with co-stimulatory molecules, TCR ligation results in the activation of the T cell for proliferation and the production and

release of cytokines. This general ability of APCs to process antigens and present Be-antigen to T cells is still poorly characterized in CBD.

Recent study shows that a mouse macrophage cell line endocytoses and degrades BeO particulates, and releases Be back into culture medium (Day et al. 2005). This suggests the possibility that APCs removal of Be particulates in the lung could release bio-available Be back into the lung microenvironment and thus perpetuate the immune response. Fine particulate Be has been shown to induce immunologic responses after inhalation by mice (Salehi et al. 2009). Characterization of Be dissolution suggests the possibility that depending on conditions within the APC and the size and chemical form of Be, intracellular degradation could be quite variable (Stefaniak et al. 2005). Thus, it is likely that Be release back into the lung microenvironment after processing by APCs may be rapid or prolonged but in both instances it would provide Be for the activation of Be-antigen specific T cells.

The lungs of CBD patients with active disease demonstrate a T cell alveolitis dominated by the presence of Be-specific $CD4^+$ T cells (Amicosante and Fontenot 2006). These T cells are found in the blood and in the bronchoalveolar lavage (BAL) fluid of CBD patients. The CBD BAL cells contain a relatively high frequency, as much as 18% of the total BAL T cells, of Be-specific $CD4^+$ T cells (Fontenot et al. 2002). In the CBD subject's lungs, these $CD4^+$ T cells display a phenotype consistent with their differentiation into T effector memory cells that persist in the lungs for prolonged periods (Fontenot et al. 1999) and demonstrate oligoclonal expansions of their Be-specific TCRs (Fontenot et al. 1999, 2002). Upon exposure to Be these T cells are activated for proliferation and the production of cytokines, including IL-2, TNF-α and IFN-γ (Pott et al. 2005). It is widely believed that these T cells play a central role in the pathogenesis of CBD. It is also widely believed that the ability of APCs to process and present Be-antigen for the activation of these T cells is necessary and sufficient. However, a recent study shows the existence of an alternate pathway resulting in the activation of these T cells. Be-specific CBD BAL $CD4^+$ T cells can be activated independent of the need for co-stimulation by the B7/CD28 pathway normally provided by APCs. The data show that co-stimulation may be provided by the expression HLA-DP and LFA-1 on the surface of Be-specific $CD4^+$ T cells (Chou et al. 2005; Fontenot et al. 2003). Moreover, Be-specific $CD28^+$ $CD4^+$ T cells present in the blood of BeS and CBD subjects become sequestered in the CBD lung where they down-regulate CD28 expression (Fontenot et al. 2003). These Be-specific $CD28^-$ T cells express HLA-DP and the co-stimulatory molecule LFA-1 on their surface. Resting CBD BAL T cells express HLA-DP and LFA-1 co-stimulatory surface molecules. These T cells are able to present Be-antigen to other T cells resulting in T cell activation and proliferation in the absence of APCs (Fontenot et al. 2006a). NMR studies show that Be binds directly to soluble rHLA-DP and that Be-pulsed rHLA-DP molecules are able to block the proliferation and IL-2 secretion induced when Be is presented by APCs (Fontenot et al. 2006b). Thus, the presence of both the HLA-DP MHC class II restricting molecule and the LFA-1 co-stimulatory molecule on the surface of Be-specific $CD4^+$ T cells, when coupled to the direct binding of Be in the HLA-DP peptide binding grove, allows these T cells to present Be-antigen to Be-specific T cells. This T–T cell Be-antigen self-presentation may bypass the need for antigen processing and presentation by APCs. Together, these studies demonstrate the existence of two pathways of Be-antigen presentation for the activation of Be-specific $CD4^+$ T cells, one that can involve APCs and a second pathway that can involve T–T cell self-presentation in CBD. While the ability of APCs to process and present Be-antigen is still poorly understood, we believe that Be-antigen T–T cell self-presentation does not rule out a role for APCs in the activation of Be-specific $CD4^+$ T cells at the site of disease activity in granulomatous inflammation and granulomas in the lungs.

The role of Be-binding molecules, such as ferritin in the movement of Be within the host and host cells was poorly characterized

Our hypothesis, that the function of APCs is important to CBD pathogenesis is supported by a current study using the Be-ferritin adduct (Price and Joshi 1983). Ferritin is a globular protein composed of heavy and light chains. Ferritin is a ubiquitous intracellular iron storage protein and an iron transport

protein found in serum and fluids associated with mucosal surfaces including the respiratory tract. Ferritin heavy chain binds to a variety of cell types via the T cell immunoglobulin mucin domain protein 2 (TIM-2) and by human transferrin receptor 1 protein, both expressed on lymphocytes and macrophages, resulting in its endocytic uptake (Chen et al. 2005; Li et al. 2010). A Be-ferritin adduct was shown to directly interact with BeS BAL and CBD BAL macrophages triggering their apoptosis, while also stimulating CBD BAL mixed cells containing both APCs and T cells, inducing Be-specific CBD BAL proliferation (Sawyer et al. 2004b). The ability of Be-ferritin to directly interact with macrophages suggests the possibility that CBD BAL APCs endocytose and process Be adducts with various molecules, such as ferritin, and are then able to present Be-antigen for the activation and proliferation of Be-specific T cells.

The effects of Be on reactive oxygen species and oxidant lung injury were unknown

In 2000, evidence suggesting that Be might alter oxygen free radicals and induce oxidant injury in CBD was limited to the single study by Comhair et al. (1999), showing increased levels of the antioxidants glutathione and glutathione peroxidase in the CBD BAL fluid. ROS are well known for their ability to mediate host defense against endocytosed microbes but the important role of ROS as signaling molecules in innate and acquired immunity is less well appreciated. Mammalian cells produce ROS in response to stimulation through a variety of receptors involved in the intracellular elimination of ingested microbes. ROS are also produced by a second pathway activated by receptors for platelet derived growth factor, epidermal derived growth factor, TCR, B cell receptor for antigen (Woo et al. 2010) and TNF receptor 1 (Yazdanpanah et al. 2009). Ligation of these receptor systems up-regulates downstream signaling cascades resulting in gene activation, transcription and protein production.

Current studies suggest that Be directly activates the production of ROS and induces oxidant injury in CBD. The ability of Be-stimulation to trigger the production of ROS was first demonstrated in mouse macrophage cell lines and primary mouse BAL resident alveolar macrophages (Sawyer et al. 2005b). Be-stimulated ROS induced macrophage apoptosis by activation of intracellular caspases. Caspase activation and ROS production were inhibited by the catalytic antioxidant porphyrin 5,10,15,20-tetrakis (benzoic acid) porphyrin manganese (III) (MnTABP). MnTBAP has superoxide dismutase and catalase activity and protects cells from hydrogen peroxide and superoxide anion toxicity. Dobis et al. (2008) first demonstrated that Be-stimulated increased levels of intracellular ROS in blood $CD4^+$ T cells from both normal healthy control subjects and CBD subjects. Of some interest, anti-oxidant intracellular thiol levels, total glutathione and cysteine, were significantly reduced in PBMCs from both CBD and BeS subjects as compared to normal healthy controls. In addition, in this study, Be-stimulated a decrease in CBD PBMC intracellular thiol levels and increased Be-specific T cell proliferation. Be-stimulated CBD PBMC T cell proliferation was inhibited by treatment with the thiol antioxidant N-acetylcysteine and MnTBAP. In a cross-over experiment, Be-stimulated oxidative stress in both APCs and autologous responder lymphocytes independently and treatment of either Be-exposed cell class with MnTBAP inhibited Be-induced T cell proliferation. Thus, Be mediates an imbalance in intracellular anti-oxidant thiol levels leading to ROS generation and increased oxidative stress that may enhance the proliferation and clonal expansion of Be-specific $CD4^+$ T cells in CBD. In a more recent study, Dobis et al. (2010) showed that sulfasalazine and its active metabolite mesalamine inhibited Be-stimulated Be-specific $CD4^+$ T cell proliferation in the BeLPT, and inhibited production of the pro-inflammatory cytokines TNF-α and IFN-γ. These drugs are used commonly to treat active inflammatory bowel disease (Crohn's disease) and arthritis and the data from this study suggest the possibility that inflammatory pathways critical to CBD pathogenesis can be inhibited, offering a potential novel approach to treating CBD and currently being studied.

Barna et al. (2002) showed that Be-stimulated CBD BAL cells produce nitric oxide (NO) in response to addition of an NO agonist in culture. This NO production was observed to attenuate Be-stimulated production of IL-18 and IFN-γ. This suggests that NO production may serve to dampen Be-stimulated cytokine production in the lungs of CBD patients and thereby slow or prolong

inflammatory responses that contribute to the progressive nature of CBD. Kokturk et al. (2009) were able to detect haem oxygenase-1 (HO-1) in the induced sputum from CBD patients, an enzyme that plays a role in defense against oxidative stress by reducing oxidation and inflammation. Together, these studies show that Be does indeed induce oxidative stress in CBD.

We did not know how Be-antigen activated $CD4^+$ T cells arise in CBD

Fontenot et al. (2002) showed that $CD4^+$ T effector memory cells were present in the blood although primarily compartmentalized in the lungs of CBD subjects. Beryllium-stimulation up-regulated the expression of Th1-type cytokines by these effector memory T cells. Interestingly, the increased expression of IFN-γ was inhibited by the presence of anti-HAL-DP monoclonal antibody, but not by antibody reagents specific for either HLA-DR or HLA-DQ in those with HLA-DPB1 βGlu69, and by antibodies to HLA-DR in those with HLA-DRB1 βGlu71. The presence of these Be-specific $CD4^+$ T effector memory cells at elevated levels in the blood of CBD patients correlated with a BAL lymphocytic alveolitis present in CBD (Fontenot et al. 2005; Pott et al. 2005). Moreover, the lungs of CBD subjects demonstrated two distinct subsets of Be-specific effector memory T cells. One effector memory T cell subset expresses the co-stimulatory molecule $CD28^+$ while a second lung compartmentalized effector memory T cells were CD28 negative (Fontenot et al. 2003). This suggests that in CBD, there is a transition from a Be-specific T central memory cell in the blood, that depends on $CD28^+$ co-stimulation for activation, to a T effector memory phenotype compartmentalized to the CBD lung that is CD28-independent. This is supported by the recent study showing that CBD BAL Be-specific $CD28^-$ T cells up-regulate LFA-1 co-stimulatory and HLA-DP surface molecules and self-present Be-antigen in the absence of APCs (Chou et al. 2005; Fontenot et al. 2006a). More extensive studies by Fontenot and his coworkers have begun to further characterize and differentiate the markers associated with the functional behavior of Be-specific CBD BAL $CD4^+$ T cells (Mack et al. 2008, 2009; Palmer et al. 2007). Mack et al. (2010) recently showed that there were significantly decreased levels of natural T regulatory T cells (T reg) in the CBD BAL lymphocyte population as compared to BeS BAL lymphocytes. The T reg cells that were present in the BAL from CBD subjects were unable to suppress T cell proliferation when stimulated by anti-CD3 antibody suggesting that the CBD BAL contains deficient levels of dysfunctional T reg that normally serve to modulate adaptive immune responses; this lack of functional T reg cells likely promotes lung inflammation in CBD. These studies demonstrate that significant progress has been made to characterize the T cell subsets in the blood and lungs of CBD patients. Comparable studies of mononuclear phagocyte phenotypes, their origins and functions are still lagging behind these studies.

The role of IL12, IL15 and IL18 and other cytokine mediator molecules had not been evaluated

Neopterin is a pteridine compound that mediates tetrahydrobioptrin synthesis and is produced by mononuclear phagocytes activated by IFN-γ (Hamerlinck 1999). Neopterin levels are increased in the serum of CBD subjects (Harris et al. 1997). Maier et al. (2003a) used an in vitro ELISA to detect increased Be-stimulated neopterin levels produced by PBMCs from CBD subjects in comparison to controls. This neopterin assay was proposed as a useful adjunct to identifying the progression from BeS to CBD, and for the diagnosis of CBD relative to other clinically similar granulomatous lung diseases such as sarcoidosis. Increased neopterin levels indicate active inflammatory and/or cell-mediated immune responses that involve mononuclear phagocytes and this assay could avoid the variability associated with measuring Be-induced cytokine levels and T cell proliferation in CBD, although confirmatory data are needed. In addition, measuring Be's ability to stimulate IFN-γ production via ELISPOT has been hypothesized as a diagnostic test to determine Be-stimulated cytokine production in conjunction with T cell proliferation. In this study, CBD subjects were shown to produce more Be-stimulated IFN-γ spot-forming units (SFU) than BeS subjects, while normals produced none (Pott et al. 2005). Whether

this test or others will provide greater sensitivity or specificity in the diagnosis of CBD and/or BeS or will function as an adjunct to the diagnosis of this disorder is still being explored.

Barna et al. (2002) observed that Be-stimulated CBD BAL cells, in addition to IFN-γ production, also produced IL-18 but did not up-regulate production of IL-12. Confirming the observation that anti-HLA-DP monoclonal antibody blocks Be-stimulated IFN-γ production by CBD PBMCs (Fontenot et al. 2002). Amicosante et al. (2002)found that Be-stimulated CBD PBMCs proliferated and produced IFN-γ, TNF-α and RANTES, but not GM-CSF, IL-4, IL-6, IL-8, IL-10 and IL-12, and anti-HLA-DP monoclonal antibody treatment blocked Be-stimulated T cell proliferation and IFN-γ production but it did not block TNF-α production. This observation is contrasted by another study showing that HLA-DP monoclonal antibody, but not HLA-DR or HLA-DQ monoclonal antibodies, blocked Be-stimulated CBD blood and BAL $CD4^+$ T cell TNF-α mRNA and protein production (Sawyer et al. 2004a). The reasons for these disparate observations relative to Be-stimulated TNF-α production are unknown, but could reflect different experimental conditions used in these two studies or perhaps differences among the CBD subjects enrolled in the different studies.

Chaudhary et al. (2004) have developed a model using normal human PBMCs incubated in the presence of autologous donor DCs to study the ability of Be to alter cytokine gene expression and production in cells not previously exposed to Be. Confirming previous studies (Maier et al. 2001b; Tinkle and Newman 1997; Tinkle et al. 1997, 1999), they observed that normal non-Be-exposed PBMC/DCs failed to up-regulate Be-stimulated production of IL-2, IFN-γ and TNF-α but interestingly did up-regulate IL-6 and IL-10 production suggesting that Be may in fact directly alter cytokine gene expression in normal cells not previously exposed to Be. Be-stimulated PBMC/DCs also up-regulated protein and mRNA expression for the chemokines MIP-1α and MIP-1β and antibodies to these chemokines attenuated Be-stimulated in vitro migration of PBMCs/DCs (Hong-Geller et al. 2006). These studies suggest that the effects of Be are not limited to only immune cells that were previously exposed to Be but that cells present in normal healthy Be workers may also be potential targets for inflammatory alterations upon Be exposure in the workplace. A study recently showed that Be treatment differentially alters intracellular signaling pathways in human PBMCs that are also exposed to bacterial lipopolysaccharide (LPS), a potent inducer of cytokine gene expression and cytokine protein production. It was observed that Be treatment inhibited LPS-stimulated IL-10 production but actually enhanced the production of the pro-inflammatory cytokine IL-1β (Silva et al. 2009). The data suggest that Be-exposure might alter host responses to bacterial infection in otherwise healthy individuals by altering intracellular signaling pathways.

An unresolved question, raised by in vitro experiments using BAL cells from CBD subjects centers on the observation that Be-stimulation induces the production of large amounts of IFN-γ (Tinkle et al. 1997) and TNF-α (Tinkle and Newman 1997) relative to those amounts normally observed when control stimuli such as SEB and LPS are used for comparisons. This observation raises the question as to whether these large amounts of Be-stimulated pro-inflammatory cytokine are physiologically relevant, or an artifact of the culture system used. Sawyer et al. (2007) determined that Be-stimulated CBD BAL $CD4^+$ T cell TNF-α mRNA and protein production is transcription dependent involving Be-stimulated up-regulation of the transcription factors NF-κB and AP-1. It seems likely then that the production of high levels of TNF-α by CBD BAL cells is not due to alternate TNF-α synthesis mechanisms such as the release of pre-formed and stored TNF-α protein or to activation-induced splicing of pre-formed pre-mRNA into mature mRNA transcripts for rapid protein synthesis (transcription-independent). Be-stimulated TNF-α pre-mRNA production and splicing into mature mRNA was normal in CBD BAL cells as compared to mRNA synthesis induced by LPS-stimulation. These data suggest that Be-stimulated TNF-α transcription and translation in CBD BAL cells occurs normally, and that a fundamental defect in the protein synthesis machinery does not explain the abnormally high levels of cytokine production. It is probable that the in vitro culture conditions, commonly used by many researchers, promote these abnormally high cytokine levels. It is possible that this is a culture artifact, given the recent observation that CBD BAL $CD4^+$ T cells are able to self-present Be-antigen and up-regulate cytokine production in vitro (Fontenot et al. 2006a). It remains a possibility

that within the CBD granuloma these Be-specific T cells could release large amounts of pro-inflammatory cytokines in the absence of APCs and in the presence of only Be_2^+, to promote the chronic inflammation and progression to fibrosis in CBD; this possibility should be explored in depth.

Be stimulated macrophage apoptosis was new, and poorly understood

Be stimulates apoptosis in mouse and human macrophage cell lines and in CBD and BeS BAL macrophages (Kittle et al. 2002; Sawyer et al. 2000). Be-stimulated macrophage apoptosis depends on the activation of intracellular caspases, is independent of Be-stimulated TNF-α production and dependent on Be-stimulated up-regulation of ROS (Sawyer et al. 2000, 2005b, 2007; Dobis et al. 2008). Apoptosis of CBD BAL macrophages occurs in response to stimulation with both Be salts and the Be-ferritin adduct, but not control aluminum salt. Of interest, CBD BAL lymphocytes do not appear to undergo apoptosis in response to these stimuli, only the macrophages (Sawyer et al. 2004b). Furthermore, Be-ferritin and Be salt up-regulate the expression of CD95 (Fas) on the surface of CBD BAL macrophages and induced the intracellular activation of caspase 3, caspase 8 and caspase 9, while CD95 surface expression and caspase activation were not observed in stimulated CBD BAL lymphocytes. Intracellular caspase activation in Be-stimulated macrophage cell lines was inhibited by the catalytic anti-oxidant MnTBAP showing that Be-stimulated ROS likely played a role in caspase activation and apoptosis (Sawyer et al. 2005b). Be-specific CBD BAL $CD4^+$ T cells may undergo cell death by other mechanisms unrelated to the Be-stimulated ROS and caspase activation, but mechanisms that eventually result in apoptosis. Palmer et al. (2007) showed that CBD BAL $CD4^+$ T cells had increased levels of CD57 surface expression, a marker associated with T cell senescence. CD57 expression correlated with CBD BAL lymphocytic alveolitis and with a reduced ability of these cells to proliferate after Be-stimulation. Be-stimulation does not trigger the apoptosis of all CBD BAL macrophages, rather a significant number of cells remain unperturbed (Sawyer et al. 2004b). It was also observed that these unaffected and fully functional CBD BAL macrophages were able to ingest the "corpses" of their apoptotic brothers. This observation raises the possibility that within the CBD granuloma there are subsets of APCs that are either sensitive to Be-stimulation or resistant to Be-stimulation. Be sensitive APCs undergo apoptosis in a manner allowing the fully functional and resistant APCs to endocytose these apoptotic cells. The intracellular dissolution of these apoptotic bodies containing Be, could then release Be back into the lung microenvironment in a form that would promote self-presentation to Be-specific $CD4^+$ T cells. That is, enhanced apoptosis may allow more effective Ag-presentation and thus progression to CBD. This possibility is supported by the demonstration of persistent Be inside CBD granulomas even decades after the initial Be-exposure has ended (Sawyer et al. 2005a). An interesting new study suggests that Be-specific T cells may play a role in Be-induced macrophage apoptosis. Palmer et al. (2008) demonstrated the expression of programmed death-1 (PD-1) on Be-specific $CD4^+$ T cells from the blood and BAL of BeS and CBD subjects. PD-1 ligands (PD-L1 and PD-L2) are expressed on BAL $CD14^+$ cells, putative lung APCs. PD-1 is a negative co-stimulatory molecule, and PD-1 inhibits an immune response by inducing apoptosis of cells expressing the PD-1 ligands. Further study will be needed to determine if Be-specific $CD4^+$ T cells expressing PD-1 mediate the apoptosis of CBD BAL macrophages that express PD-1 ligands.

Role for Be persistence in the lung was unknown

Butnor et al. (2003) demonstrated Be particles inside the cytoplasm of multinucleate giant cells in CBD lung granulomas. The highly sensitive method of secondary ion mass spectroscopy was employed to show the retention of Be inside CBD lung granulomas (Sawyer et al. 2005a). Be was detected in the lungs of CBD patients who had ceased exposure to Be almost a decade previously suggesting that Be persists in the lungs of Be-exposed individuals for very long time periods.

- *A new model of CBD pathogenesis*: Based on these new data, we can now hypothesize a

potential mechanism that would sustain chronic inflammation and granuloma formation in CBD. Persistent Be, that is never actually eliminated from the CBD lung granuloma, continues to be endocytosed by granuloma macrophages. These macrophages undergo ROS- and caspase-dependent apoptosis. The apoptotic macrophages are endocytosed by unperturbed granuloma macrophages. Dissolution of these Be containing apoptotic bodies releases Be in a manner that promotes self-presentation to Be-specific $CD4^+$ T cells. These activated T cells proliferate and release pro-inflammatory cytokines that maintain chronic inflammation by promoting the entry (Hong-Geller 2009) of mononuclear phagocytes and Be-specific $CD4^+$ T effector memory cells from the blood into the granuloma. This vicious cycle continues within the lung granulomas of Be workers for decades resulting in increased lung inflammation, impaired lung function and progression to fibrosis and may ultimately be due to the inability of both innate and acquired immune defense systems to eliminate Be from the lung. This model helps focus attention on the interplay between persistent Be and cells of the innate and acquired immune systems with the lung and the CBD granuloma, but it does not address the important contribution of the host's genetic background to these processes.

- This model is also consistent with a possible role for APCs in CBD granuloma formation. Be-exposure (or worker re-exposure) likely results in the uptake of Be by airway DCs, skin DCs and by resident alveolar macrophages. Dissolution of intracellular Be particles generates the expression of surface HLA-DP-Be-antigen complexes in association with surface co-stimulatory molecules ($B7^+$). These APCs traffic to regional lymph nodes where they are able to activate naïve T cells, in a manner dependent on HLA-DP-Be-antigen expression and B7/CD28 co-stimulation. Naïve T cells activated in this manner rearrange their TCRs, proliferate and expand their TCRs in a Be-specific oligoclonal manner becoming Be-specific T effector memory cells. These $CD28^+$ T effector memory cells enter the blood stream of BeS subjects and traffic to the lungs where, along with lung APCs they form interstitial mononuclear cell infiltrates. Here they down-regulate the expression of CD28 and up-regulate HLA-DP and LFA-1 expression. These $CD28^-$ Be-specific $CD4^+$ T cells in the granuloma are now fully able to self-present Be-antigen within the granuloma and as modeled above.

Genetic components were just being identified, but were limited and worker population based studies were needed

Several studies on the genetic background have now been performed on CBD worker based populations. These studies confirm previous studies showing the persistence of Be exposure and disease in the Be workplace and confirm the association between HLA-DP-βGlu69 and CBD. A study of current Be workers in a ceramics plant compared the prevalence of BeS and CBD determined in 1992 to that determined in 1998 (Henneberger et al. 2001). Despite efforts to reduce Be exposures between 1992 and 1998, there was no decline in either BeS or CBD. Rouleau et al. (2005) showed that very fine particles of Be metal are found in workplace dust and because the threshold amount of Be needed to induce sensitization and disease is still unknown it may be impractical to reduce workplace Be below this putative threshold. Rossman et al. (2002) compared the HLA genotypes in Be-exposed workers with no evidence of BeS or CBD as compared to workers with Bes or CBD. HLA-DP-βGlu69 was the most important marker associated with BeS and CBD but HLA-DQ and HLA-DR genotypes when associated with HLA-DP-βGlu69 were also associated with CBD. The study showed that specific HLA amino acids associated with HLA-DP contributed to disease progression in CBD. Maier et al. (2003b) in a case control study of CBD, BeS and Be exposed workers without sensitization or disease also demonstrated a high frequency of HLA-DR-βGlu69 in BeS and CBD subjects as compared to the Be exposed subjects without disease. They found that HLA-DR and HLA-DQ were associated with CBD in the presence of HLA-DP-βGlu69, confirming the study by Rossman et al. (2002).

New studies have examined genotypes, other than HLA genotypes, associated with CBD. An early report using a limited number of subjects suggested

an association between TNF-α promoter region single nucleotide polymorphisms (SNPs), high levels of Be-stimulated TNF-α protein production and disease severity in CBD (Maier et al. 2001a). McCanlies et al. (2007) determined the TNF-α promoter SNPs in and expanded study of 886 Be workers and found no association of TNF-α SNPs in workers who were Be exposed with no sensitization or disease and in workers with BeS or CBD. Sato et al. (2007a) confirmed this finding in a similar cohort of Be workers but also showed that there was a significant association between specific TNF-α promoter SNPs and the levels of Be-stimulated BAL cells TNF-α as previously reported (Maier et al. 2001a). How SNPs in the TNF-α promoter region might result in higher levels of Be-stimulated TNF-α protein production by CBD BAL cells is unknown, but could explain in part the consistent observation of high TNF-α in these cultures reported previously (Tinkle and Newman 1997).

Bekris et al. (2006) studied the presence of SNPs in the gene encoding glutamate cysteine ligase, the rate limiting enzyme in glutathione synthesis, and found that that one SNP (GCLM-588 C/C) was associated with CBD susceptibility but there were no associations found in BeS study subjects suggesting that modulation of glutathione synthesis could play a role in CBD pathogenesis. This conclusion is supported by the studies discussed above, showing an imbalance in the regulation of oxidative stress in CBD BAL cells (Dobis et al. 2008). Genetic variation in Butyrophilin-like 2 (BTNL2) is believed to predispose patients to sarcoidosis independent of MHC class II alleles (Rybicki et al. 2005). BTNL2 is a member of the immunoglobulin gene superfamily of proteins structurally similar to the B7 co-stimulatory molecule present on APCs that recognizes CD28 on antigen-specific $CD4^+$ T cells. Ligation of the B7/CD28 co-stimulatory pair plus ligation of MHCII-antigen-TCR complexes is necessary for the full activation and differentiation of naïve $CD4^+$ T cells into functional subsets (Rybicki et al. 2005). Sato et al. (2007b) were unable to confirm a similar association of BTNL2 in CBD to that found in sarcoidosis despite the fact that the granulomatous lung inflammation in these two disorders is virtually indistinguishable. TGF-β1 is an important regulatory cytokine that plays a role in immunomodulation and in the pathogenesis of many diseases including sarcoidosis (Limper et al. 1994). Jonth et al. (2007) found that specific TGF-β genotypes are associated with more severe disease in both sarcoidosis and CBD and two of the loci identified are associated with lower production of TGF-β protein suggesting the possibility that decreased TGF-β mediated down-regulation of inflammation and the immune response in CBD could promote disease progression. Specifically, these TGF-β genotypes may play a role in the dysfunctional T reg cells observed in CBD (Mack et al. 2010), as a possible explanation for its functional effects, although more study is needed to determine if this occurs in the lung. Determining these potential links and others may provide potential targets for therapy to slow the progression of CBD.

It is well known that animal models of CBD recapitulate human lung disease poorly. Thus, the relevance of data obtained from animal models of CBD may be limited. Recently, Gordon and his co-workers (Tarantino-Hutchison et al. 2009) attempted to overcome this limitation by creating HLA-DP transgenic mice. It is still early in the development of this mouse model of CBD and at present, caution is warranted in the interpretation of preliminary data obtained thus far, although the early studies appear to hold some promise.

There were only few and only preliminary studies of the role of Be in pulmonary fibrosis

Pulmonary fibrosis remains a poorly studied area in CBD. Be directly affects not only cells of the innate and acquired immune systems but directly alters the function of many other cell classes. Coates et al. (2007) and Gorjala and Gary (2010) reported that Be-stimulation induced proliferation arrest and premature senescence in human fibroblasts and cancer cells. How senescent CBD fibroblasts interact with other cell classes in the lung, especially with mononuclear phagocytes including lung DCs is unknown. It may be that Be-induced fibroblast senescence makes these cell susceptible to corticosteroids explaining the ability of corticosteroid therapy to stop the progression of pulmonary fibrosis in CBD (Marchand-Adam et al. 2008). Simonian et al. (2006) in a mouse model of pulmonary fibrosis showed that in mice deficient in γ–δ T cells, repeated lung exposure to killed bacteria dramatically increased lung $CD4^+$ T cells, collagen

deposition and increasing pulmonary fibrosis suggesting that this poorly understood subset of T cells may serve to down regulate T cell accumulation and fibrosis in the lungs. The role of γ–δ T cells in CBD is unstudied. In a second model of hypersensitivity pneumonitis characterized by $CD4^+$ T cells infiltration, collagen deposition and pulmonary fibrosis, Simonian et al. (2009) demonstrated that TH17 polarized $CD4^+$ T cells producing IL-17A were critical to the disease progression to pulmonary fibrosis. The role of the TH17 $CD4^+$ T cell subset in CBD is unknown. Together, these two studies suggest the possibility that in CBD disease progression from lung granulomatous inflammation to pulmonary fibrosis might involve the function of γ–δ T cells and/or polarized TH17 $CD4^+$ T cells. Marchal-Somme et al. (2007) showed that immature DCs accumulate in areas of epithelial cell hyperplasia associated with fibroblast foci in human fibrotic interstitial lung disease. Taken with the studies above, we speculate on the possibility that emigrant inflammatory DCs subsets might play an important role that affects the levels of γ–δ T cells and drives the polarization of $CD4^+$ T cells into the TH17 phenotype promoting progression to fibrosis in CBD.

Summary

Progress has been made to fill in some of the gaps that we felt were important to understanding CBD pathogenesis (Sawyer et al. 2002). CBD is one of the few immune-mediated lung disorders for which the causative antigen, Be, is known. Despite this, there remain many unanswered questions and many unexplored and fruitful areas of investigation. The studies reviewed above suggest a need for studies using Be-stimulated cell systems as hypothesis generating models to identify key pathways for the direct effects of Be on various specific cell classes. These types of studies not only avoid the need for cells and tissues obtained directly from human patients with beryllium disease but they could serve to identify novel pathways and directions that can be brought back to studies using cells isolated from human subjects. There is a clear need for expanded studies using the HLA-DP-βGlu69 transgenic (or similar) mouse models, and we hope that this model system will become generally available to investigators. Interesting new data suggest a possible role for DCs and novel T cell subsets, such as γ–δ T cells and TH17 T cells, in CBD disease progression. Based on the current literature, we propose a new model to explain how persistent Be perpetuates granulomatous inflammation in CBD, but obvious gaps remain. We still do not know how Be traffics within APCs or if and how Be-antigen is processed and presented in association with surface MHCII complexes and whether other accessory innate immune pathways are involved in this process. The recent characterization of the HLA-DP-βGlu69 crystal (Dai et al. 2010) and the demonstration of Be-antigen self-presentation (Fontenot et al. 2006a) have moved this field of research forward, but the role of phenomenon such as Be-induced macrophage apoptosis in CBD pathogenesis remain speculative, while studies on pulmonary fibrosis in CBD have not been performed. It is our hope that young investigators, new to the field of CBD research, will benefit from this updated review of the progress in this area and identification of the as yet remaining gaps that need to be explored.

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