ABSTRACT

BOST, PHILLIP CHAPMAN. Bis(maltolato)oxovanadium(IV) Activation of STAT-1 Signaling in Fibroblasts as a Potential Therapy for Pulmonary Fibrosis. (Under the direction of James C. Bonner).

The purpose of this research was to investigate the potential of a therapeutic vanadium compound in the modification and regulation of common repair mechanisms associated with pulmonary fibrosis. We hypothesized that bis(maltolato)oxovanadium(IV) (BMOV) has therapeutic potential for the treatment of pulmonary fibrosis. BMOV was formulated for the treatment of diabetes and has insulin mimetic properties, but whether it has crossover therapeutic value for the treatment of pulmonary fibrosis is currently unknown. Previous work with toxic forms of vanadium, demonstrated that despite their toxicity, these compounds exhibited interferon (IFN)-like properties which aids in regulating repair processes in fibrotic lung wounds. We sought to explore whether BMOV could represent a new and effective therapy for the treatment of pulmonary fibrosis by examining its impact on key fibrogenic pathways in lung fibroblasts *in vitro*.

To test our hypothesis, we first demonstrated in chapter 1 that when used to treat isolated pulmonary fibroblasts, BMOV behaves in a way similar to that of IFN-γ as defined by the phosphorylation of STAT-1 and upregulated expression of CXCL10. This ability to over-express CXCL10 was lost when lung fibroblasts from STAT-$1^{-/-}$ mice were treated with BMOV. These data indicate that BMOV initiates production of CXCL10 through a STAT-1-dependent mechanism and suggest that BMOV may have therapeutic effects when used to treat interstitial lung fibrosis.

In chapter 2, we refined our *in vitro* model to better reflect the signaling events that take place in a lung undergoing fibrotic remodeling. To accomplish this we co-treated lung fibroblasts with both BMOV and interleukin-13 (IL-13) which is a ligand common to both asthmatic and fibrotic lungs. IL-13 acts through STAT-6 dependent signaling to mediate the production of growth factors that drive fibrosis. We found that this co-treatment significantly reduced the BMOV-induced phosphorylation of STAT-1 and subsequent production of CXCL10. Contrarily, BMOV co-treatment also reduced phosphorylation of IL-13-induced STAT-6 signaling compared to treatments of IL-13 alone. Using mouse lung fibroblasts, we attempted to determine if this co-regulation of STAT pathways occurred at or downstream of the respective phosphorylated STAT signals. To accomplish this line of inquiry we utilized fibroblasts cultured from wild type mice, STAT-$1^{-/-}$ mice, and STAT-$6^{-/-}$ mice. We found that in the absence of STAT-1, BMOV lost its ability to mitigate STAT-6 phosphorylation indicating that STAT-1 plays a fundamental role in that down-regulation. In addition, using STAT-$6^{-/-}$ fibroblasts we determined that IL-13 was not able to diminish STAT-1 phosphorylation or CXCL10 production.

We conclude that the therapeutic potential of BMOV regarding treatment of fibrosis may be blunted if the disease has already taken root. Further studies used to investigate BMOV treatment of fibrosis should utilize whole mouse models, both knock-out and wild type. Understanding the cross talk between opposing STAT-signaling pathways will provide important insights into the fibrotic process. These studies suggest that BMOV may have therapeutic potential by virtue of its specificity and prolonged activation of STAT-1 phosphorylation.

Bis(maltolato)oxovanadium(IV) Activation of STAT-1 Signaling in Fibroblasts as a Potential Therapy for Pulmonary Fibrosis

by
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DEDICATION

I would like to dedicate this work to my family:

To my wife Erin, the strongest woman in the world.

To my mother, for constantly reaffirming my faith in humanity.

To my father, for his prescient advice and constant stream of wisdom.

To my big sister Catherine, to whom I will always look up.

To my little brother Kevin, who makes me smile *this big* every time I think of him.

You have each been displaced by this process in different and difficult ways. Thank you so much for being patient with me.

BIOGRAPHY

On November 12th, 1982, Phillip Chapman Bost was born to proud, exhausted parents David and Pattie. Phillip grew up in Greenville, SC in the best neighborhood a kid could want – winding creeks, tunnels, trails, an older sister to show him the ropes, and the freedom to explore. About the time puberty came around (quite possibly to distract him from the angst welling up within), Phillip was enrolled in a scientific summer camp at Furman University. This exposure to gyroscopes, snakes, and mild explosives sparked a scientific curiosity that still burns hot to this day. Biology, chemistry, computer, and logic courses in high school shortly followed, leading him to study biology and chemistry at Mars Hill College. It was there, nestled in the chilly mountains of western North Carolina, where Phillip met his future wife. They both ran cross-country, and despite his abysmally slow times, Erin thought he was alright. Meanwhile, Phillip found himself mentored by a tight-knit group of scholars, including his first real scientific mentor, Dr. William White. Dr. White pushed him harder and further than he thought he could go. During summers and weekends, Phillip was first exposed to scientific lab work at a small water toxicology lab which utilized the indicator species *Pimephales promelas* and *Ceriodaphnia dubia*. Immediately following graduation, Phillip haphazardly moved to Raleigh, NC, sure he would find scientific work immediately. When that didn't happen, he sat around for a while until stumbling luckily into yet more work in toxicology. Using GC/MS analysis, he worked in a lab that tested for the presence of illicit drugs in urine samples. When he finally grew tired of the smell of other peoples' urine, Phillip decided to pursue graduate school in the discipline that had thankfully

employed him for so many years. During his time in the Toxicology department at North Carolina State University, he scraped together some time to be married on the hottest day of the year. After a nap and a quick shower, the couple got back to work. Phillip and Erin currently live in Raleigh with a very strange puppy, Rayme, and the one-eyed cat known as Bok-Bok, who is, not surprisingly, even stranger.

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INTRODUCTION

Pulmonary fibrosis

Pulmonary fibrosis is a debilitating form of chronic interstitial pneumonia that is difficult to diagnose and treat (ATS/ERS, 1999). Diagnosis typically requires surgical lung biopsy and subsequent identification of a histological pattern known as usual interstitial pneumonia (Katzenstein *et al*., 2008). Treatment options are severely limited and the typical mortality rates range from between 3.2 and 5 years after the initial diagnosis (Panos *et al*., 1990; Moeller *et al*., 2009). The physical characteristics of pulmonary fibrosis include, but are not limited to, decreased overall function of the lung, hardening and scarring of lung tissues, and extensive tissue remodeling (Crystal *et al*., 1976). Most chronic fibrotic lung diseases are the ultimate result of a persistent irritant that disrupts an appropriate repair process. Exposure to exogenous particles implicated in pulmonary fibrosis often occurs in occupational settings (Glazer *et al*., 2004). These irritants can subsequently induce the production of a litany of growth factors, cytokines, and reactive oxygen species that exacerbate the fibrotic injury (Bonner, 2007). During appropriate wound healing, tightly regulated re-epithelialization of the injured site coupled with appropriate collagen deposition by myofibroblasts is followed by apoptosis of the myofibroblasts. When the myofibroblasts fail to undergo apoptosis, excessive extracellular matrix can be deposited, ultimately leading to fibrosis (Tomasek *et al*., 2002).

The precise disease etiology has not yet been elucidated partly due to the fact that a wide array of stimuli that can induce pulmonary fibrosis including, particulate inhalation, radiation, and even some forms of chemotherapy (Khalil *et al*., 2007; Abratt *et al*., 2004;

Daba *et al*., 2004). Another possible reason that the disease etiology has not been fully delineated might be due to inherent flaws in multiple signaling pathways involved in wound repair that ultimately lead to an aberrant healing process (Maher *et al*., 2007). It is also possible that fibrosis is not caused by a single gene, but rather due to multiple genes or gene defects acting in concert.

Once diagnosed with interstitial lung fibrosis, patients are limited to very few treatment options. Historically, corticosteroids and other anti-inflammatory treatments have been the primary treatment despite a lack of proven efficacy (Kim *et al*., 2008). Of the current options available, lung transplantation has the best record for disease outcome (Shah *et al*., 2005) and idiopathic pulmonary fibrosis (IPF) patients currently account for more than 25% of all lung transplantations (Davis *et al*., 2007). Despite this fact, those individuals with IPF assume the most post-transplantation risk compared to transplantation candidates with different diseases (Mason *et al*., 2007). Until the root causes of this disease outcome are elucidated and exploited, victims of pulmonary fibrosis will be limited to proven ineffective treatments, risky invasive surgery, or taking their chances with clinical trials.

The pulmonary fibroblast as a central effector cell in fibrosis

Compared to the composition of a healthy lung, fibrotic lesions have a distinct cellular makeup. This is partly due to typical healing mechanisms that follow injury, but because of dysregulated wound healing processes, fibroblasts can assume an insidious role rather than an efficacious one. A developing fibrotic lesion exhibits recruitment of myofibroblasts, the primary effector cell in fibrogenesis (Scotton *et al*., 2007). These cells

are responsible for the rampant deposition of extracellular matrix (ECM) and can develop from multiple sources. One source is the recruitment of resident fibroblasts already present in the lung. Another source is the recruitment of circulating progenitor mesenchymal or epithelial cells (Barth *et al*., 2007; Gomperts *et al*., 2006). The third source of myofibroblasts comes from a phenomenon known as epithelial-mesenchymal-transition (EMT) in which resident epithelial cells undergo a transformation to phenotypically become myofibroblasts (Willis *et al*., 2005).

Myofibroblasts are contractile and similar to smooth muscle cells in that they contain α-smooth muscle actin (SMA). Myofibroblasts have a tendency to migrate to active regions of fibrosis, such as injury sites and fibroblastic foci. The fibroblastic foci are made up of an interconnected three-dimensional nexus of fibroblasts which have converged from multiple sources. During this migration to the foci, the myofibroblasts deposit collagen in the presence of transforming growth factor (TGF)- β1 (Fan *et al*., 1999). This collagen is deposited in an aligned, coordinated manner that assists with wound contraction and resolution (Nedelec *et al*., 2000).

In a normal repair process the recruitment and migratory behavior of myofibroblasts will eventually cease which, in effect, causes the fibroblastic foci to diminish. The lung then returns to normal function with minimal scarring. In the absence or mis-timing of normal apoptosis-inducing signals however, the foci persist and deposit more ECM than is necessary for repair, thus creating a separate, fibrotic wound (Wynn, 2008). Indeed, IPF patients with the greatest number of fibroblastic foci in their histological samples possess the worst prognoses for disease outcome and survival (King *et al*., 2001).

IL-13 as a mediator for pulmonary fibrosis

Interleukin-13 (IL-13) is a major mediator of pulmonary fibrosis and asthma (Wills-Karp *et al*., 1998; Kaviratne *et al*., 2004; Zhu *et al*., 1999). IL-13 levels are increased in both asthmatic and fibrotic lungs relative to those of healthy patients (Hancock *et al*., 1998). IL-13 signals principally through signal transducer and activator of transcription (STAT)-6 to increase pro-fibrogenic growth factors such as platelet-derived growth factor (PDGF) and TGF-β1 (Lee *et al*., 2001; Ingram *et al*., 2003; Fichtner-Feigl *et al*., 2006). STAT-6-deficient mice are protected against lung remodeling that occurs during allergic lung inflammation. These mice lack all aspects of lung remodeling (e.g., airway fibrosis, increased airway smooth muscle mass, mucous cell hyperplasia) that occurs during the pathogenesis of asthma (Kuperman *et al*., 1998).

The proliferation and persistence of myofibroblasts at the site of injury is a key feature of fibrotic lungs. Polypeptide growth factors act to exacerbate myofibroblast proliferation at the site of injury (Bonner *et al*., 1991). This enhanced propagation of myofibroblasts is enabled by an excess of PDGF. Tyrosine kinase inhibitors that act on PDGF receptors have been shown to decrease pulmonary fibrosis in rats by reducing the downstream mitogenic effect that PDGF has on cells of mesenchymal origin such as myofibroblasts (Rice *et al*., 1999; Bonner, 2004). TGF-β1 is similarly overly expressed in the fibrotic lung and can increase the number of myofibroblasts by way of EMT (Willis *et al*., 2007). TGF-β can also stimulate the myofibroblasts to express and secrete an excessive amount of collagen that comprises the fibrotic scar tissue (Gharaee-Kermani *et al*., 2009).

The anti-fibrotic action of interferons and interferon-inducible chemokines

STATs are phosphorylated by the same class of intracellular non-receptor tyrosine kinases known as Janus kinase (JAK) proteins (Levy *et al*., 2002). Once phosphorylated by their respective JAK protein, STATs typically homo- or hetero-dimerize through reciprocal Src homology 2 (SH2) interactions and translocate to the nucleus where they bind to DNA and activate specific target genes (Yang *et al*., 2008).

While the seven proteins that make up the STAT family of transcription factors are nearly identical structurally, they remain functionally distinct. Just as STAT-6 induces expression of pro-fibrogenic genes such as growth factors, STAT-1 induces anti-fibrogenic proteins that function to resolve fibrosis and limit the fibrotic response. STAT-1 is activated by interferons (IFNs) which are cytokines that mediate growth arrest and apoptosis. Activated STAT-3 and STAT-5, however, promote cell survival and proliferation, particularly in cancer cells (Thomas *et al*., 2004; Chin *et al*., 1997; Yu *et al*., 2004). Studies have shown that STAT-$1^{-/-}$ mice lack all biological responses to IFNs, which underscores the central role of STAT-1 in IFN signal transduction (Akira, 1999). In addition, STAT-$1^{-/-}$ mice are more susceptible to pulmonary fibrosis compared to wild type mice (Walters *et al*., 2005).

Among the target genes for STAT-1 is the chemokine (C-X-C motif) ligand 10 (CXCL10). CXCL10 has been shown to attenuate the effects of bleomycin induced pulmonary fibrosis by interfering with the neovascularization that drives the formation of the developing fibroblastic foci (Keane *et al*., 1999). In addition, the receptor for the CXCL10

ligand, CXCR3, plays a key role in fibrosis as well. CXCR3-deficient mice suffer greater mortality than that of wild type mice when pulmonary fibrosis is induced (Jiang *et al*., 2004). Angiogenesis is a critical component to the development of pulmonary fibrosis which can be brought on by the presence of potent angiogenic proteins such as the vascular endothelial growth factor (VEGF). Even when VEGF is present, however, the presence of CXCL10 and subsequent activation of CXCR3 is enough to block the development of new blood vessels (Bodnar *et al*., 2009). IFNs initiate this antifibrotic signaling axis by transcriptionally activating STAT-1 in fibroblasts, which then increase their production of CXCL10 (Bromberg *et al*., 1996).

The dichotomy of vanadium analogs: toxicity versus therapy

Vanadium is a bioactive metal that is known to be both a toxic pollutant and, more recently, a potential therapeutic. Vanadium pentoxide (V_2O_5) is an industrial byproduct found in fuel oil exhaust and is also used as a catalyst for the manufacture of sulfuric acid. During fuel combustion and use as a catalyst, workers are regularly exposed to V_2O_5 via inhalation (Nemery, 1990). It has been shown that V_2O_5 is carcinogenic in rodents and can also cause DNA damage in humans, making it a potential carcinogen in humans as well (Ress *et al*., 2003; Erlich *et al*., 2008).

We have previously demonstrated that, when inhaled by both rats and mice, V_2O_5 can cause fibrotic lesions and pulmonary fibrosis that partially resolve, possibly due to the activation of the STAT-1 signaling axis (Antao-Menezes *et al*., 2008). This reveals the dualistic nature of vanadium as both a potentially harmful compound and as a potential

therapeutic depending on the context and duration of exposure. The scientific literature regarding vanadium oxides as they relate to lungs has typically only reflected on their role as an occupational hazard and pollutant that can cause lung disease. There is an entirely separate body of literature that focuses on the therapeutic potential vanadium analogs have for treating diabetes.

One of these vanadium analogs that is being assessed for the treatment of diabetes is bis(maltolato)oxovanadium(IV) (BMOV). Vanadium in drinking water has been known to lower elevated blood glucose for over twenty years, but the toxicity of available vanadium analogs have limited the application of this finding (Heyliger *et al*., 1985; Ramanadham *et al*., 1989). BMOV was formulated for the purpose of lowering toxicity, increasing potency, and improving gastrointestinal absorption (McNeill *et al*., 1992). Though not its designed purpose, BMOV may retain some of the antifibrotic behavior that is featured after V_2O_5; principally the activation of STAT-1 signaling to promote fibroblast growth arrest and apoptosis to favor resolution of the fibrogenic response.

BMOV may also promote unanticipated fibrogenic activity that could limit or prohibit its use as a potential therapy for diabetes. One characteristic that BMOV shares with other vanadium based compounds that could possibly pose problems is its tendency to inhibit protein-tyrosine phosphatase proteins (Swarup *et al*., 1982; Burke *et al*., 1998; Krejsa *et al*., 1998). Since overactive tyrosine kinases are implicated in many cancers, prolonging their phosphorylation by introducing a non-specific phosphatase inhibitor, such as BMOV, could be dangerous (Fujiki *et al*., 2009). An additional problem that may be encountered is the tendency for vanadium compounds to induce VEGF production (Ingram *et al*., 2007). This

could also serve to undermine BMOV's potential as a therapeutic treatment for fibrosis or diabetes, or worse, promote growth of pre-existing cancers which tend to thrive in environments where neovascularization might be enhanced.

The central hypothesis of this thesis is that BMOV activates STAT-1 to reduce pro-fibrogenic pathways in fibroblasts by increasing levels of the protective interferon-inducible chemokine CXCL10, and further that BMOV-activated STAT-1 counteracts the pro-fibrogenic activity of STAT-6 that is activated by IL-13. This hypothesis was tested *in vitro* using lung fibroblasts as these cells are the principal cell type in the control and modulation of pulmonary fibrosis. In the first chapter, we investigated the activity of BMOV as a STAT-1 activator and inducer of CXCL10. Importantly, direct evidence was provided to show that BMOV-induced CXCL10 expression requires STAT-1. In the second chapter, we showed that BMOV antagonizes IL-13-induced STAT-6 activation and this antagonism was proven to be due to the action of STAT-1.

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CHAPTER 1

The Therapeutic Vanadium Analog Bis(maltolato)oxovanadium(IV) Increases CXCL10 Production by Lung Fibroblasts via the STAT-1 Transcription Factor

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Running Head: Effect of therapeutic vanadium analog on lung chemokine production

ABSTRACT

Bis(maltolato)oxovanadium(IV) (BMOV) is a bioavailable vanadium-based compound that has potential therapeutic value for the treatment of diabetes. BMOV was specifically formulated to be taken up into the bloodstream via absorption across the gastrointestinal barrier. While BMOV shows promise as therapy for diabetes due to its effect in stabilizing hepatic blood glucose metabolism, it is currently unknown whether this vanadium analog has any beneficial or deleterious effects on other organ systems, including the lungs – which would be predictably sensitive to such compounds.

The signal transducer and activator of transcription (STAT) family of transcription factors are important in mediating tissue remodeling in response to injury. STAT-1 mediates growth arrest and apoptosis in fibroblasts and regulates production of the anti-fibrotic chemokine (C-X-C motif) ligand 10 (CXCL10). In this study we report that BMOV caused a time-dependent increase in STAT-1 phosphorylation and CXCL10 protein secretion by isolated human lung fibroblasts. Interferon-γ (IFN-γ) also induced CXCL10 production in human lung fibroblasts. BMOV stimulated STAT-1 phosphorylation and CXCL10 secretion in STAT-$1^{+/+}$ mouse lung fibroblasts but not in lung fibroblasts isolated from the lungs of STAT-$1^{-/-}$ mice. These data indicate that BMOV activates CXCL10 production by lung fibroblasts through a STAT-1-dependent mechanism and suggests that BMOV has potentially beneficial interferon-like effects in whole lung.

INTRODUCTION

Pulmonary fibrosis is often characterized by decreased lung function, hardening and scarring of lung tissues, and extensive tissue remodeling (Crystal *et al*., 1976). Although the causal elements of pulmonary fibrosis can differ, most chronic fibrotic diseases result from the presence of a persistent irritant which generates reactive oxygen species to induce the production of a host of growth factors, cytokines, and chemokines (Tomasek *et al*., 2002). After the toxic insult, fibroblasts accumulate and proliferate at the site of injury (Bonner, 2007). Fibroblasts, in conjunction with signaling events provided by other cell types such as epithelial cells and macrophages, deposit an excess amount of collagen (Wynn, 2008). The deposition of collagen by fibroblasts is an example of the aberrant wound healing that is a major recurring theme in lung fibrosis (Scotton *et al*., 2007). An individual's life expectancy after diagnosis with pulmonary fibrosis typically ranges from 2 to 5 years (Panos *et al*., 1990; Moeller *et al*., 2009). Importantly, there is currently no affective treatment strategy for pulmonary fibrosis (ATS/ERS, 2002).

We have previously shown that vanadium pentoxide, a toxic by-product of the industrial burning of fuel oil, causes lung injury and airway fibrosis in rats and mice, but these fibrotic lesions resolve, possibly through activation of the STAT-1 signaling pathway (Antao-Menezes *et al*., 2008). This pathway regulates apoptosis and growth arrest in part by up-regulating CXCL10 (Kanda *et al*., 2007). Fibroblasts play a key role in either the resolution or development of pulmonary fibrosis and STAT-1 activation could alter the wound repair process to favor resolution (Bodnar *et al*., 2009).

In contrast to the toxic effects of inhaled vanadium oxides on the lung, chemically modified vanadium analogs have been developed as therapeutic strategies for the treatment of diabetes. One such analog, BMOV, was specifically formulated to maximize therapeutic potency and bioavailability while simultaneously minimizing the toxic effects of other common vanadium supplements (McNeill *et al*., 1992). This form of vanadium can be administered via drinking water where it is then systemically delivered and maintains greater levels of tissue deposition relative to vanadyl sulfate (Setyawati *et al*., 1998).

In the present study, we show that BMOV caused a time-dependent increase in STAT-1 phosphorylation and CXCL10 protein secretion by isolated human lung fibroblasts. Interferon-γ (IFN-γ) also induced CXCL10 production in human lung fibroblasts. BMOV stimulated STAT-1 phosphorylation and CXCL10 secretion in STAT-$1^{+/+}$ mouse lung fibroblasts but not in lung fibroblasts isolated from the lungs of STAT-$1^{-/-}$ mice.

MATERIALS AND METHODS

Reagents:

BMOV was synthesized as described by McNeill *et al*. (1992) and provided by Chris Orvig at the University of British Columbia. Total STAT-1 antibody and phospho-STAT-1 (Tyr701) antibody that detects phosphorylated tyrosine 701 of p91 STAT-1 were purchased from Cell Signaling Technology (Beverly, MA).

Cell Culture:

Normal diploid human lung fibroblasts (HLF) (CCD-16Lu) were purchased from American Type Culture Collection (ATCC) and were originally obtained by autopsy. More detailed information on these lung fibroblasts can be obtained from the ATCC website (www.atcc.org). HLF 16Lu were used due to our previous work with these cells in elucidating mechanisms of vanadium-induced cell signaling and gene expression. Lung fibroblasts were isolated from STAT-$1^{-/-}$ and wild type STAT-$1^{+/+}$ as described previously (Walters *et al*., 2005). Cryogenically preserved second passage cells (10^6) were seeded into 75 cm^2 tissue culture flasks and covered with DMEM supplemented with 10% FBS and antibiotics. Once cells had grown to at least 90% confluence they were treated with trypsin and seeded into separate 60 mm tissue culture dishes. Once confluent, cells were rendered quiescent for 24 hours in serum-free and chemically defined media (SFDM) supplemented with 1ml insulin, transferin, and selenium mixture (ITS). After a 24 hour incubation following SFDM replacement, cells were treated once with BMOV (10 μg/cm^2), IL-13 (10

ng/μl), or both without changing the media. Control dishes were not treated, but were collected in the same way as treated cells.

Western blot analysis:

Cell lysates were collected at specific time points by washing the cells once with PBS on ice, followed by 200 μl of lysis buffer. The lysed cells were scraped from the dish, briefly sonicated, spun in a microfuge at maximum speed for 2 minutes to fractionate insoluble DNA and chromatin proteins from the soluble cellular proteins. Samples were separated by SDS-PAGE, transferred to nitrocellulose membranes, and blocked for 1 hour in 5% nonfat milk in TBST (20 mM Tris, 137 mM NaCl and 0.1% Tween 20). The blot was then incubated at 4°C overnight in a 1/1000 dilution of primary antibody followed by incubation for 1 h in a 1/2000 dilution of HRP-conjugated secondary antibody. The immunoblot signal was detected and visualized through enhanced chemiluminescence (ECL). ECL Plus™ Western Blotting Reagent was purchased from GE Healthcare (formerly Amersham Biosciences). Lysis buffer was made from the following components: 20 mM Tris-HCL (TRIZMA-HCL), 150 mM NaCL, 1mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM Na_3VO_4 (Sodium Orthovanadate), 1% Halt™ Protease Inhibitor Cocktail 100X.

CXCL10 ELISA:

CXCL10 protein in fibroblast-conditioned medium was measured using commercially available ELISA kits (R&D Systems).

Statistics:

All statistical data were plotted and analyzed using the GraphPad Prism software. A one-way ANOVA was used to test for significance between treatment groups and post-hoc analysis was performed utilizing the Tukey multiple comparison test. A value of $p < 0.05$ was considered to be significant as indicated in figures by the presence of asterisks.

RESULTS

CXCL10 expression in human lung fibroblasts is increased by BMOV. Confluent, quiescent cultures of human lung fibroblasts secreted CXCL10 into culture media in a time and dose-dependent manner after BMOV treatment (Fig. 1). IFN-γ also increased the secretion of CXCL10 by human lung fibroblasts (Fig. 2).

BMOV activates STAT-1 in human lung fibroblasts. Cell lysates isolated from HLF 16 Lu were subjected to PAGE and Western blot analysis performed with antibodies specific for phosphorylated STAT-1 or total STAT-1 protein. BMOV treatment caused phosphorylation of STAT-1 in a dose and time-dependent manner in human lung fibroblasts (Fig. 3). However, BMOV treatment did not change the overall levels of STAT-1 protein during the designated time course.

Deletion of STAT-1 abolishes BMOV induced CXCL10 secretion in mouse lung fibroblasts. Similar to results with human lung fibroblasts, STAT-1 was phosphorylated in wild type mouse lung fibroblasts after BMOV exposure (Fig. 4). As expected, no STAT-1 protein was detected by Western blot analysis in STAT-$1^{-/-}$ mouse lung fibroblasts. CXCL10 was completely absent in conditioned media from STAT-$1^{-/-}$ mouse lung fibroblasts and was not induced by BMOV (Fig. 5).

DISCUSSION

Many vanadium analogs (e.g., vanadium oxides) that are generated as by-products of industrial processes are highly toxic when inhaled into the lungs and cause pulmonary fibrosis in rats and mice. Other vanadium analogs modified by the addition of organic moieties have potential therapeutic value for the treatment of diabetes when delivered orally for uptake into the bloodstream via the gastrointestinal tract. As such, two bodies of scientific literature have developed along divergent lines; one that has focused on vanadium as a toxic pollutant that causes lung disease and another on vanadium as a therapy for diabetes. It is unknown whether therapeutic vanadium analogs have any benefit in treating fibrotic lung disease or on the contrary, have any potential toxicity to the lung. Ours is the first study to our knowledge that begins to address the biologic effect of a therapeutic vanadium analog (BMOV) on specific pro-fibrogenic pathways modeled in cultured lung fibroblasts – key cells in the fibrogenic process.

We found that human and mouse lung fibroblasts treated with BMOV secrete CXCL10, a lymphocyte chemokine that has been reported to have protective anti-fibrotic and angiostatic effects on the lung (Jiang D *et al*., 2004). Moreover, BMOV-induced CXCL10 production by fibroblasts was dependent on the transcription factor STAT-1 as evidenced by the complete ablation of CXCL10 production in fibroblasts isolated from the lungs of STAT-$1^{-/-}$ mice. We previously reported that STAT-$1^{-/-}$ mice develop more severe pulmonary fibrosis when treated with the chemotherapeutic agent bleomycin (Walters *et al*., 2005), which indicated that STAT-1 is also protective against pulmonary fibrogenesis. This

coincides with other studies that have shown that $CXCL10^{-/-}$ mice are also more susceptible to bleomycin induced pulmonary fibrosis (Tager *et al*., 2004).

How STAT-1 is activated and regulated during a fibrotic response could be a key determinant in the progression or resolution of fibrotic lung disease. This is partially to do with one of the STAT-1 target genes CXCL10, which is an important regulator of growth arrest and apoptosis (Strieter *et al*., 1995). We previously reported that vanadium compounds are potent activators of STAT-1 via an IFN-β autocrine loop (Antao-Menezes *et al*., 2008). Therefore, we postulate that BMOV also activates STAT-1 and subsequent CXCL10 production via an IFN-dependent mechanism. It is through this activation of anti-fibrotic proteins that make BMOV a candidate to treat pulmonary fibrosis.

Given that the extent of fibroblastic foci present in histological analyses can predict the survival outcome of IPF patients (King *et al*., 2001), any treatment that may induce apoptosis for infiltrating and persisting myofibroblasts may prove to be a fruitful treatment strategy (Gabbiani, 2003). This may be of profound importance since current treatment strategies for pulmonary fibrosis, which mainly target inflammation, are largely unsuccessful (Wynn, 2004). Various other treatment strategies are currently being investigated. Targeted interferon-gamma (IFN-γ) treatment (Ziesche *et al*., 1999), platelet-derived growth factor (PDGF) receptor inhibition utilizing an imatinib treatment (Abdollahi *et al*., 2005), and stem cell replacement represent a few of the many alternative antifibrotic strategies (Wynn, 2007). Whether BMOV is a potential therapeutic antagonist or harmful protagonist for pulmonary

fibrosis remains to be determined. It will also be important to clarify whether BMOV used in the treatment of diabetes will have pro-fibrotic side effects in the lung.

Further studies with *in vivo* use in mice will be needed to determine whether BMOV can reduce lung fibrosis in appropriate animal models.

ACKNOWLEDGEMENTS

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FIGURE LEGENDS

Fig. 1 **CXCL10 secretion from 16 Lu human lung fibroblasts is induced by BMOV.** CXCL10 expression and secretion from human lung fibroblasts is stimulated following BMOV-induced phosphorylation of STAT-1. Data from an ELISA assay demonstrates a significant increase in secreted CXCL10 beginning twelve hours after a one-time treatment of BMOV. The results are expressed as CXCL10 in pg/ml concentrations. Measurements were made in cell supernatant made up of serum-free and chemically defined media and secreted proteins. Statistical significance was determined by ANOVA followed by the Tukey post hoc test. Each treatment group was compared to its time matched control. *** indicates $p<0.001$.

Fig. 2 **Induction of CXCL10 secretion follows interferon-γ (IFN-γ) exposure by human lung fibroblasts.** CXCL10 expression and secretion from human lung fibroblasts is stimulated following interferon-induced phosphorylation of STAT-1. ELISA data demonstrates an increase in CXCL10 following a one-time treatment of IFN-γ. Results are expressed in pg/ml.

Fig. 3 **Western blot analysis of phosphorylated STAT-1 (pSTAT-1) and total STAT-1 protein in human lung fibroblasts treated with BMOV.** Following a one-time treatment of human lung fibroblasts with BMOV, STAT-1 undergoes prolonged phosphorylation. Time points measured, in hours after initial exposure, were, 0.1, 6, 12, 18, and 24. Cell lysates were separated using SDS-PAGE, transferred to nitrocellulose membranes, and

probed for phospho-STAT-1. Total STAT-1 was also probed for as a loading control. Phosphorylation is prolonged and begins at 6 hours following exposure. Non-treated cells expressed no phospho-STAT-1, but exhibited total STAT-1 levels comparable to that of the treatment group.

Fig. 4 **Western blot analysis of phosphorylated STAT-1 (pSTAT-1) and total STAT-1 protein in wild type mouse lung fibroblasts treated with BMOV and absence of STAT-1 in STAT-1-deficient mouse lung fibroblasts.** BMOV activates STAT-1 phosphorylation in wild type mouse lung fibroblasts, but not STAT-$1^{-/-}$ fibroblasts. Additionally, no total STAT-1 is found in STAT-$1^{-/-}$ fibroblasts either.

Fig. 5 **BMOV induction of CXCL10 protein secretion is blocked in STAT-1 null mouse lung fibroblasts.** A) CXCL10 expression and secretion from wild type mouse lung fibroblasts is stimulated following BMOV-induced phosphorylation of STAT-1. A significant increase in secreted CXCL10 begins at twelve hours after initial treatment with BMOV. Statistical significance was determined by ANOVA followed by the Tukey's test. Each treatment group was compared to its respective time-matched control. *** indicates $p<0.001$. B) In STAT-$1^{-/-}$ fibroblasts CXCL10 is not significantly expressed.

FIGURES

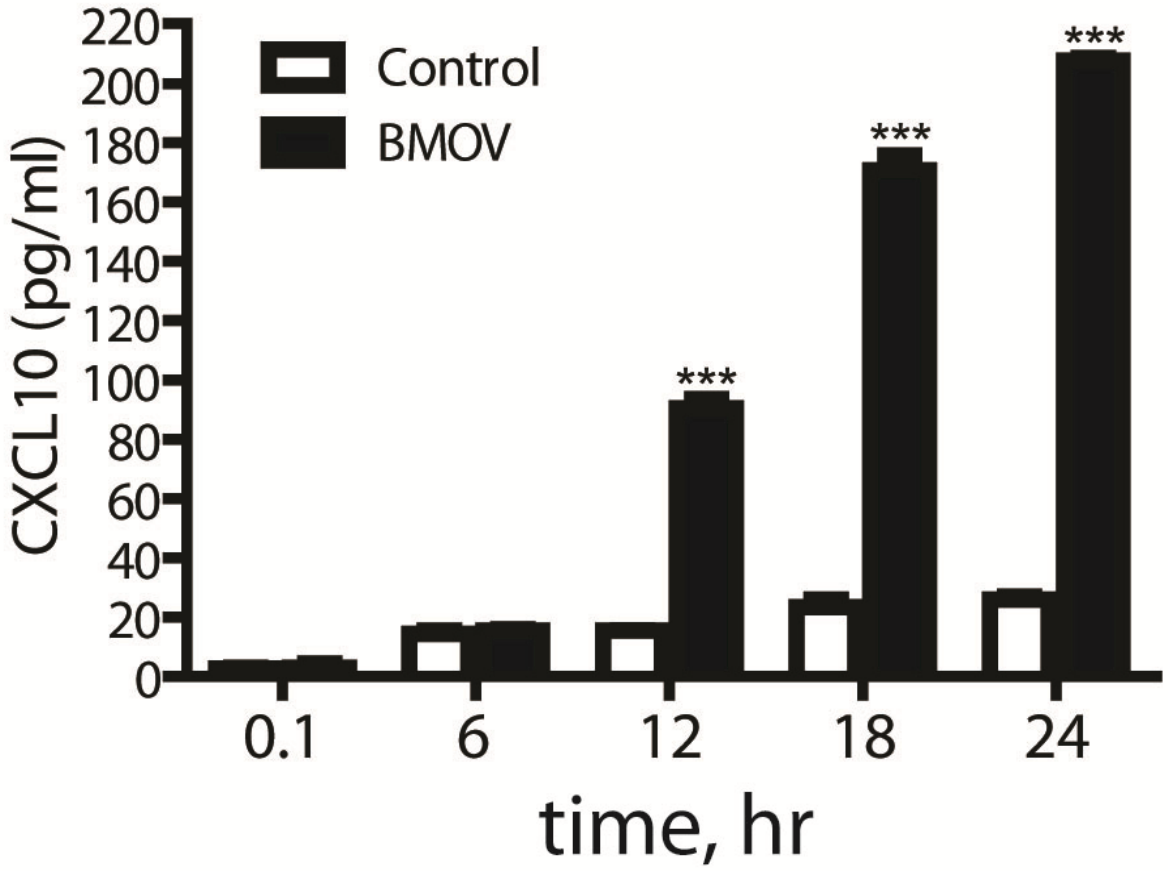

Fig. 1 CXCL10 secretion from 16 Lu human lung fibroblasts is induced by BMOV.

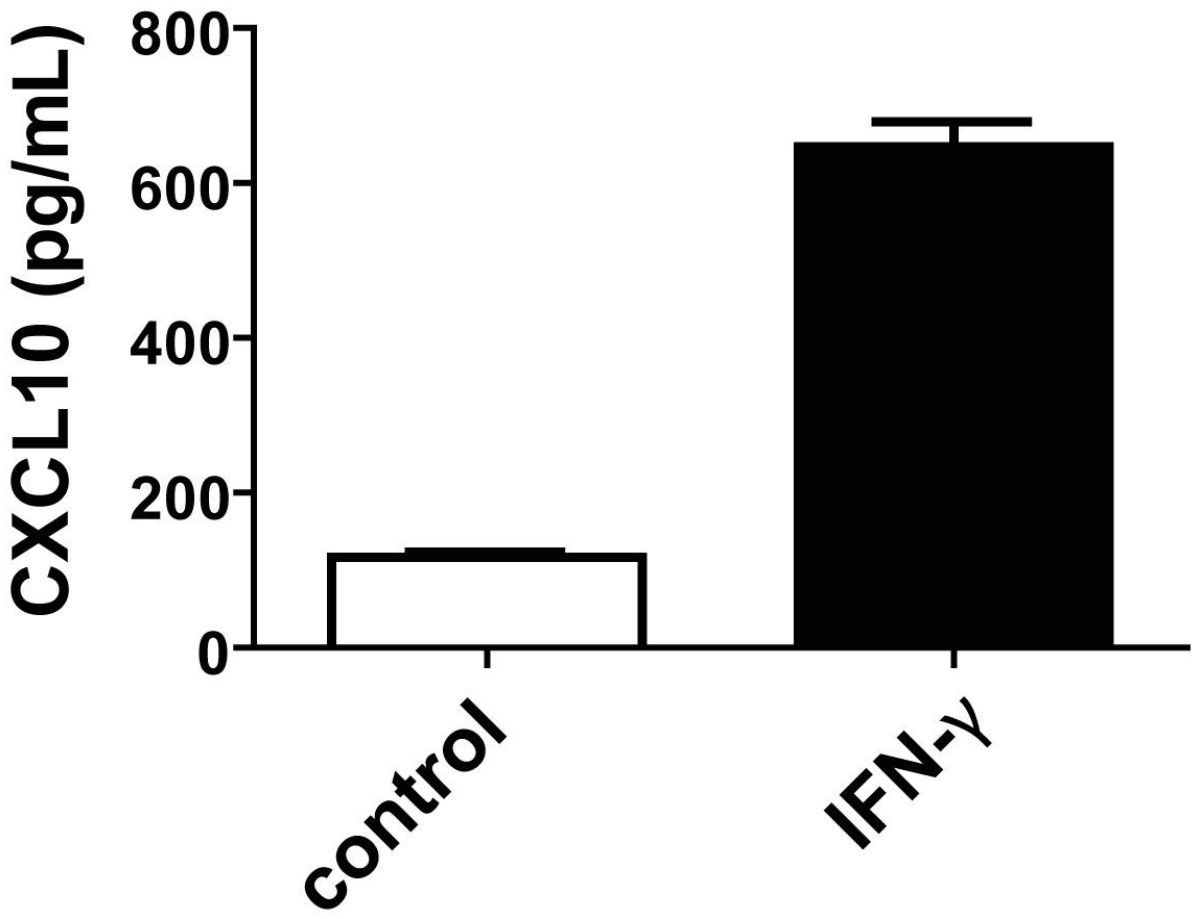

Fig. 2 Induction of CXCL10 secretion follows interferon-γ (IFN-γ) exposure by human lung fibroblasts.

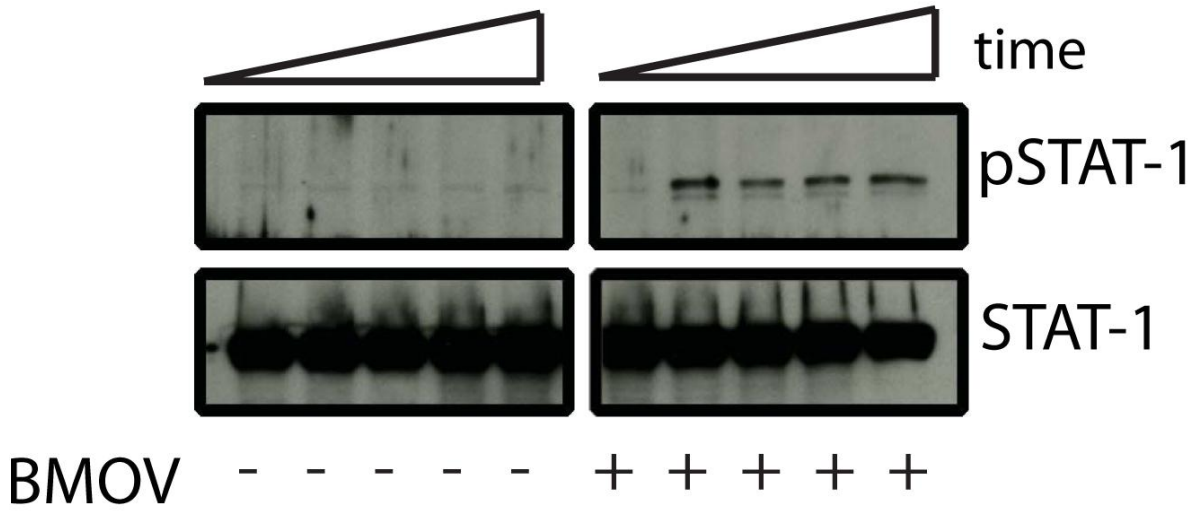

Fig. 3 Western blot analysis of phosphorylated STAT-1 (pSTAT-1) and total STAT-1 protein in human lung fibroblasts treated with BMOV.

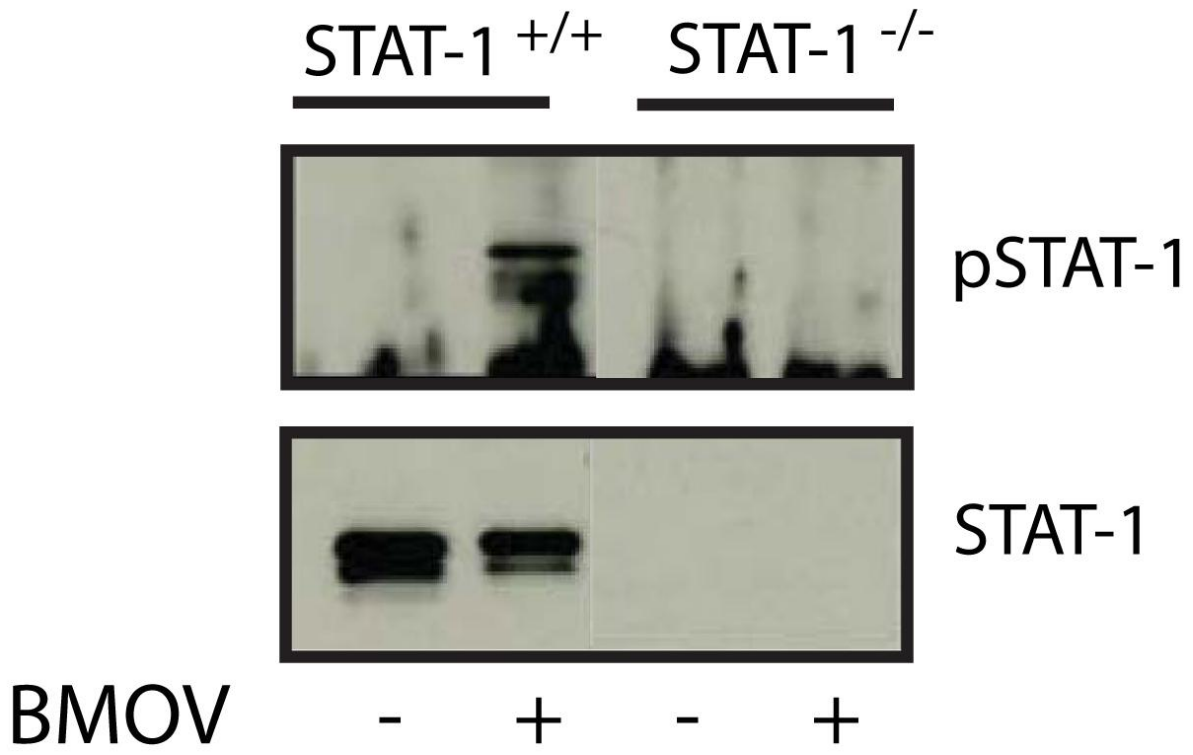

Fig. 4 Western blot analysis of phosphorylated STAT-1 (pSTAT-1) and total STAT-1 protein in wild type mouse lung fibroblasts treated with BMOV and absence of STAT-1 in STAT-1-deficient mouse lung fibroblasts.

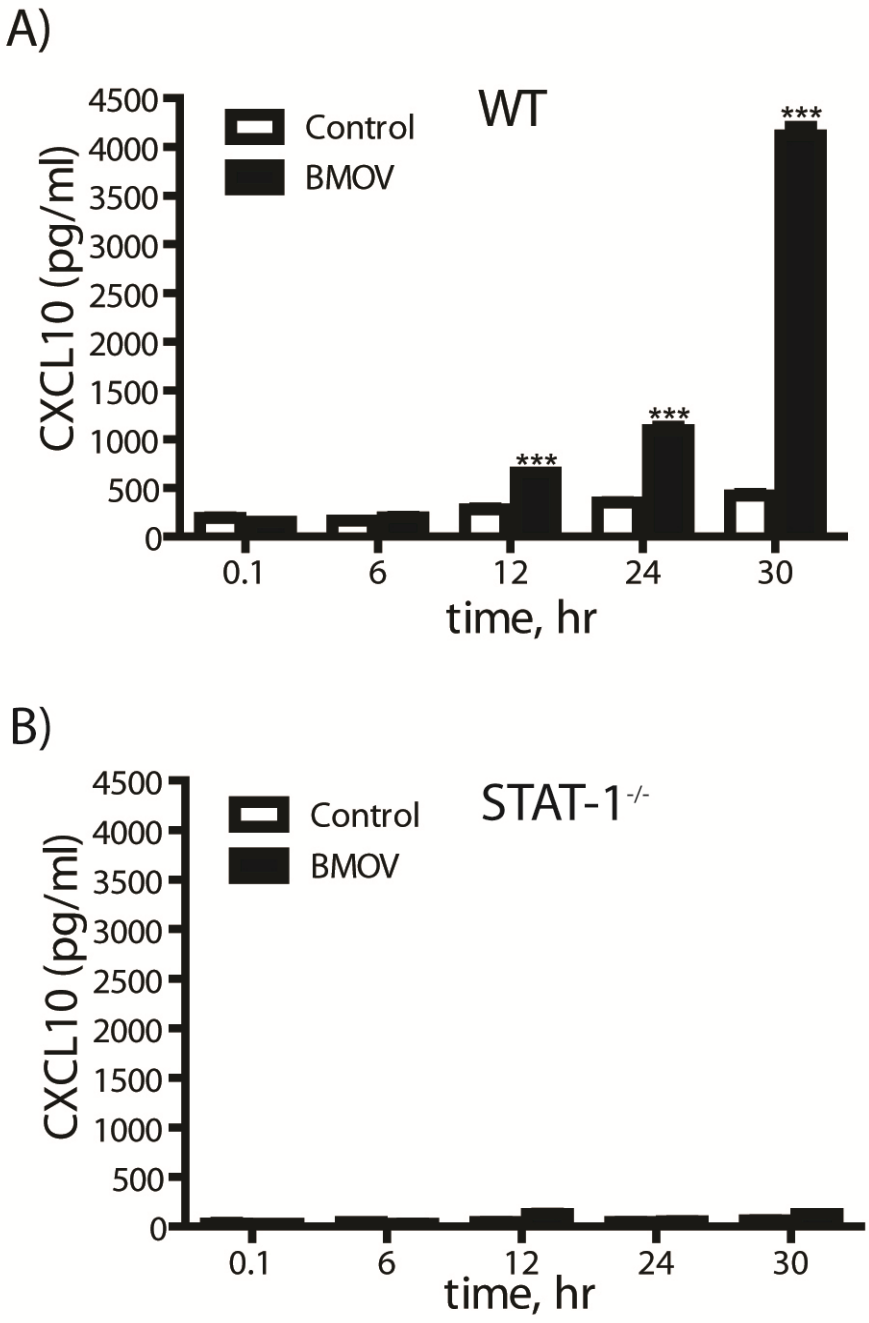

Fig. 5 BMOV induction of CXCL10 protein secretion is blocked in STAT-1 null mouse lung fibroblasts.

CHAPTER 2

CXCL10 Production by Lung Fibroblasts Induced by the Therapeutic Vanadium Analog Bis(maltolato)oxovanadium(IV) is Antagonized by Interleukin-13 (IL-13) via a STAT-6-Dependent Mechanism

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Funding: This work was funded by NIH grant R21-ES015801-01.

Running Head: Effect of therapeutic vanadium analog on lung chemokine production

ABSTRACT

Bis(maltolato)oxovanadium(IV) (BMOV) is a bioavailable vanadium-based compound that, when delivered orally, has potential therapeutic value for the treatment of diabetes. We previously discovered that BMOV mimics the action of anti-fibrogenic interferons (IFNs) to activate the signal transducer and activator of transcription (STAT)-1 and increase secretion of the IFN-inducible chemokine CXCL10 by cultured lung fibroblasts, suggesting that BMOV might serve as a possible anti-fibrogenic agent. The purpose of this investigation was to determine whether BMOV could induce CXCL10 production in the presence of interleukin-13, a major cytokine that acts through STAT-6-dependent signaling to mediate pro-fibrogenic responses in isolated lung fibroblasts. The addition of IL-13 to cultures of human lung fibroblasts significantly reduced BMOV-induced CXCL10 production and BMOV-induced STAT-1 phosphorylation. Conversely, BMOV reduced IL-13-induced STAT-6 phosphorylation in human lung fibroblasts. IL-13 also reduced BMOV induced STAT-1 activation and subsequent CXCL10 secretion in fibroblasts isolated from the lungs of normal mice, but not in fibroblasts isolated from the lungs of STAT-6-deficient mice. Collectively, these data suggest that the IFN-like properties of BMOV such as activation of STAT-1 and increases in CXCL10 production could be limited in the presence of IL-13 and therefore further study should focus on determining whether BMOV delivered orally could reduce pulmonary fibrosis *in vivo* in mice.

In addition, BMOV antagonized STAT-6 signaling initiated by interleukin-13 (IL-13), a major mediator of pulmonary fibrosis and asthma. IL-13 reduced BMOV-induced

STAT-1 activation and CXCL10 production. Our findings of mutual antagonism between BMOV and IL-13 indicate that BMOV could serve as a potential anti-fibrotic therapy.

INTRODUCTION

Pulmonary fibrosis is a debilitating disease with poor prognoses and an undefined etiology (Moeller *et al*., 2009; Maher *et al*., 2007). The causal elements may vary to a large degree but one common cause is the presence of an unusually persistent irritant (Bonner, 2007).

IL-13 is used in this study to model the tissue environment of the fibroblast in the asthmatic lung. IL-13 induces production of many of the same growth factors and mitogenic cytokines that usually precede aberrant and excessive remodeling of the extracellular matrix known to occur in these conditions (Zhu *et al*., 1999).

It has been demonstrated that IFNs can mitigate the effects of pulmonary fibrosis (Chin *et al*., 1997). We have previously shown that a therapeutic vanadium analog, known as bis(maltolato)oxovanadium(IV) (BMOV), which is typically reserved for the treatment of diabetes, can also mimic the effects of IFN (Bost *et al*., Thesis Chapter 1). Both compounds are capable of activating the STAT-1 signaling axis, which in the context of pulmonary fibrosis, translates into appropriate regulation of repair mechanisms. Other more common vanadium compounds, such vanadium pentoxide (V_2O_5) found in various occupational settings, have been shown to cause pulmonary fibrosis. In mouse models, the fibrotic lesions that result from V_2O_5 exposure partially resolve, indicating a dual nature for vanadium compounds (Antao-Menezes *et al*., 2008).

We have sought to elucidate whether the antifibrotic mechanisms associated with certain vanadium compounds can be divorced from the fibrotic mechanisms of others. One potential path to achieving this goal would be to simultaneously lower the toxicity and alter

the route of exposure of vanadium compounds by attaching innocuous functional groups. In 1992 researchers crafted a vanadium analog with these criteria by coupling vanadium between two maltol groups, which is a functional group commonly found in food additives (McNeill *et al*., 1992). While this type of vanadium compound has been used in diabetes treatment due to its capacity to mimic insulin, exploitation for use in the treatment of other unrelated diseases has been limited (Thompson *et al*., 2009).

In the present study, we show that BMOV and IL-13 activate STAT-1 and STAT-6, respectively, in isolated lung fibroblasts. The activation of STAT-1 by BMOV decreases STAT-6 phosphorylation in response to IL-13. The activation of STAT-6 dampens STAT-1 phosphorylation and decreases the downstream expression and secretion of CXCL10 in both human and mouse lung fibroblasts. Lung fibroblasts from STAT-$6^{-/-}$ mice lose the ability to inhibit STAT-1 phosphorylation and transcription. This implies that in order for STAT-1 inhibition to take place via IL-13, STAT-6 must be present. Taken together, these data suggest that STAT-1 and STAT-6 are mutually antagonistic and regulate one another's respective downstream effects. This intersection between two competing transcription factors may represent a crucial fulcrum in the progression or remediation of pulmonary fibrosis.

MATERIALS AND METHODS

Reagents:

BMOV was synthesized as described by McNeill *et al*. (1992) and provided by Chris Orvig at the University of British Columbia. Recombinant IL-13 was purchased from R&D Systems, Inc. (Minneapolis, MN). Total STAT-1 antibody and phospho-STAT-1 (Tyr701) antibody that detects phosphorylated tyrosine 701 of p91 STAT-1 were purchased from Cell Signaling Technology (Beverly, MA). Total STAT-6 antibody and phospho-STAT-6 (Tyr641) antibody that detects phosphorylated tyrosine 641 of p110 STAT-6 were purchased from Cell Signaling Technology (Beverly, MA).

Cell Culture:

Normal diploid human lung fibroblasts (CCD-16Lu) were purchased from American Type Culture Collection (ATCC) and were originally obtained by autopsy. More detailed information on these lung fibroblasts can be obtained from the ATCC website (www.atcc.org). HLF 16Lu were used due to our previous work with these cells in elucidating mechanisms of vanadium-induced cell signaling and gene expression. Mouse lung fibroblasts were isolated from STAT-$1^{-/-}$ and wild type STAT-$1^{+/+}$ in addition to STAT-$6^{-/-}$ and wild type STAT-$6^{+/+}$ as described previously (Walters *et al*., 2005). Cryogenically preserved second passage cells (10^6) were seeded into 75 cm^2 tissue culture flasks and covered with DMEM supplemented with 10% FBS and antibiotics. Once cells had grown to at least 90% confluence they were treated with trypsin and seeded into separate 60 mm tissue culture dishes. Once confluent, cells were rendered quiescent for 24 hours in serum-free and

chemically defined media (SFDM) supplemented with 1ml insulin, transferin, and selenium mixture (ITS). After a 24 hour incubation following SFDM replacement, cells were treated once with BMOV (10 μg/cm^2), IL-13 (10 ng/μl), or both without changing the media. Control dishes were not treated, but were collected in the same way as treated cells.

Western blot analysis:

Cell lysates were collected at specific time points by washing the cells once with PBS on ice, followed by 200 μl of lysis buffer. The lysed cells were scraped from the dish, briefly sonicated, spun in a microfuge at maximum speed for 2 minutes to fractionate insoluble DNA and chromatin proteins from the soluble cellular proteins. Samples were separated by SDS-PAGE, transferred to nitrocellulose membranes, and blocked for 1 hour in 5% nonfat milk in TBST (20 mM Tris, 137 mM NaCl and 0.1% Tween 20). The blot was then incubated at 4°C overnight in a 1/1000 dilution of primary antibody followed by incubation for 1 h in a 1/2000 dilution of HRP-conjugated secondary antibody. The immunoblot signal was detected and visualized through enhanced chemiluminescence (ECL). ECL Plus™ Western Blotting Reagent was purchased from GE Healthcare (formerly Amersham Biosciences). Lysis buffer was made from the following components: 20 mM Tris-HCL (TRIZMA-HCL), 150 mM NaCL, 1mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM Na_3VO_4 (Sodium Orthovanadate), 1% Halt™ Protease Inhibitor Cocktail 100X.

Densitometry:

Western blot images were evaluated by densitometry using Adobe Photoshop CS3 using the 'rectangular marquee tool' and by selecting the 'record measurements' option under the 'analysis' menu. Densitometric values for phospho-STATs (pSTATs) were normalized against the total STAT signal for each experiment to derive a pSTAT/STAT ratio. The data were expressed as relative densitometric units.

CXCL10 ELISA:

CXCL10 protein in fibroblast-conditioned medium was measured using commercially available ELISA kits (R&D Systems).

RESULTS

CXCL10 expression in human lung fibroblasts was increased in a time dependent manner following treatment with BMOV from two distinct sources. Treatment with IL-13 exhibits a similar CXCL10 expression profile to that of the negative, but in co-treatments of BMOV and IL-13, CXCL10 expression is knocked down relative to treatment with BMOV alone (Fig. 1). This data suggests that BMOV's ability to induce production of CXCL10 is mitigated due to the downstream effects of IL-13 in isolated fibroblasts.

A Western blot of phosphorylated STAT-1 from cells exposed to the same treatments reveals that IL-13 suppressive effect of CXCL10 occurs because of decreased phosphorylation of STAT-1 (Fig. 2). STAT-1 is the primary transcription factor that regulates CXCL10. IL-13 is a potent activator of STAT-6. When human lung fibroblasts are exposed to IL-13, potent phosphorylation of this transcription factor occurs within minutes and decreases over time (Fig. 3).

In addition to the suppressive effects IL-13 stimulation has on STAT-1, BMOV also acts to suppress STAT-6. When human lung fibroblasts are co-exposed to both BMOV and IL-13, STAT-6 phosphorylation is decreased relative to IL-13 alone (Fig. 4). Densitometric analysis of phosphorylated bands indicates a two-fold suppression by BMOV. This suggests that STAT-1 and STAT-6 may play a mutually antagonistic suppressive role for one another.

STAT-6 was phosphorylated after IL-13 exposure in wild type mouse lung fibroblasts, but not STAT-$6^{-/-}$ fibroblasts (Fig. 5). STAT-$6^{-/-}$ MLF were unaffected by IL-13 exposure (Fig. 6). CXCL10 expression following exposure to BMOV has been demonstrated in both human and mouse lung fibroblasts contingent on the presence of STAT-1. In

addition, when co-exposed with BMOV, IL-13 suppresses the expression of CXCL10. In the absence of STAT-6 however, IL-13 loses its ability to suppress the BMOV induced CXCL10 (Fig. 7).

DISCUSSION

Vanadium compounds are known to be toxic components of air pollution particles (Erlich *et al*., 2008) and are encountered occupationally by individuals working in the petrochemical industry (Nemery, 1990). In the latter case, there is clear epidemiologic evidence linking bronchitis and reduced lung function to vanadium exposure (Irsigler *et al*., 1999). Therefore, it was initially surprising that some vanadium compounds had been developed as therapeutic agents for the treatment of diabetes. However, as with many metals, there is generally a balance between therapeutic activity and toxicity. We previously showed that BMOV is a potent activator of STAT-1 and it is through this pathway that CXCL10 is ultimately up-regulated (Bost *et al*., Thesis Chapter 1). In this study, we sought to determine whether BMOV-induced STAT-1 signaling in isolated fibroblasts would be antagonized by IL-13, a potent activator of STAT-6 that mediates multiple disease phenotypes that characterize pulmonary fibrosis (Fichtner-Feigl *et al*., 2006) and asthma (Wills-Karp *et al*., 1998; Kuperman *et al*., 2002). We further investigated whether BMOV might act in a reciprocal manner to counteract the effects of IL-13. Indeed, upon co-exposure with both BMOV and IL-13, STAT-6 phosphorylation was decreased suggesting antagonistic cross-talk between STAT-1 and STAT-6 pathways. Importantly, IL-13 significantly reduced BMOV-induced CXCL10 production by lung fibroblasts, suggesting that any therapeutic effect of BMOV might be limited by the pro-fibrogenic activity of IL-13.

How STAT-1 is activated and regulated during a fibrotic response could be a key determinant in the progression or resolution of fibrotic lung disease. Several lines of evidence indicate that STAT-1 is protective against the development of pulmonary fibrosis.

First, STAT-1 is well-known to be an important regulator of growth arrest and apoptosis in cells of mesenchymal origin, including fibroblasts (Thomas *et al*., 2004). Growth arrest and apoptosis serves to limit a fibrotic response in the lung or other tissues by decreasing the available number of collagen-producing fibroblasts. Second, STAT-1-deficient mice develop a more severe fibrotic reaction after injury with bleomycin as compared to wild type mice of the same strain and background (Walters *et al*., 2005). That study showed that susceptibility of STAT-1-deficient mice was due to significant increases in lung collagen and the lack of fibroblast growth inhibition by interferons. Third, interferons exert their protective anti-fibrogenic properties through STAT-1 (Chin *et al*., 1997). In fact, interferon-γ has been extensively explored as an anti-fibrotic therapy for the treatment of patients with idiopathic pulmonary fibrosis (Bouros *et al*,. 2006).

In addition to playing a central role in the growth arrest of fibroblasts, STAT-1 appears to be required for induction of CXCL10, which is a primary lymphocyte chemokine that functions to reduce fibrotic reactions (Strieter *et al*., 1995). Our own studies have shown that STAT-1 is required for BMOV-induced CXCL10 production by lung fibroblasts (Bost *et al*., Chapter 1). Furthermore, our laboratory previously reported that vanadium pentoxide increases CXCL10 in human lung fibroblasts through an interferon-dependent mechanism (Antao-Menezes *et al.*, 2008). Work from other investigators has shown that mice deficient in CXCR3, the receptor for CXCL10, develop more severe bleomycin-induced pulmonary fibrosis. CXCR3 deficiency resulted in both a reduced early burst of IFN-γ production and decreased expression of CXCL10 after lung injury. The fibrotic phenotype of CXCR3-deficient mice was reversed by the administration of exogenous interferons, which suggested

that CXCR3 activation by CXCL10 acts through a feedback loop to further increase the production of interferons (Jiang *et al*., 2004). Finally, an investigation of CXCL10 and CXCR3 levels in cells collected from patients with or without idiopathic pulmonary fibrosis (IPF) by bronchoalveolar lavage contained significantly lower levels of CXCL10 and CXCR3 compared to those from normal individuals (Pignatti *et al*., 2006). Collectively, these studies emphasized the protective role of the CXCL10 receptor, CXCR3, against pro-fibrogenic responses by fibroblasts.

The demonstration that BMOV reduces IL-13-induced STAT-6 signaling suggests that BMOV could have potential anti-fibrotic activity. However, BMOV-induced STAT-1 activation and CXCL10 secretion were reduced by IL-13, which suggests that the pro-fibrotic effect of IL-13 might overwhelm the anti-fibrotic properties of BMOV. IL-13 promotes fibrosis by increasing levels of growth factors that stimulate fibroblast proliferation and collagen deposition (Ingram and Bonner, 2006). Our laboratory previously reported that IL-13 stimulates fibroblasts to produce PDGF through a STAT-6-dependent mechanism (Ingram *et al*., 2006). Other investigators have shown that IL-13 stimulates the production of TGF-β1, a principal growth factor in fibrosis that increases collagen deposition by fibroblasts (Lee *et al*., 2001). Therefore, it has been well-established that IL-13 promotes fibrosis by increasing profibrogenic factors. However, to our knowledge it has not been established that IL-13 promotes fibrosis by suppressing the production of protective factors such as CXCL10. Therefore, our findings from this study are important in that they provide a new pathologic mechanism for IL-13 in suppressing CXCL10 levels that are increased by BMOV.

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FIGURE LEGENDS

Fig. 1 **CXCL10 is secreted by 16 Lu human lung fibroblasts in response to BMOV.** CXCL10 expression and secretion from human lung fibroblasts is stimulated following BMOV-induced phosphorylation of STAT-1. ELISA data indicates that CXCL10 is increased in a time dependent manner following a one-time dose of BMOV (10 $\mu g/cm^2$). IL-13 (10ng/ml) alone had no observable effect on CXCL10 concentrations, but when co-treated with BMOV, CXCL10 is diminished. The results are expressed as CXCL10 in pg/ml concentrations. Measurements were made in cell supernatant made up of serum-free and chemically defined media and secreted proteins. Time points measured, in hours after initial exposure, were, 0.1, 6, 12, 18, and 24. A) Results with BMOV from Germany source. B) Results with BMOV from Canadian source.

Fig. 2 **IL-13 suppresses BMOV-induced STAT-1 phosphorylation in human lung fibroblasts.** A) Following a one-time treatment with BMOV, IL-13, neither, or both, phosphorylated-STAT-1 was measured using western blot analysis. Total STAT-1 was used as a loading control and was unaffected by treatment. B) Phosphorylation bands found in the western blot were densitometrically analyzed and compared their respective loading control. BMOV treatment alone phosphorylated STAT-1 the most, but was that effect was greatly diminished upon co-treatment with IL-13.

Fig. 3 **Western blot of phospho-STAT-6 and total STAT-6 protein in 16 Lu human lung fibroblasts treated with 10 ng/ml IL-13.** Following a one-time treatment with IL-13,

phosphorylated STAT-6 was measured using western blot analysis. Time points measured, in hours after initial exposure, were, 0.1, 6, 12, 18, and 24. STAT-6 was phosphorylated immediately after treatment and remained active in decreasing intensity as time progressed.

Fig. 4 **BMOV suppresses IL-13-induced STAT-6 phosphorylation in human lung fibroblasts.** A) Following a one-time treatment with BMOV, IL-13, neither or both, phosphorylated-STAT-6 was measured using western blot analysis. Total STAT-6 was used as loading control and was unaffected by treatment. B) Densitometric analysis of the phosphorylated bands relative to their respective loading control confirm that IL-13 potently activates STAT-6. BMOV does not appear to have any influence on STAT-6 phosphorylation when used as an isolated treatment on fibroblasts. Co-exposures with IL-13, however, demonstrated that BMOV negatively regulates STAT-6 phosphorylation.

Fig. 5 **IL-13 phosphorylation of STAT-6 occurs in STAT$^{+/+}$ mice, but not in STAT-6$^{-/-}$ mice.** IL-13 activates STAT-6 phosphorylation in wild type mouse lung fibroblasts, but not STAT-6$^{-/-}$ fibroblasts. Additionally, no total STAT-6 is found in STAT-6$^{-/-}$ fibroblasts either.

Fig. 6 **Western blot for phospho-STAT-1 and total STAT-1 protein in fibroblasts isolated from the lungs of wild type or STAT-6 deficient mice.** A) Western blots probing for phospho-STAT-1 and total STAT-1 were carried out on lysates obtained from wild type and STAT-6$^{-/-}$ mouse lung fibroblasts. Both sets of fibroblasts were given identical treatments of BMOV, IL-13, neither, or both. B) Densitometric analysis of the western blots show that comparable phosphorylation occurs with one exception. When STAT-6$^{-/-}$ mouse

lung fibroblasts were co-treated with BMOV and IL-13, STAT-1 phosphorylation levels were comparable to those that were treated with BMOV alone. This demonstrates that STAT-6 must be present for IL-13 to dampen phosphorylation of STAT-1.

Fig. 7 **CXCL10 secretion by fibroblasts isolated from the lungs of A) wild type or B) STAT-6-deficient mice.** Comparison of wild type and STAT-$6^{-/-}$ CXCL10 secretions in a representative ELISA demonstrate that STAT-6 must be present for IL-13 to suppress CXCL10. The inhibition of CXCL10 secretions present in wild type mice are absent in STAT-6 null mice.

FIGURES

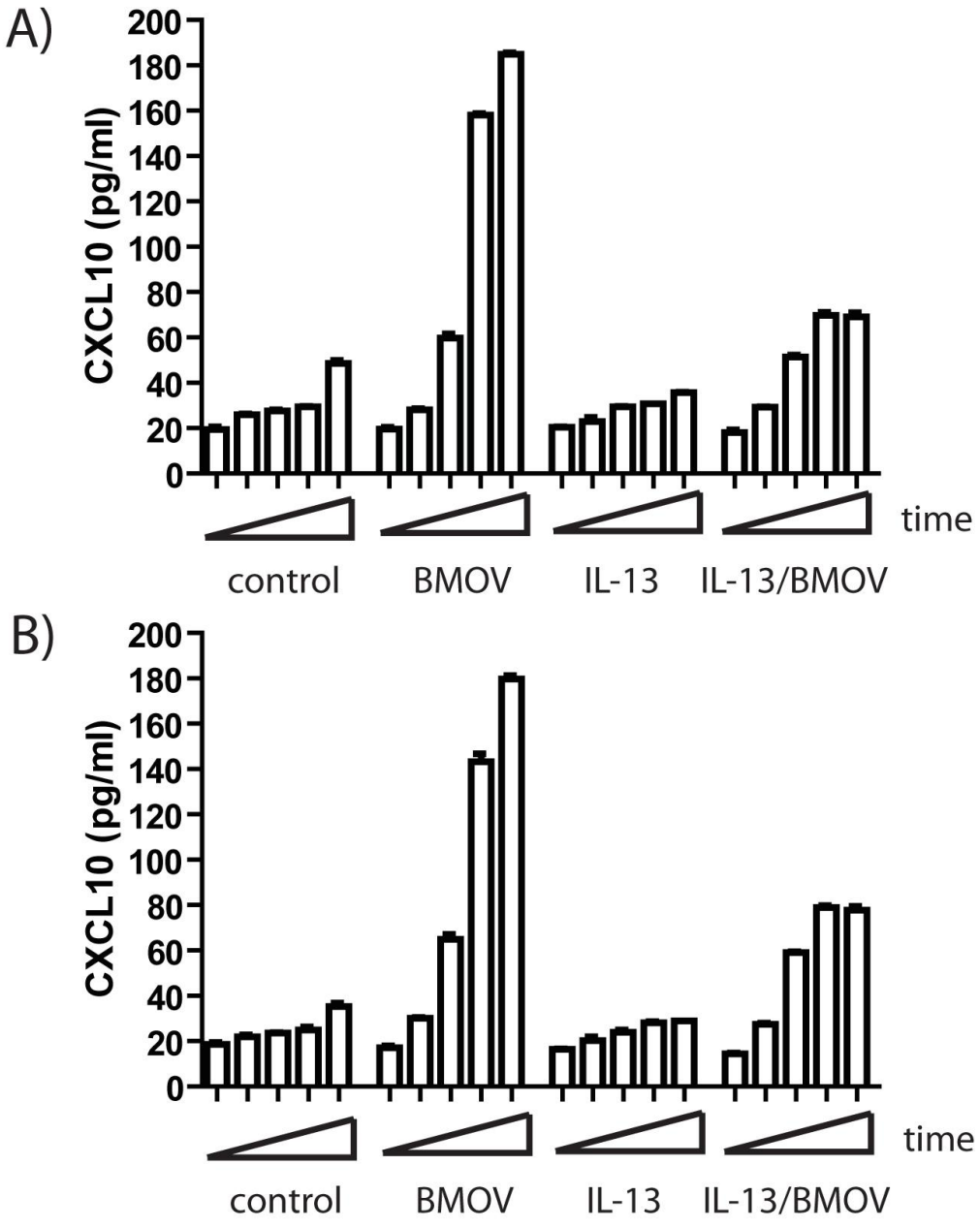

Fig. 1 CXCL10 is secreted by 16 Lu human lung fibroblasts in response to BMOV.

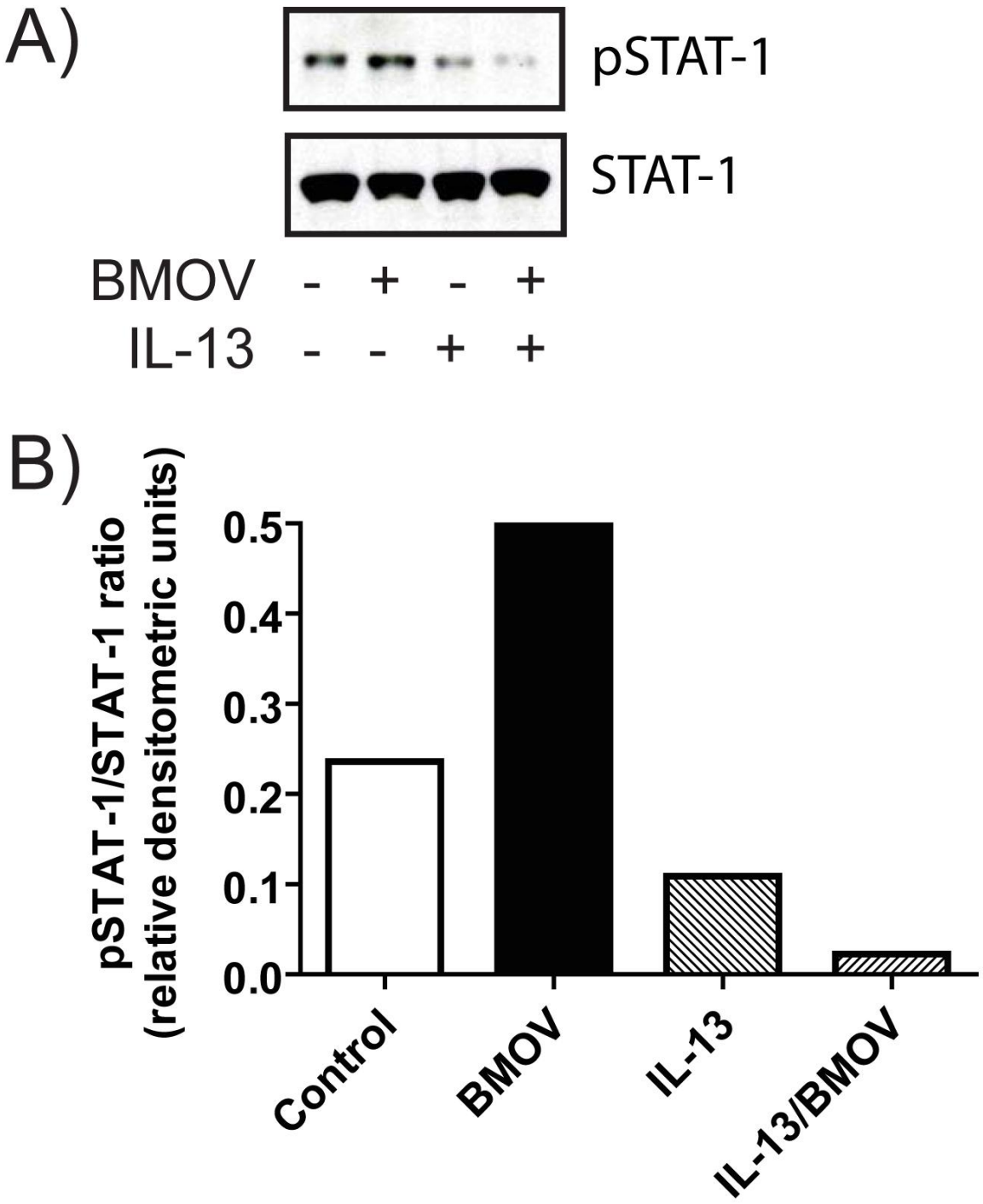

Fig. 2 IL-13 suppresses BMOV induced STAT-1 phosphorylation in human lung fibroblasts.

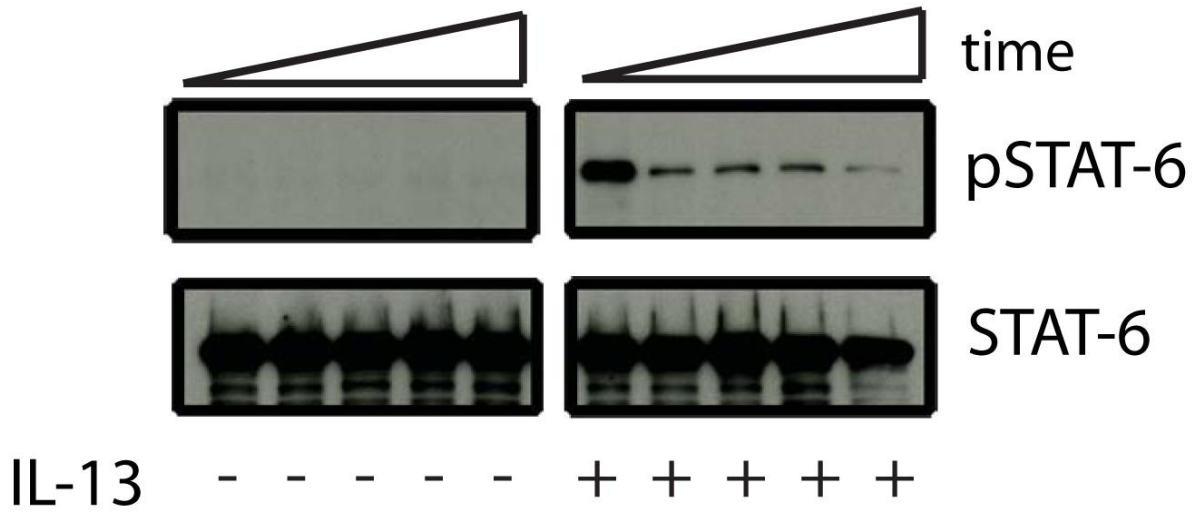

Fig. 3 Western blot of phospho-STAT-6 and total STAT-6 protein in 16 Lu human lung fibroblasts treated with 10 ng/ml IL-13.

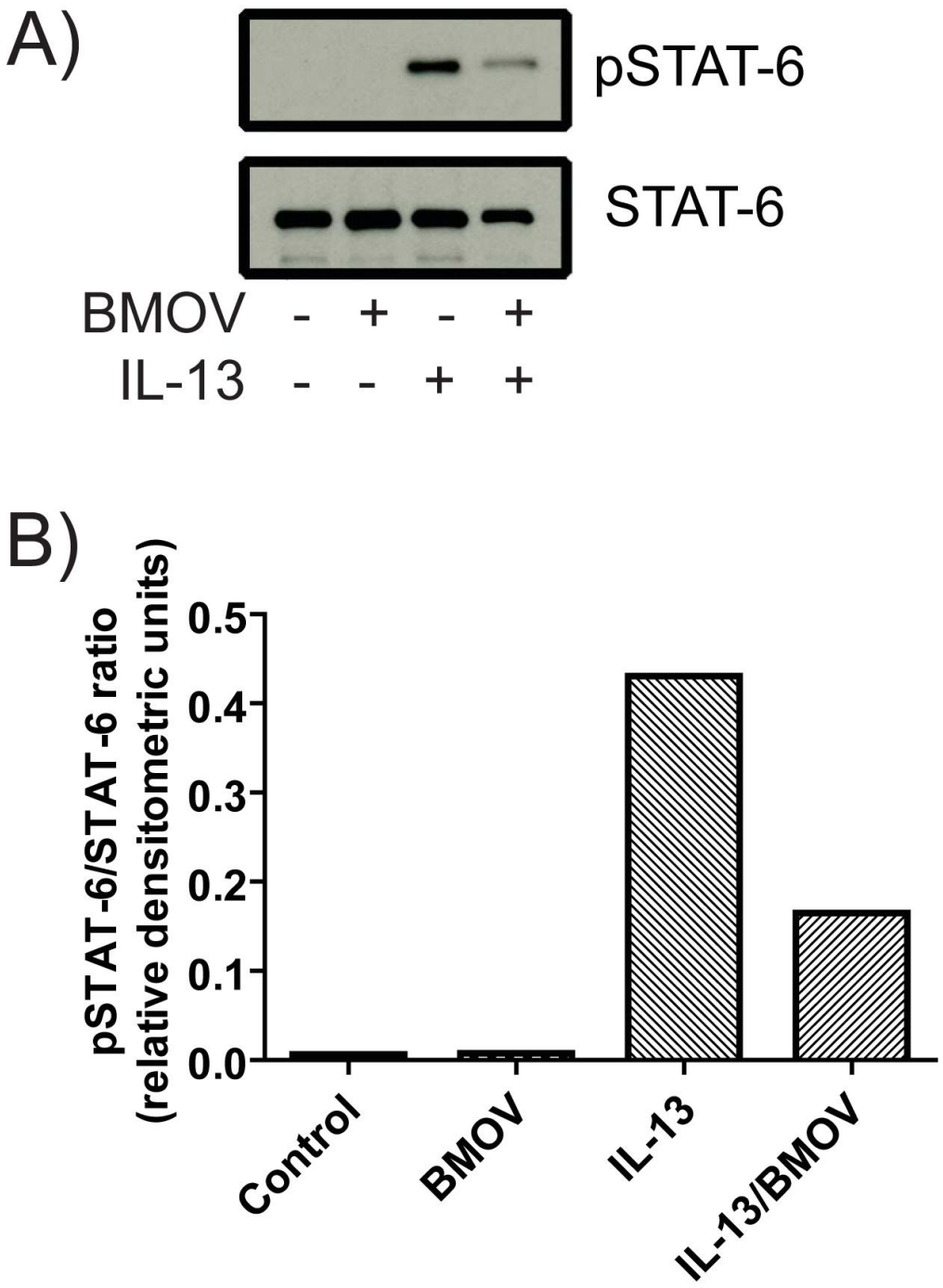

Fig. 4 BMOV suppresses IL-13-induced STAT-6 phosphorylation in human lung fibroblasts.

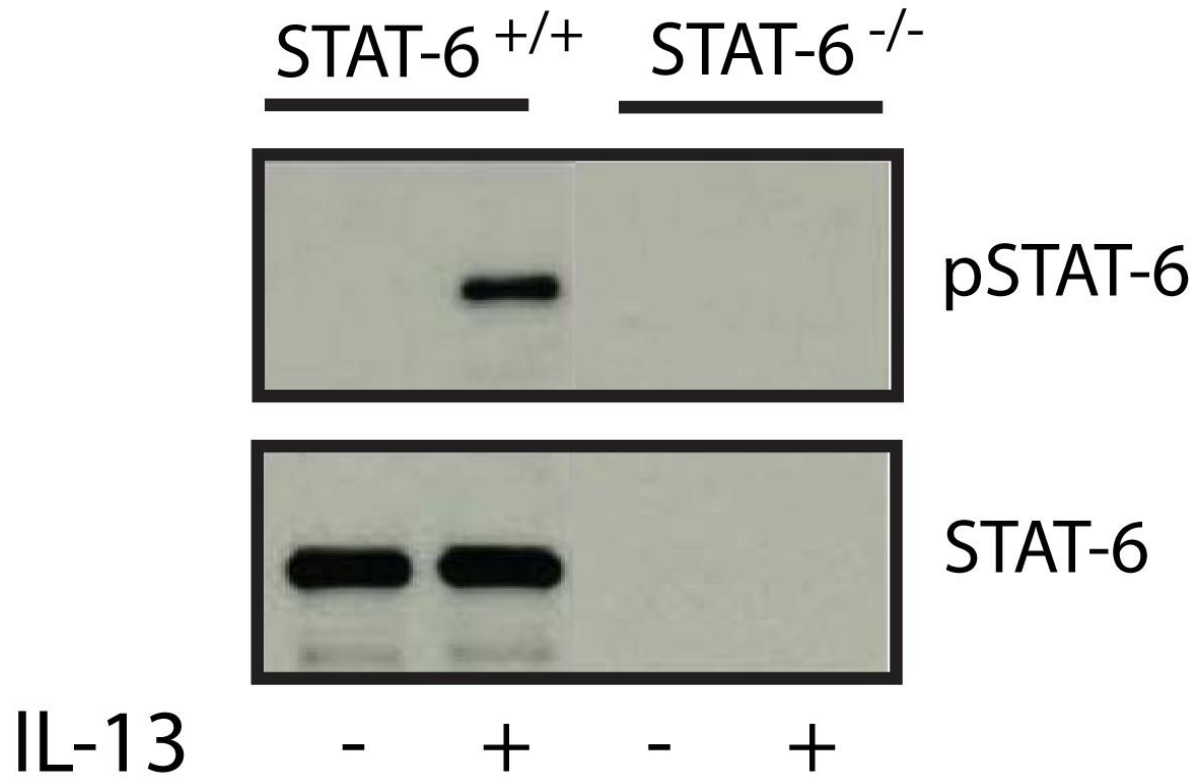

Fig. 5 IL-13 phosphorylation of STAT-6 occurs in STAT$^{+/+}$ mice, but not in STAT-6$^{-/-}$ mice.

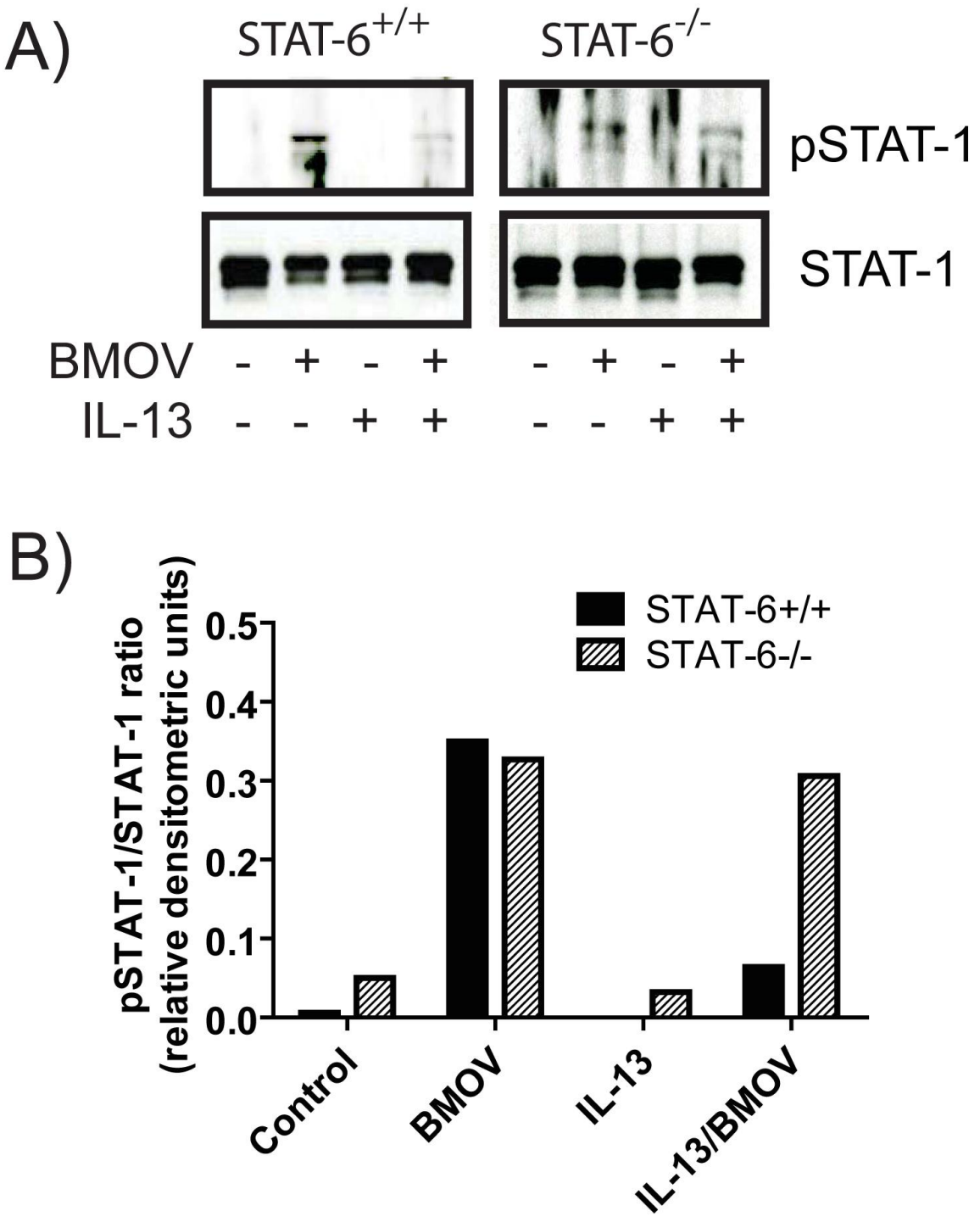

Fig. 6 Western blot for phospho-STAT-1 and total STAT-1 protein in fibroblasts isolated from the lungs of wild type or STAT-6 deficient mice.

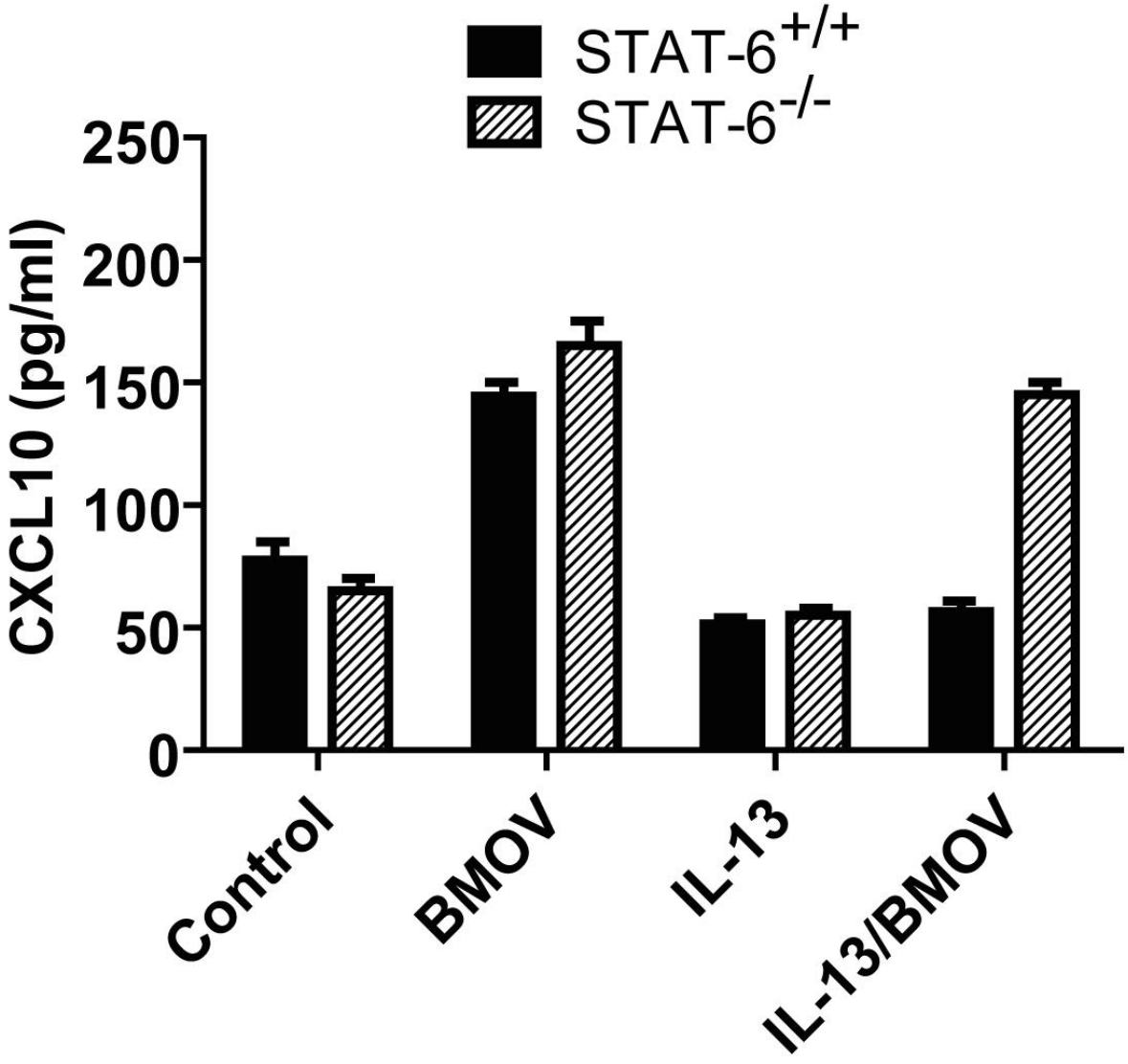

Fig. 7 CXCL10 secretion by fibroblasts isolated from the lungs of A) wild type or B) STAT-6-deficient mice.

CONCLUSION

In chapter 1 we reported that BMOV induces phosphorylation of STAT-1 in a time-dependent fashion using human lung fibroblasts *in vitro*. This, in turn, induced CXCL10 up-regulation and secretion. This imitates the responses to interferon-γ (IFN-γ) which also induce CXCL10 production. Using mouse lung fibroblasts, we showed that BMOV stimulates CXCL10 expression in wild type mouse cells, but not STAT-$1^{-/-}$ mice. Taken together, these data indicate that BMOV is capable of inducing upregulation of CXCL10, but only in the presence of STAT-1. This suggests that BMOV activation of CXCL10 in lung fibroblasts is through a STAT-1 dependent mechanism and may prove to have beneficial interferon-like effects.

In chapter 2 we investigated whether BMOV retained its ability to induce production and secretion of CXCL10 in the presence of IL-13 in isolated fibroblasts. IL-13 and BMOV act through different STAT-dependent axes that have very different outcomes. While IL-13 acts through STAT-6 to mediate pro-fibrogenic pathways, BMOV acts through STAT-1 to mediate CXCL10, an anti-fibrotic protein. We sought to investigate whether these competing pathways interacted and how. We reported that by adding IL-13 to human lung fibroblast cell cultures, BMOV-induced CXCL10 is significantly reduced. In addition, BMOV activation of STAT-1 phosphorylation is also attenuated. In a reciprocal manner, BMOV reduces IL-13-induced STAT-6 phosphorylation in human lung fibroblasts indicating a point of convergence in these two pathways. In wild type mouse lung fibroblasts similar results were seen regarding the reduction of BMOV induced STAT-1 phosphorylation and CXCL10 secretion when cells were co-treated with IL-13. In STAT-6 deficient mice,

however, IL-13 lost its ability to interfere with the STAT-1 signaling axis. In addition, BMOV demonstrated inhibition of IL-13-induced STAT-6 phosphorylation demonstrating mutual antagonism between STAT-1 and STAT-6. Taken together, these data indicate that even though BMOV has interferon-like properties as demonstrated in its ability to activate the STAT-1 pathway, these beneficial effects could be restricted in the presence of IL-13. Extrapolation to *in vivo* models may demonstrate that mice with preexisting or induced asthma are less susceptible to fibrosis treatment. Future studies should explore an *in vivo* approach which may then elucidate whether BMOV delivered orally can effectively alter the pulmonary fibrosis disease outcome. In addition, *in vivo* studies may explore the interplay between STAT-1 and STAT-6 using a variety of mouse models available.

While certain vanadium compounds (e.g., vanadium pentoxide) are capable of causing acute inflammation which may lead to pulmonary fibrosis in humans in addition to rodents, these compounds also possess STAT-1 activating features much like interferons, which act to resolve fibrosis. Therefore, most vanadium compounds represent a "double-edged sword" where one edge represents toxicity and pro-fibrogenic activity while the other edge represents anti-fibrogenic activity. BMOV retains the beneficial "interferon-like" STAT-1 activation profile while lacking both the toxicity. However, a typical route of exposure for BMOV is oral administration while most toxic vanadium compounds found in pollutants are generally inhaled.

The potent protein tyrosine phosphatase 1 B (PTP1B) inhibition mediated by BMOV may play a role in the prolonged activation of STAT-1. We hypothesize that BMOV will retain the anti-fibrotic behavior found in V_2O_5 while lacking the causal elements of fibrosis.

BMOV's potential as an antifibrotic agent is demonstrated in its ability to (1) increase production of the protective interferon-inducible chemokine CXCL10, and (2) antagonize IL-13 induced STAT-6 phosphorylation which is a primary pathway involved in fibrogenesis. In addition to specifically activating the STAT-1 signaling axis, BMOV also has non-specific phosphatase inhibition properties which could possibly undermine its therapeutic potential. For example, prolonged phosphorylation of mitogenic kinase pathways are implicated in various cancers. This fact coupled with vanadium's proven ability to activate VEGF suggests that there is more work to be done regarding investigation into the use of therapeutic metals.

The need for novel treatments in the fight against pulmonary fibrosis is great. Current options are limited, highly risky, and have dubious effectiveness. The elucidation of the roles that STAT signaling play in the progression or remediation of fibrosis after the initial epithelial wound may yet lead to promising discoveries and improved treatment options for affected patients. Given BMOV's exciting potential in the treatment of diabetes, more toxicity data will likely become available from studies which capitalize this insulin mimetic utility.