Published in final edited form as:

Hum Immunol. 2014 December ; 75(12): 1155–1162. doi:10.1016/j.humimm.2014.10.010.

p38 Mitogen-Activated Protein Kinase in beryllium-induced dendritic cell activation

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Abstract

Dendritic cells (DC) play a role in the regulation of immune responses to haptens, which in turn impact DC maturation. Whether beryllium (Be) is able to induce DC maturation and if this occurs via the MAPK pathway is not known. Primary monocyte-derived DCs (moDCs) models were generated from Be non-exposed healthy volunteers as a non-sensitized cell model, while PBMCs from BeS (Be sensitized) and CBD (chronic beryllium disease) were used as disease models. The response of these cells to Be was evaluated. The expression of CD40 was increased significantly ($p < 0.05$) on HLA-DP Glu69+ moDCs after 100 μ M BeSO₄-stimulation. BeSO₄ induced p38MAPK phosphorylation, while I κ B- α was degraded in Be-stimulated moDCs. The p38 MAPK inhibitor SB203580 blocked Be-induced NF- κ B activation in moDCs, suggesting that p38MAPK and NF- κ B are dependently activated by BeSO₄. Furthermore, in BeS and CBD subjects, SB203580 downregulated Be-stimulated proliferation in a dose-dependent manner, and decreased Be-stimulated TNF- α and IFN γ cytokine production. Taken together, this study suggests that Be induces non-sensitized Glu69+ DCs maturation, and that p38MAPK signaling is important in the Be-stimulated DCs activation as well as subsequent T cell proliferation and cytokine production in BeS and CBD. In total, the MAPK pathway may serve as a potential therapeutic target for human granulomatous lung diseases.

Keywords

DCs; CBD; Lymphocyte proliferation; p38MAPK; NF- κ B

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Conflict of interest statement

None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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1. Introduction

Chronic beryllium disease (CBD) develops in up to 16% of individuals exposed to beryllium (Be) and is characterized by granulomatous inflammation and the accumulation of T cells in the lung [1]. Using the Be lymphocyte proliferation test (BeLPT) we have identified workers with BeS, demonstrating an immune response to Be with an abnormal BeLPT, but no evidence of CBD [2–4]. While BeS precedes CBD, the immune changes that cause progression from BeS to CBD are unclear. However, at some point accumulation of CD4+ T cells are noted in the lung, and once these Be-specific CD4+ T cells are activated, they clonally proliferate and secrete cytokines such as IL-2, IFN- γ , and TNF α but not IL-4 [5–7]. This process results in granulomatous inflammation and perpetuation of this Be-immune response. Susceptibility to CBD and BeS is strongly associated with the MHC class II molecule, HLA-DPB1, with a negatively charged glutamic acid residue at the 69th position of the β -chain (Glu69+)[8–12]. Studies have indicated that HLA-DP is important in antigen presentation, as antibodies to HLA-DP are able to block both proliferation noted on the BeLPT, along with Be-stimulated cytokine production in CBD [13]. The features of the antigen presenting cells (APCs) responsible for antigen presentation via Glu69 are unclear at this time, but probably contribute to the development of the Be immune response.

DCs are unique professional APCs whose primary function is to capture, process, and present antigens to unprimed T cells. Immature Dendritic cells (iDCs) reside in nonlymphoid tissues where they can capture and process antigens. Thereafter, DCs migrate to the T cell areas of lymphoid organs where they lose Ag-processing activity and mature to become potent immunostimulatory cells [14–16]. Fully mature DCs show a high surface expression of MHC class II and costimulatory molecules (e.g. CD40, CD80, CD83 and CD86) but a decreased capacity to internalize Ags [17–19]. In addition, DCs play a major role in the regulation of immune responses to a variety of haptens, while haptens in turn participate in the process of DCs maturation [20, 21]. The role of DCs function and maturation in development of BeS and CBD is currently not well understood.

Haptens such as nickel and 1-chloro-2, 4-dinitrobenzene induce members of the Mitogen-activated protein kinase (MAPK) family, namely, extracellular regulated kinases (ERKs), jun kinases (JNK), and p38 MAPK in DC maturation [22, 23]. Our understanding of the role of these pathways in various biological processes has been improved by the availability of ERK- and p38-specific inhibitory drugs used to block these pathways in research studies [24, 25]. In particular, the p38 MAPK-specific inhibitor, the pyridinyl imidazole compound SB203580, has facilitated extensive investigation of the role of the p38 MAPK pathway in these inflammatory responses. In contrast to the MAPK pathway, the involvement of the NF- κ B pathway in response to metallic hapten exposure is still poorly understood. However, both the MAPK and NF- κ B pathways are involved in DC maturation induced by danger signals such as toll-like receptor (TLR) agonists [26].

Based on the above information, the aim of this study was to elucidate if Be is able to induce DCs activation in a manner similar to nickel and other haptens, as well as proinflammatory cytokines and danger signals such as lipopolysaccharide (LPS), which use common signal

transduction pathways including MAPKs and NF- κ B. Thus, we hypothesized that MAPKs and NF- κ B are stimulated by Be, and result in DC maturation, activation and cell proliferation and cytokine production, filling a gap in our understanding of the development of BeS and CBD.

2. Materials and Methods

2.1. Study populations

Eight patients with a diagnosis of CBD and eight with BeS were enrolled in this study at National Jewish Health (NJH). The diagnosis of CBD was established using previously defined criteria, including the presence of granulomatous inflammation on lung biopsy, and a positive proliferative response of blood and/or BAL T cells to BeSO₄ in vitro (5–7). The diagnosis of BeS was established based on a positive proliferative response of blood cells to BeSO₄ in vitro using the BeLPT, as well as the absence of granulomatous inflammation or other abnormalities on lung biopsy noted at the time of their enrolment in this study. Active smokers were excluded from enrollment. To evaluate the maturation of DCs, we utilized WBCs derived from normal healthy non-smoking blood donors without known prior exposure to Be. Buffy coats were obtained from these normal healthy volunteers (n = 8), enrolled at the University of Colorado Denver (UCD). Genotyping for HLA-DP β 1 was obtained for the blood donors and yielded 4 subjects with HLA-DP β 1 Glu69+ genotype and 4 without.

Informed consent was obtained from each patient, and the protocol was approved by the Human Subject Institutional Review Boards at UCD and NJH.

2.2. Cell isolation

2.2.1—Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient centrifugation. The viability of obtained PBMCs was always >95%, as determined by trypan blue staining. The viable cells were quantified in a Neubauer chamber (Zeiss, Oberkochen, Germany) and leftover cells were cryopreserved in liquid nitrogen.

2.2.2. Generation of moDCs—DCs were generated from buffy coats from the normal healthy volunteers [27]. In brief, PBMCs were seeded (1×10^6 cells/cm²) into culture flasks (Nunc, Roskilde, Denmark) in RPMI1640 medium supplemented with 10% heat-inactivated calf serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 units/ml streptomycin. After 2 h of incubation at 37°C, non-adherent cells were removed and the adherent CD14+ blood monocytes (purity >80% by FACS analysis) were cultured in RPMI1640 medium supplemented with 100 ng/ml human GM-CSF (Leukomax, Novartis, Basel, Switzerland), 1,000 IU/ml IL-4 (Strathmann, Hannover, Germany) and 10% heat-inactivated calf serum. For the maturation of the cells, the medium was changed on day 6. The new medium contained GM-CSF and IL-4 in the concentrations given above, as well as 50 ng/ml TNF- α (Strathmann, Hannover, Germany). The DC cultures were fed with fresh medium and cytokines every three days and cell differentiation was monitored using inverse light microscopy.

2.3. SSP-PCR determination of the HLA-DP β 1 loci

Genomic DNA was prepared from blood PAXgene tubes according to the manufacturer's instructions. HLA-DP β 1 genotyping was performed with blinding to the subject's disease status using SSP-PCR methodology as described by Bunce et al. [28] and Gilchrist et al. [29] providing specific HLA-DP β 1 alleles. Individuals were classified as Glu69+ or –.

2.4. Western blotting

MoDCs (obtained at day 7) were exposed to 10 μ M or 100 μ M BeSO₄ for 0 min, 30 min, 1h and 2h. Cells were treated with TNF α for 30 min as a positive control. For whole cell lysate preparation, 2 – 3 $\times$ 10⁶ cells were lysed using RIPA (Radioimmunoprecipitation) buffer containing protease inhibitors (Santa Cruz, CA). Cell lysates were left on ice for 30 min and vortexed 3 times briefly and followed by centrifugation at 17,600 g at 4°C for 20 min. Nuclear fractionation of cells was performed using the NE-PER Nuclear and Cytoplasmic Extraction kits (Thermo Fisher Scientific) following the manufacturer's protocol. Protein concentration was determined using the bicinchoninic acid (BCA) assay (Sigma). 20 μ g proteins were subjected to 10% SDS-PAGE. The proteins were transferred onto PVDF membranes (Amersham Biosciences, Les Ulis, France) and the membranes were probed with the rabbit anti-I κ B- α polyclonal Ab (C-21, Santa Cruz Biotechnology, Santa Cruz, CA), anti-NF- κ B p50 (NLS, Santa Cruz Biotechnology, Santa Cruz, CA) or the rabbit anti-phospho-p38 MAPK (Thr 180/Tyr 182) monoclonal Ab (3D7, Cell Signaling Technology, Ozyme, St-Quentin-en-Yveline, France) followed by goat anti-rabbit polyclonal Ab conjugated to horse radish peroxidase (Cell Signaling Technology). The membranes were stripped for the primary Abs and reprobed with Ab raised against total p38 MAPK as a loading control (p38 N20, Santa Cruz Biotechnology). The immunoblots were visualized by enhanced chemiluminescence (Amersham Biosciences). Densitometric analysis of the blots was performed using the Quantity One Software (Bio-Rad Laboratories, CA).

2.5. Flow cytometry analysis

After 24h treatment with 10 μ M or 100 μ M BeSO₄, moDCs were washed in FACS medium (PBS containing 1% BSA) and stained at 4°C for 20 min by antibodies directly conjugated with FITC or PE. Thereafter cells were washed three times with PBS and analyzed by FACScalibur (Becton Dickinson, Heidelberg, Germany) using the CellQuest software (Becton Dickinson). The following antibodies were used: FITC-labeled anti-mouse IgG, anti-human HLA-DR, CD40, CD83, as well as PE-labeled anti-mouse IgG, anti-human CD80 and CD86 (Becton Dickinson). Double staining was performed using pairs of PE- and FITC-labeled antibodies.

2.6. PBMC culture and Beryllium-induced lymphocyte proliferation testing (BeLPT)

Isolated PBMCs were adjusted to 1 $\times$ 10⁶ cells/ml in RPMI complete medium with 10% heat-inactivated calf serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 units/ml streptomycin. 200 μ l aliquots were then cultured in four wells per treatment condition, using 96-well round-bottom plates (Costar 3799; Corning, Inc., Corning, NY), to yield a final concentration of 0.2 $\times$ 10⁶ cells per well. Cells were treated by 0, 10 μ M or 100 μ M BeSO₄.

0, 1 μ M or 10 μ M SB203580 (dissolved in distilled water, Calbiochem) was added 30 min before treatment with BeSO₄ for the p38 MAPK inhibitor blocking experiments. Cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂ for 4 days. Proliferation was measured as 0.5 ICi [³H]thymidine (Amersham, Arlington Heights, IL) incorporation for 6 h. Radioactivity levels were determined by a liquid scintillation counter-1205 Betaplate (Wallac) and proliferation was expressed as ratio (stimulation index, SI) of the average Be stimulated cpms divided by control cpms.

2.7. Cytokine measurements

TNF- α and IFN- γ production were measured in supernatants collected 24 h after treatment. Levels of TNF- α and IFN- γ were determined using ELISA kits (R & D Systems, Minneapolis, MN) with a sensitivity of 7.8 pg/ml as previously described [30]. Matched antibody pairs and standards (capture and detection) for TNF- α and IFN- γ was obtained from R & D Systems (Minneapolis, MN).

2.8. Statistical Analysis

The Kruskal-Wallis test was used to determine the effect of treatments. After the data were checked for significant treatment differences, individual contrasts were calculated to compare treatment means of interest. All other comparisons were made using Wilcoxon signed rank test to assess the statistical significance of experimental data for continuous variables. Statistical analysis of the data was performed using SAS version 9.4. A $p < 0.05$ was used to determine statistical significance.

3. Results

3.1. Clinical parameters, blood and BAL fluid data

The demographics of the BeS and CBD patients are shown in Table 1. No difference was seen in the age of the BeS and CBD patients enrolled in this study. The majority of both subject groups were male, typical of Be industries. No significant difference in the estimated duration of Be exposure was observed in BeS and CBD subjects. CBD subjects had a statistically significant increase in the percentage of BAL lymphocytes compared with BeS patients (median 28.5, range 9.5–57, versus median 6.05, range 1.0–13; $P < 0.05$). It was shown the high frequencies of individuals carrying HLA-DP β 1 Glu69+ genotype from CBD (88%) and BeS (100%). These data are consistent with published data on the frequency of the Glu69 marker in the CBD.

3.2. BeSO₄ induces phenotypical changes of moDCs

MoDCs generated from 4 HLA-DP β 1 Glu69+ and 4 Glu69– normal healthy volunteer subjects were cultured for 7 days with GM-CSF and IL-4 and incubated in the presence of BeSO₄. CD40, CD80, CD86 and HLA-DR were measured after 24h. Based on our previous data demonstrating that BeSO₄ is toxic to cells at concentrations greater than 100 μ M, and our studies showing optimal cytokine production between 10–100 μ M concentrations, we determined the optimal concentrations of Be in this range for DC activation. As seen in Fig 1, the expression of CD40 on DC with HLA-DP β 1 Glu69+ genotype were induced by BeSO₄, with a significant increase $p < 0.05$ after 100 μ M BeSO₄-stimulation (Fig 1a). CD80,

CD86 and HLA-DR expression were not significantly up-regulated by Be (Fig 1b, 1c, 1d). The expression of all of these cell surface markers on DCs with HLA-DP β 1 Glu69– genotypes were unchanged by BeSO₄ (data not shown).

3.3. Both NF- κ B and p38MAPK are stimulated in Be-induced Glu69+ moDCs

The level of I κ B and the phosphorylation of p38MAPK were evaluated by Western blotting. Total p38MAPK levels were determined as a loading control and a ratio of I κ B to total p38MAPK in normal healthy controls moDCs. Results showed that I κ B- α (dashed line) was increased after 30 min of 100 μ M BeSO₄ treatment and 10 μ M BeSO₄ treatment (Figure. 2b), with a peak at 30 min for 100 μ M BeSO₄ and 1 hour after 10 μ M BeSO₄ treatment but only in Glu69+ subjects. Phosphorylation of p38 (straight line) was detected at 30 min of 100 μ M BeSO₄ treatment and at 1h of 10 μ M BeSO₄ treatment, again only in Glu69+. There was no significant change in subjects with the Glu69– genotype (data not shown). These results suggest that both NF- κ B and p38MAPK were stimulated by Be but only in Glu69+ subjects.

3.4. The p38 MAPK regulates Be-stimulated NF- κ B activation in Be-induced Glu69+ moDCs

To further elucidate the signaling pathway involved in NF- κ B activation, we studied the relationship between p38MAPK and NF- κ B activation in normal healthy Glu69+ moDCs pretreated with 10 μ M SB203580, a p38 MAPK-specific inhibitor, for 1 h and further stimulated with 10 μ M or 100 μ M BeSO₄ for 1h. As shown in Figure 3, 10 μ M SB203580 significantly decreased the nuclear NF- κ B p50 signal after stimulation with Be. This suggests that p38MAPK regulates Be-induced NF- κ B in Glu69+ moDCs.

3.5. The p38 MAPK Inhibitor SB203580 down regulates both the Be-stimulated CBD and BeS PBMCs proliferation

The proliferation of Be-specific PBMCs from CBD (n = 8) and BeS (n = 8) subjects was determined using the BeLPT. Be-stimulation with 10 μ M BeSO₄ induced the proliferation of CBD PBMCs (average SI = 3.3 ± 1.1) to a greater extent than BeS PBMCs (average SI = 1.8 ± 0.8), and 100 μ M BeSO₄ induced proliferation of CBD PBMCs (average SI = 4.5 ± 1.9 , Figure 4a) to a greater extent than BeS PBMCs (average SI = 1.8 ± 0.4 , Figure 4b). After treatment with different concentration of SB203580 (1 or 10 μ M), both CBD and BeS PBMCs proliferation was inhibited in a dose-dependent manner, although 10 μ M SB203580 fully inhibited the BeLPT response (Figure 4). Specifically, the SI was significantly decreased in CBD PBMCs stimulated with 10 μ M and 100 μ M BeSO₄ and treatment with 10 μ M SB203580 (SI = 1.0 ± 0.2 and 1.6 ± 0.6 , $p < 0.01$ versus Be-stimulation alone, Figure 4a). Similarly, a decrease in proliferation was observed among BeS PBMCs stimulated with both 10 μ M and 100 μ M BeSO₄ and treated with 10 μ M SB203580 (SI = 0.6 ± 0.1 and 0.6 ± 0.1 , $P < 0.01$ versus Be-stimulated alone).

3.6. The p38 MAPK Inhibitor SB203580 down regulates the Be-stimulated CBD PBMCs cytokine production

Be-stimulated CBD PBMCs TNF α (Fig 5a) and IFN γ (Fig 5b) cytokine production was also decreased by SB203580 in a dose-dependent manner (n=3). The ability of PBMCs to produce 10 μ M BeSO₄-stimulated TNF α (median: 254 pg/mL) was significantly reduced by

approximately 70% to 32% of the initial amount, when cells were co-treated with 1 μ M SB203580 ($p<0.01$) and by approximately 85%, to 16% of the initial amount with 10 μ M SB203580 ($p<0.05$). While not statistically significantly, the 10 μ M BeSO₄-stimulated IFN γ (median: 261 pg/mL) was reduced by approximately 20% when co-treated with 1 μ M SB203580 and by approximately 40% with 10 μ M SB203580, with a similar trend noted with 100 μ M Be-stimulation. Be-stimulated BeS PBMCs ($n=3$) TNF α and IFN γ cytokine levels were not detectable (data not shown).

4. Discussion

The objective of the present study was to understand whether Be induces DC maturation and T-cell proliferation via the MAPK and NF- κ B pathways in non-sensitized and sensitized cell models and to confirm the impact of these pathways in CBD. Using normal healthy controls, we were able to demonstrate that Be-stimulates a marker of DC maturation, specifically CD40. Interestingly, Be-stimulated DC maturation as indicated by CD40 upregulation, was only observed in Glu69+ control cells and not Glu69– cells. This may be a key step in the development of sensitization, suggesting the limitation of this response by Glu69 status. Subsequently, we determined that p38MAPK signaling was upregulated by Be in those who were Glu69+, a potential link between DC activation and the Be-stimulated immune response. We also demonstrated that Be-stimulated upregulation of NF- κ B is inhibited by the p38MAPK inhibitor SB20358 only in those who were Glu69+, confirming the role of p38MAPK and Glu69 in this response. Finally, we confirmed the importance of the p38MAPK pathway in CBD and BeS T cell proliferation and cytokine production, demonstrating that a p38MAPK inhibitor was able to reduce key cellular responses triggered by Be. This data suggests that the p38MAPK pathway is likely important in the immune response to Be, potentially by impacting the ability of DCs to mature in response to Be, and to augment the immune response to Be in CBD. These data advance our current level of understanding of the Be-induced inflammatory processes in the development of BeS and CBD.

DCs play a major role in the regulation of immune responses to a variety of haptens. In addition, these haptens appear to participate in the process of DC maturation [23, 26]. While it is known that Be acts as a hapten and modify the immune response, Be's impact on DC maturation and the mechanism of this maturation has not been well understood. Studies to date indicate that Be-exposure in non-diseased human and animal cell models may modify and stimulate a variety of cellular responses, including cell migration [31], cytokine regulation [32], and growth inhibition [33]. These immunomodulatory activities suggest that Be may trigger an innate immune response in non-sensitized individuals. There is also evidence that Be can act as an adjuvant that promotes IFN- γ production in BALB/c mice immunized with soluble leishmanial antigens (SLA) and IL-12, however, no specific response were observed to Be alone [34]. Acting as an adjuvant, Be may amplify the immune response in a non-specific manner and/or once BeS and or CBD has developed as well. Although this response may not necessarily lead to the manifestation of disease in healthy individuals, the induction of lung inflammation and injury upon Be-stimulation may contribute to the toxicity and pathogenesis of Be in genetically susceptible individuals, i.e. in Glu69+ workers and may play a role in the development of BeS. A number of animal

studies [35,36] and recent studies of Glu69 transgenic mice [37] suggest that differences in MHC genes between strains can influence the immunotoxicity of Be. Since mice have no HLA-DP1 Glu69 equivalent alleles and demonstrate a limited immune response to Be, it supports our notion that Be-stimulated Glu69-DC maturation response is limited by genetics.

To address these questions, we utilized a non-sensitized primary moDCs model which was generated from Be unexposed healthy volunteers with either an HLA-DP β 1 Glu69+ or Glu69- genotype, since CBD and BeS susceptibility has been strongly associated with this MHC class II molecule. The following markers were selected to assess the MoDC phenotype: CD40, CD80, CD86, and HLA-DR, as these markers on DCs are known to promote lymphocyte activation. Our data showed that 100 μ M BeSO₄ significantly upregulated the expression of CD40 ($p < 0.05$), while CD80, CD86, and HLA-DR were unchanged. Among HLA-DP β 1 Glu69- DCs, these markers were unchanged or slightly decreased by BeSO₄. These results suggest that even in cells that are not sensitized to Be, that Be is able to induce markers of macrophage maturation in those with an HLA-DPB1 Glu69. This could be an early step initiating or perpetuating the immune response to Be in Be-exposed individuals, ultimately allowing them to develop an adaptive immune response to Be and potentially BeS.

Both in vivo and in vitro studies have shown the importance of MAPKs and NF- κ B in DC maturation induced by TLR agonists or by inflammatory cytokines [26, 38]. Moreover, metallic haptens (e.g. NiSO₄) appear to induce the activation of both pathways along with DC maturation [20, 21, 24]. We showed that I κ B- α was upregulated and degraded after 30 min of 100 μ M BeSO₄ treatment and 1h of 10 μ M BeSO₄ treatment in our Glu69+ but not Glu69- MoDC model. Similarly, phosphorylation of p38 was detected at 30 min of 100 μ M BeSO₄ treatment and 1h of 10 μ M BeSO₄ treatment. These results suggest that both NF- κ B and p38MAPK maybe stimulated by Be during DC maturation; it is also possible that they are key regulators of DC maturation. As it has previously been reported that the p38 pathway can influence NF- κ B activation, at least partly, through the physical association of MKK6 and IKK α [39], we examined the relationship between p38MAPK activation and NF- κ B. Our results demonstrating that inhibition of p38 MAPK with 10 μ M SB203580 significantly decreased NF- κ B signal, strongly suggests that p38MAPK regulates Be-induced NF- κ B activation.

Recently it was reported that metals like Cobalt containing alloys, and nickel and palladium can trigger an inflammatory response by directly activating human Toll-like receptor 4 (TLR4) on DC (40, 41, 42, 43). The DC activation was monitored by assessment of release of the pro-inflammatory mediator interleukin (IL)-8, a major downstream result of TLR ligation (40, 41,42). In addition, some metal haptens, such as titanium particles (Ti) and diet with nickel chloride (NiCl₂) can down-regulate the expression of TLR4 (44, 45, 46). However, in our study, there was no significant change in IL-8 after Be stimulation (data not shown). Furthermore, Be does not appear to alter the TLR4 mRNA expression levels in either PBMCs or BAL cells in CBD, BeS or healthy controls nor alter the TLR4 protein expression on the moDC (data not shown). It does not appear that Be can activate TLR4 expression directly, and our data suggests that the inflammatory response triggered by Be

occurs through another path, distinct from these metals. However, whether it is involved in other TLR signaling and or alteration is a goal of future studies.

Once an adaptive immune response to beryllium is established, Be-stimulates activation and proliferation of a subset of Be-specific CD4⁺ pathogenic T cells and secretion of pro-inflammatory cytokines that in-turn amplifies granuloma formation in CBD [5, 47]. To investigate the role of p38MAPK in this process of Be-related disease, we evaluated the impact of the p38MAPK inhibitor SB203580 on the Be-specific adaptive immune response. Specifically, we determined that Be-stimulated proliferation was downregulated in CBD and BeS by the p38 inhibitor, SB203580, in a dose-dependent manner in BeS and CBD, as was Be-stimulated CBD PBMCs TNF- α and IFN- γ cytokine production. In another clinically and pathologically similar granulomatous disease, of unknown cause, sarcoidosis a recent study by Rastogi et al [48] demonstrated the importance of the p38MAPK pathway in granulomatous inflammation finding basal activation of p38MAPK in sarcoidosis alveolar macrophages, that was increased further by TLR4 or NOD1 stimulation. Coupled with functional assays, this study showed that inhibition of p38MAPK abrogated the production of key cytokines relevant to sarcoidosis in response to TLR4 and NOD1 ligands; these results are similar to ours demonstrating that p38 inhibition downregulates Be-stimulated cytokine production in CBD. While the sarcoidosis study used a non-specific stimulant of the immune response (eg TLR4 and NOD1) and we used an Ag-specific stimulant, Be, these data together suggest that p38MAPK is upregulated in granulomatous inflammation and likely plays a pivotal role in supporting chronic inflammation in these granulomatous diseases.

In conclusion, our study suggests that Be induces DC maturation along with the p38MAPK pathway in Glu69⁺ subjects. In turn, this pathway may further enhance Be-induced DC activation and once an adaptive immune response is established, T cell proliferation and cytokine production. Future studies will focus on the role of other aspects of the MAPK pathway in Be-induced DC activation and T cell proliferation, such as ERK and JNK, as well as their possible interactions. It will be necessary to clarify whether these signaling differences are unique to CBD and sarcoidosis or are generalizable to other processes characterized by Th1 cytokine expression. These subsequent studies may help reveal the function of MAPKs in the Be disease and raise the hope that the therapeutic benefits of selectively modulating MAPK activity may become clinically applicable to the treatment of granulomatous lung diseases in the future. Based on our data we are optimistic that the MAPK pathway may serve as a potential therapeutic target for rational drug development with the goal of inhibiting p38 activation for human granulomatous lung diseases. In addition, the results provided in our study and future studies may improve our understanding of factors involved in the development of BeS and CBD and serve as a model of exposure-related immunologic diseases, including sarcoidosis with application to other environmentally induced diseases.

Acknowledgments

This work was supported by NIH K12 HL090147-01, K01ES020857-01, P01 ES11810-06A1, and UL1 TR000154 from NIH/NCATS.

Abbreviations

APCs	antigen presenting cells
Be	Beryllium
BeLPT	Be lymphocyte proliferation test
BeS	Be sensitized
CBD	Chronic beryllium disease
DCs	Dendritic cells
iDCs	Immature Dendritic cells
ERKs	extracellular regulated kinases
JNK	jun kinases
LPS	lipopolysaccharide
MAPK	Mitogen-activated protein kinase
moDCs	monocyte-derived DC
PBMCs	Peripheral blood mononuclear cells
TLR	toll-like receptor
RIPA buffer	Radioimmunoprecipitation buffer

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Fig 1.

The effect of BeSO₄ on the phenotype of moDCs with HLA-DP β 1 Glu69+ genotype. At day 7, moDC were treated with 10 or 100 μ M BeSO₄ for 24 h. Cell surface markers were measured using flow cytometry. Numbers represent the percentage of positive cells after Be treatment. The expression of (1a) CD40 was increased significantly from moDCs in Glu69+ subjects after after 100 μ M BeSO₄ stimulation for 24h on day 7 (* p<0.05, n=4). There was no significant difference on the expression of (1b) CD80, (1c) CD86 and (1d) HLA-DR after Be-stimulation.

Fig 2.

Both NF- κ B and p38MAPK were stimulated by Be in the monocyte DC Glu69+ cell models. At day 7, moDC were treated for different time periods (0, 30 min, 1h and 2h) with 10 μ M or 100 μ M BeSO₄. The levels of IKB and the phosphorylation of p38MAPK were evaluated by Western blotting. Total p38MAPK levels were determined as a loading control. Results of one representative subject out of four are shown, with similar results noted for other subjects. The conditions presented by lane are as follows: 1. DC Control, 2. DC with 10 μ M BeSO₄ for 30 min, 3. DC with 100 μ M BeSO₄ for 30 min, 4. DC with 10 μ M BeSO₄ for 1h, 5. DC with 100 μ M BeSO₄ for 1h, 6. DC with 10 μ M BeSO₄ for 2h, 7. DC with 100 μ M BeSO₄ for 2h.

Fig 3.

The p38 Mitogen-Activated Protein Kinase Inhibitor SB203580 regulates Be-induced NF-κB activation in Glu69+ subjects. At day 7, moDCs were treated for different time periods with 10μM or 100μM BeSO₄ in the presence or absence of 10μM SB203580. The nuclear level of NF-κB p50 and the actin were evaluated by Western blotting. actin levels were determined as a loading control and results of one representative subject are shown, with similar results noted for the other subjects. The conditions presented by lane are as follows: 1. DC Control, 2. DC with TNF for 30 min; 3. DC with 10μM SB203580, 4. DC with 10 μM BeSO₄ for 1h, 5. DC with 10μM SB203580 and 10μM BeSO₄ for 1h, 6. DC with 100 μM BeSO₄ for 1h, 7. DC with 10μM SB203580 and 100μM BeSO₄ for 1h.

Figure 4.

The p38 Mitogen-Activated Protein Kinase Inhibitor SB203580 inhibits the Be-stimulated (a) CBD and (b) BeS PBMCs proliferation. The stimulation index (SI) of Be-stimulated (a) CBD PBMCs (n=8) and (b) BeS PBMCs (n=8) treated with 10µM or 100µM BeSO₄ and 1µM or 10µM SB203580. Values are represented as mean stimulation index (SI). **p<0.01.

Figure 5.

The p38 Mitogen-Activated Protein Kinase Inhibitor SB203580 treatment inhibits Be-stimulated CBD PBMC cytokines (a) TNFα and (b) IFNγ production (n=3). PBMCs were treated with 10μM or 100μM BeSO₄ and 1μM or 10uM SB203580. The data values were normalized according to each subject's 10μM Be-stimulated TNFα and IFN γ levels.

*p<0.05, **p<0.01.

Table 1

Clinical characteristics of BeS and CBD subjects.

	BeS (n=8)	CBD (n=8)
Age (yr)	59 (47–78)	60 (49–79)
Gender (M/F)	6/2	7/1
Race (W/AA/H)	7/0/1	6/1/1
Smoking status (CS/FS/NS)	0/0/8	0/0/8
HLA-DPβ1 Glu69 (+/-)	8/0	7/1
Beryllium exposure (yr)	24.5 (12–47)	39 (29–44)
BAL cells		
WBC count ($\times 10^6$)	25 (9.7–41)	25.5 (7.9–104)
Lymphocytes (%)	6.05 (1–13)	28.5 (9.5–57)

Data are expressed as median (range). W, white; AA, African American; H, Hispanic; CS, current smoker; FS, former smoker, NS, never smoker