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Cereblon expression in human peripheral blood mononuclear cells in response to *in vitro* stimulation with *M. leprae*

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Summary
Background The host immune responses associated with the clinical phenotypes of *Mycobacterium leprae* infection are not fully understood. The inflammatory complications of leprosy, leprosy reactions, particularly erythema nodosum leprosum (ENL), present therapeutic challenges. Thalidomide is an effective drug for ENL but is not widely available due to teratogenicity. Thalidomide binds cereblon (CRBN), a substrate receptor for the E3 ligase complex, promoting ubiquitination. Thus, we investigated the CRBN expression in human peripheral blood mononuclear cells (PBMCs) in response to *in vitro* stimulation with *M. leprae* with/without CRBN blockade peptide.
Methods Blood samples were obtained from apparently BCG-vaccinated and BCG-unvaccinated healthy volunteers. PBMC was isolated and stimulated with irradiated *M. leprae* with or without CRBN blockade peptide. CRBN, NF-kB, and *PARK2* proteins were determined by ELISA, and their gene expression by qPCR.
Results Stimulation with *M. leprae* significantly increased *CRBN* gene expression and protein production. Incubation of PBMCs with *M. leprae* with *CRBN* blockade significantly increased *NF-kB* expression. In a subgroup analysis, *CRBN* and *NF-kB* gene expression following stimulation with *M. leprae* ($p \leq 0.05$) was significantly higher in PBMCs from *Mycobacterium bovis* bacillus Calmette–Guérin (BCG)-vaccinated individuals compared to those from unvaccinated participants. *PARK2* gene expression and parkin protein were significantly decreased in PBMCs stimulated with *M. leprae* compared to unstimulated PBMCs ($p \leq 0.05$). In a subgroup analysis, *PARK2* gene expression and parkin protein were decreased in the PBMCs from BCG-unvaccinated individuals following incubation with *M. leprae* compared to those from BCG-vaccinated individuals. Stimulation of the PBMCs with *M. leprae* with CRBN blockade increased *PARK2* gene expression and parkin protein production ($p \leq 0.05$).
Conclusion The findings are evidence that CRBN may have a role in modulating *PARK2* and *NF-kB* gene expression in response to *M. leprae* infection. This needs

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further investigation in individuals with leprosy. The differential gene expression of *CRBN* and *PARK2* in BCG-vaccinated and BCG-unvaccinated individuals could be further explored to understand the mechanism of BCG protection against leprosy.

Keywords: Cereblon, leprosy, *Mycobacterium leprae*, *NF-kB*, *PARK2, parkin*

Introduction

Leprosy is caused by infection with *Mycobacterium leprae* or *Mycobacterium lepromatosis*. It mainly affects the skin and peripheral nerves.[1,2] Approximately 40% of individuals with leprosy develop acute immune-mediated inflammatory complications known as leprosy reactions.[3] Leprosy reactions are immunologically mediated and can occur even after successful completion of antibacterial multi-drug therapy (MDT). The reactions may cause severe and irreversible nerve damage and require prompt treatment.

There are two types of leprosy reactions: Type-1 reactions and erythema nodosum leprosum (ENL). Leprosy reactions may occur before, during, or after successful completion of antimicrobial therapy. ENL complicates lepromatous leprosy (LL), where affected individuals have high mycobacterial antigen loads. ENL is associated with severe impact on health-related quality of life.[4]

In most leprosy-endemic countries, ENL is treated with corticosteroids, which are associated with major adverse effects, including death.[5] Thalidomide was reported to be an effective treatment for ENL in 1965 but is not widely available in many leprosy-endemic countries. Thalidomide and its analogues are now used in the management of myeloma. Recent studies have demonstrated that the immunomodulation of thalidomide is through binding to a protein called cereblon (CRBN).[6,7] Low CRBN expression is associated with thalidomide resistance in myeloma.[8,9] Currently, a low CRBN expression level is used to predict resistance to immunomodulatory monotherapy in myeloma. CRBN is a predictive biomarker for survival: high levels serve as a surrogate marker for low risk of mortality in myeloma.[10,11]

CRBN is a cytoplasmic and nuclear protein located in tissues.[6,12] CRBN is a component of the E3-ubiquitin-ligase complex[13] and interacts with the proteasome for non-lysosomal protein degradation in cells.[6] It forms an E3-ubiquitin-ligase complex, which ubiquitinates proteins and hence degrades unwanted proteins such as microbial antigens and tumour cells.

PARK2 gene polymorphism has been identified as a major risk factor for leprosy.[14,15] *PARK2* is expressed in Schwann cells and macrophages.[16] These cells are the primary sites where *M. leprae* resides. *PARK2* genes code for parkin, which degrades unwanted proteins by tagging them with ubiquitin. Tagged ubiquitin, parkin, and CRBN form the E3-ubiquitin-ligase complex.[17]

E3-ubiquitin-ligases also regulate clonal anergy and immune self-tolerance, and CRBN is found to be a novel E3-ubiquitin-ligase regulator.[8,9] T cell anergy (unresponsiveness) is a characteristic of individuals with LL,[18] and reversal of T cell anergy has been implicated in ENL reaction.[19,20]

CRBN deficiency increased $CD4^+$ T-cell activation and enhanced IL-2 secretion, helping $CD4^+$ T-cells differentiate into Th17 cells.[21] CBRN blockade enhances effector memory cell activation and causes T-cell-mediated autoimmune disorders and delayed-type hypersensitivity reactions in experimental animals.[22,23] CRBN downregulates the Toll-like receptor 4 (TLR4)-mediated signalling pathway and reduces NF-κB expression and pro-inflammatory cytokine production.[24] *M. leprae* activates TLR4 in macrophages and dendritic cells.[25]

There is circumstantial evidence that CRBN expression has biological significance in leprosy. CRBN regulates the PARK2 gene, a gene described as a risk factor for leprosy. CRBN also regulates ubiquitination through binding to E3 ligases and hence affects the outcome of intracellular pathogens or their antigens. It serves as a receptor protein for thalidomide, a drug used for ENL treatment. CRBN gene variants are associated with the adverse effects of thalidomide treatment in individuals with ENL.[26] Hence, understanding CRBN expression in *M. leprae*-stimulated human peripheral blood mononuclear cells (PBMCs) may provide insights to understand how *M. leprae* infection progresses to clinical disease. We investigated the expression of CRBN in *in vitro* stimulated human PBMCs with *M. leprae* in BCG-vaccinated and BCG-naive individuals.

Materials and methods

ETHICAL STATEMENT

Informed written consent for blood samples was obtained from participants following approval of the study by the London School of Hygiene & Tropical Medicine Research Ethics Committee (LSHTM17786), UK. All participant data were analysed and reported anonymously.

BLOOD SAMPLE COLLECTION AND PBMC ISOLATION

Children below 18 years old, adults above 65 years old, pregnant and lactating females, and individuals on any medication were excluded from the study. Thirty mL of venous blood was collected from 33 healthy volunteers into sterile BD Heparinized Vacutainer tubes (BD, Franklin Lakes, NJ, USA) and used for PBMC isolation. The volunteers were from 14 countries: UK (14), Tanzania (2), Ghana (2), Ethiopia (2), India (2), Gambia (2), South Sudan (2), USA (2), Brazil (1), South Africa (1), Mali (1), Spain (1), Eritrea (1), and Sudan (1).

PBMCs were separated by density gradient centrifugation at 850 × g for 20 min on Ficoll–Hypaque (Histopaque, Sigma Aldrich, UK) as described earlier.[27] Cells were washed three times in sterile phosphate-buffered saline (Sigma Aldrich, UK) and resuspended with 1 mL of Roswell Park Memorial Institute (RPMI medium 1640 + GlutaMAX + Pen-Strep GBICO, Life Technologies, UK). Cell viability was determined using 0.4% sterile Trypan Blue solution (Sigma Aldrich, UK); the viability was between 96 and 100%. PBMC freezing was performed using a cold, freshly prepared freezing medium composed of 20% foetal bovine serum (FBS, heat inactivated, endotoxin tested <5 EU/ml, GIBCO Life Technologies, UK) and 20% dimethyl sulfoxide in RPMI medium 1640. Cells were kept at −80 °C for 2 to 3 days and transferred to liquid nitrogen until use. Cell thawing was done as described by.[28] The procedure is briefly described as follows: cells were incubated in a water bath (37 °C) until thawed halfway and re-suspended in 10% FBS in RPMI medium 1640 (1×) (37 °C) containing 1/10,000 benzonase until completely thawed, washed two times (5 min each), and counted. The percentage viability obtained was above 90%.

PBMCS STIMULATION TESTS WITH OR WITHOUT CRBN BLOCKADE

CRBN blocking peptide, a synthetic peptide (accession #: Q96SW2) that blocks CRBN antibody (NBP1-56305), was purchased from Fitzgerald, USA. Total PBMCs (200,000 cells/well) were added in triplicate into 96-well U-bottom tissue culture plates and cultured with 10 mg/mL irradiated armadillo-derived *M. leprae* whole cell sonicate (obtained through BEI Resources, NIAID, NIH: *Mycobacterium leprae*, Strain NHDP, Gamma-Irradiated Whole

Cells (lyophilized), NR-19326) before and after CRBN blockade, 1 mg/mL phytohemagglutinin (PHA), or AIM-V medium at 37 °C with 5% CO and 70% humidity. Unstimulated cells and PHA-stimulated cells were used as a negative and positive control, respectively, for each sample. After 6 days, supernatants were collected and kept frozen until used in enzyme-linked immunosorbent assay (ELISA). Two groups of cells and two antigens (PHA and *M. leprae*) were used. In group (i), PBMCs (200,000 cells) were cultured in 200 µl of medium containing either PHA, *M. leprae,* or AIM-V medium. In group (ii), cells were incubated with 2 µg/ml CRBN blocking peptide and then incubated with 10 mg/mL irradiated *M. leprae* whole cell sonicate. Cells from each group were cultured in triplicate. Supernatants were collected and used for ELISA assay. The cells were stored in RNAlater (Ambion, Austin, Texas) for RNA isolation.

ELISA

Supernatants were tested for CRBN and human PARK2 (E3 ubiquitin-protein ligase parkin), and cell lysates were tested for human nuclear kappa B (Nf-kB) Ready, Set, Go! ® Sandwich ELISA. Thermo Scientific NE-PER Nuclear and Cytoplasmic Extraction Kits were used for cell lysis and extraction of nuclear protein (Nf-kB). Capture and biotinylated detection antibodies directed against CRBN, PARK2, and NF-kB were purchased from ABclonal Technology Co., Ltd., USA; Wuhan Fine Biotech Co., Ltd., China; and Reddot Biotech, Canada, respectively. 96-well flat-bottom Nunc MaxiSorp® ELISA plates (Affymetrix, eBioscience, UK) were used. Standards for each analyte were prepared by serial dilution as recommended by the suppliers. Detection was performed with avidin-horseradish peroxidase (Avidin-HRP) conjugated with tetramethylbenzidine following the supplier's procedure (Affymetrix, eBioscience, UK). For all plates, the optical density (OD) at 450 nm was measured using an ELISA plate reader (Microplate reader; Bio-Rad, Richmond, CA, USA). A curve fit was applied to each standard curve according to the manufacturer's manual. Sample concentrations were interpolated from these standard curves. The assays were sensitive to overconcentration ranges from 0.313 to 20 ng/ml for CRBN and 0.156 ng to 10 ng/ml for PARK2 and NF-kB.

RNA ISOLATION AND REVERSE TRANSCRIPTION

Isolation of RNA from cells was performed using the PAXgene Blood RNA Kit and RNeasy kit (QIAGEN Crawley, West Sussex, United Kingdom) according to the manufacturer's protocol. DNase I (QIAGEN) was included for all RNA preparations for DNA digestion. RNA yield was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Epsom, UK), and integrity was checked by agarose gel electrophoresis. For all samples, complementary DNA (cDNA) was synthesized on the same day to avoid the risk of RNA degrading during storage. cDNA was synthesised from RNA (200 ng/reaction mixture) using the ReadyScript™ cDNA reverse transcriptase kit (SIGMA-ALDRICH, USA). Reactions were incubated in an AB19700 programmable thermal cycler (Applied Biosystems, Foster City, California) for 5 min at 25°C, followed by 30 min at 42 °C and 5 min at 85°C, and then cooling to 4 °C.

PRIMERS AND QUANTITATIVE POLYMERASE CHAIN REACTION

Primers between 20 and 20–24 nucleotides long were designed across intron/exon boundaries on mRNA sequences obtained from the National Centre for Biotechnology Information database (NCBI) to give a product between 100 and 500 bp. All primer sequences were blasted on the NCBI data bank to confirm their specificity. For the reference gene, GAPDH glyceraldehyde-3-phosphate dehydrogenase (GADPH) was used. Custom synthesis

of oligonucleotide primers was performed by Sigma-Life Science and provided in desalted form. The nucleotide sequences of the forward and reverse primers, respectively, used in this study were as follows:

CRBN (F): 5′-GCCTGCAGAAAGGAGTGAGCAAC-3′; CRBN(R):5′-ATGTGATGTCGGCAGACTGG-3′
GAPDH(F):5′-AATGGGCAGCCGTTAGGAAA-3′; GAPDH(R);5′-GCGCCCAATACGACCAAATC-3′
NF-KB1(F): 5′-GCAGCACTACTTCTTGACCACC-3′; NF-KB1(R): 5′-CTGCTCCTGAGCATTGACGTC-3′
PARK2(F):5′-CCAGAGGAAAGTCACCTGCGAA-3′; PARK2(R):5′-CTGAGGCTTCAAATACGGCACTG-3′.

Real-time quantitative PCR (qPCR) for all genes was performed on the QIAquant real-time programmable thermal cycler (Qiagen, Crawley, UK) using the SYBR Green PCR Kit (Qiagen, Crawley, UK). The qPCR conditions were set as follows: The initial activation step (polymerase activation) was achieved by incubating at 95 °C for 5 min, 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 45 s, extension at 72 °C for 45 s, and fluorescence acquisition for 5 s at 72 °C. The primer-dimer formation was checked by melting curve analysis. Melting point data were obtained by increasing the temperature from 50 °C to 99 °C by 1 °C at each step. The interval between increases in temperature was 30 s for the first step and then 5 s for subsequent steps. An assay control was included from mRNA extraction to the amplification steps. For mRNA extraction, one assay control per batch was used. The assay control included all buffers except the sample and was processed under identical conditions with the samples. The same assay control was used during cDNA synthesis and real-time quantitative PCR.

CALCULATING THE RELATIVE GENE EXPRESSION

The relative gene expression (fold change) was analysed by using the $2^{-\Delta\Delta CT}$ method (Cikos et al., 2007). The difference in threshold number for the amplification of the target gene (ΔCT) was obtained by subtracting the CT of the target gene from the CT of the control gene. Relative gene expression was calculated by subtracting the ΔCT of the unstimulated cells from the ΔCT of the target gene (ΔCT target gene - ΔCT unstimulated cells = ΔΔCT). Then, the fold change (FC) was obtained by using the formula $FC = 2^{-\Delta\Delta CT}$.

DATA ANALYSIS

ELISA data. The optical density of each sample for each protein was obtained by the ELISA reader. The OD was converted to concentration (pg/mL) by Microplate Manager 6. An unpaired t-test was used to compare the relative concentration of the proteins of interest to the controls (unstimulated PBMC). For comparing each protein concentration before and after CRBN blockade, a paired t-test was used. Results are presented as mean ± standard error of the mean (SE) with P-values with a cutoff of 0.05. SE was chosen since the primary objective of the study was to measure how the mean of the sample is related to the mean of the underlying population. SE takes standard deviation and sample size into account.

Real-time quantitative PCR. For the mRNA gene expression of target genes, the relative threshold cycle value (CT) comparison method was used. The fold change of each target gene expression was used for statistical analysis. An unpaired t-test was used to compare the fold change of each target gene compared to control gene expression for unstimulated PBMCs. To

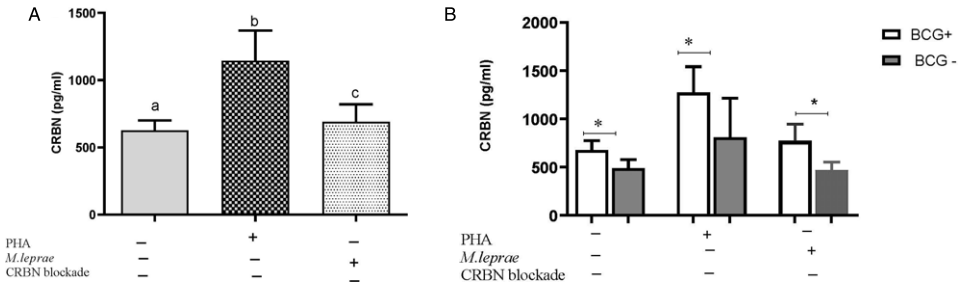

Figure 1. Amount of CRBN produced in the PBMCs in response to *M. leprae* whole cell stimulation Different letters show statistically significantly different at 95% Confidence interval and 5% level of significance. (A) CRBN production in *M. leprae* stimulated versus unstimulated PBMCs irrespective of the BCG vaccination status. (B) CRBN production in BCG vaccinated (BCG^+) and BCG unvaccinated (BCG^-) PBMCs samples in response to *M. leprae* stimulation.

compare the expression level of the desired gene before and after CRBN blockade, a paired t-test was used.

Results

DEMOGRAPHIC FACTORS

Thirty-two healthy volunteers were recruited, and 50% were female. The mean age of participants was 38.69 ± 1.78 standard error of the mean (SE) years, with a range of 27 to 63 years. 17 (53.1%) participants were *Mycobacterium bovis* bacillus Calmette–Guérin (BCG) vaccinated, and 15 (46.9%) had no history of BCG vaccination.

CRBN GENE EXPRESSION AND PROTEIN PRODUCTION

PBMC samples from participants were stimulated with *M. leprae* with and without CRBN blocking present. Unstimulated PBMC and PHA were used as negative and positive controls, respectively. PBMC stimulation with *M. leprae* had increased CRBN production (773.3 pg/ml) compared to unstimulated PBMCs (673.7 pg/ml) ($p \leq 0.05$). Similarly, CRBN gene expression by PBMCs was increased with *M. leprae* stimulation (Figure 1A, Table 1). In a subgroup analysis, CRBN production and gene expression were significantly increased in BCG-vaccinated individuals compared to the unvaccinated group in both unstimulated and *M. leprae*-stimulated samples ($p \leq 0.05$) (Figure 1B, Table 1).

NF-KB PRODUCTION AND GENE EXPRESSION

NF-kB was significantly increased in *M. leprae*-stimulated PBMCs (137.40 ng/ml) compared to unstimulated PBMCs (19.66 ng/ml) ($p \leq 0.05$). Stimulation of PBMCs with *M. leprae* after CRBN blockade was associated with greater than four-fold augmentation of NF-kB production (629.20 ng/ml) compared to PBMCs stimulation by *M. leprae* before CRBN blockade (137.40 ng/ml), which is statistically significant ($p \leq 0.05$) (Figure 2A, Table 1).

In a subgroup analysis, NF-kB production was significantly higher in BCG-vaccinated individuals (77.43 ng/ml) compared to BCG-unvaccinated individuals (1.23 ng/ml) ($p \leq 0.05$) in unstimulated PBMC samples (Figure 2B). *M. leprae* stimulation has increased NF-kB production to 322.70 ng/ml and 7.26 ng/ml in BCG-vaccinated and BCG-unvaccinated PBMC samples, respectively ($p \leq 0.05$). CRBN blockade did not significantly change NF-kB production and its gene expression in both BCG^+ and BCG^- PBMCs samples (Figure 2B).

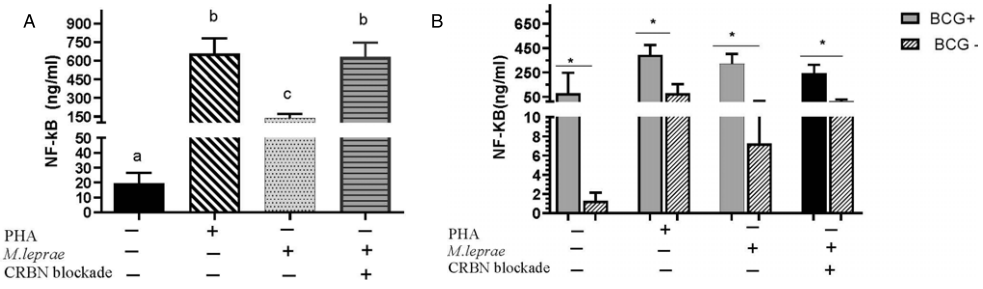

Figure 2. Amount of NF-kB produced in the PBMCs in response to *M. leprae* whole cell stimulation. Different letters show statistically significantly different at 95% Confidence interval and 5% level of significance. (A) NF-kB production in *M leprae* stimulated versus unstimulated PBMCs irrespective of the BCG vaccination status. (B) NF-kB production in BCG vaccinated (BCG^+) and BCG unvaccinated (BCG^-) PBMCs samples in response to *M. leprae* stimulation.

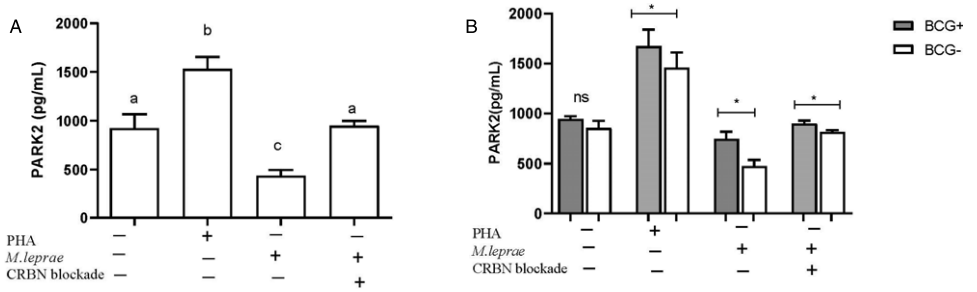

Figure 3. Amount of PARK2 produced in the PBMCs in response to *M. leprae* whole cell stimulation. Different letters show statistically significantly different at 95% Confidence interval and 5% level of significance. (A) PARK2 production in *M. leprae* stimulated versus unstimulated PBMCs irrespective of the BCG vaccination status. (B) NF-kB production in BCG vaccinated (BCG^+) and BCG unvaccinated (BCG^-) PBMCs samples in response to *M. leprae* stimulation.

PARK2 GENE EXPRESSION AND PARKIN PROTEIN PRODUCTION

Parkin production was significantly decreased in *M. leprae*-stimulated PBMCs (436.7 pg/ml) compared to unstimulated PBMCs (925 pg/ml) ($p \leq 0.05$). CRBN blockade has increased parkin production (948.80 pg/ml) in *M. leprae*-stimulated PBMCs. CRBN blockade reversed the negative impact of *M. leprae* on parkin production and *PARK2* gene expression (Figure 3A and Table 1).

The level of parkin production and *PARK2* gene expression were similar in unstimulated BCG^+ (947.40 pg/ml) and BCG^- (855.10 pg/ml) PBMCs samples ($p \geq 0.05$). However, after stimulation with *M. leprae,* parkin and *PARK2* gene expression have significantly increased in BCG^+ samples (748.50 pg/ml) compared to BCG-naïve (476.20 pg/ml) ($p \leq 0.05$) PBMCs samples. CRBN blockade further increased parkin production and *PARK2* gene expression in both BCG^+ (897.50 pg/ml) and BCG-naïve samples (817.30 pg/ml) PBMCs samples ($p \leq 0.05$) (Figure 3B and Table 1).

Table 1. *CRBN, PARK2* and *NF-κβ* gene expression in the *M. leprae* stimulated PBMCs from healthy individuals with or without *CRBN* blockade. (1A) Gene expression in *M. leprae* stimulated versus unstimulated PBMCs. (1B) Gene expression in BCG vaccinated (BCG^+) and BCG unvaccinated (BCG^-) PBMCs

1A				
Gene	PBMC stimulation	ΔΔCT	FC	*P* value
CRBN	Media only	−1.67	3.182145935	<0.001
	M. leprae	−3.42	10.70342044	
NF-κβ	*M. leprae*	−3.57	11.87618857	<0.001
	$CRBN^B$ + *M. leprae*	−4.15	17.75311155	
PARK2	*M. leprae*	2.73	0.190782401	<0.001
	$CRBN^B$ + *M. leprae*	0.13	0.463294031	

1B					
Gene	PBMC stimulation	BCG status	$\Delta\Delta C_T$	FC	*P* value
CRBN	Media only	BCG^+	−2.62	6.147500725	0.007
		BCG^-	−1.51	2.848100391	
	M. leprae	BCG^+	−3.98	15.77972327	<0.001
		BCG^-	−1.71	3.271608234	
NF-κβ	*M. leprae*	BCG^+	−2.17	4.500233939	0.001
		BCG^-	−3.02	8.111675838	
	$CRBN^B$ + *M. leprae*	BCG^+	−2.12	4.34693945	0.005
		BCG^-	−1.29	2.445280555	
PARK2	*M. leprae*	BCG^+	0.48	0.716977624	0.041
		BCG^-	0.87	0.547146851	
	$CRBN^B$ + *M. leprae*	BCG^+	0.03	0.979420298	0.0854
		BCG^-	0.45	0.732042848	

Statistical test: unpaired t-test; $\alpha = 0.05$; $\Delta\Delta C_T$ = delta delta Ct; Ct = threshold value; FC = fold change; ^{B} = blockade. GAPDH was used as a housekeeping gene; BCG^+ = BCG-vaccinated, BCG^- = BCG- unvaccinated.

Discussion

The *PARK2* gene product, parkin, regulates several pathways of the immune system in infectious diseases. A single nucleotide polymorphism of this gene has been reported as a major risk factor for susceptibility to leprosy.[29] *PARK2* is regulated by *CRBN* through the E3-ubiquitin-ligase complex,[30] but the exact mechanism is yet to be elucidated. Two circumstantial factors have attracted our attention to investigate *CRBN* and *PARK2* gene expression and their corresponding protein production in response to human PBMCs stimulation with *M. leprae*. Firstly, *PARK2* gene polymorphism has been identified as a major risk factor for leprosy, and *PARK2* is expressed in Schwann cells and macrophages, the intracellular niche of *M. leprae*. Secondly, *CRBN* has been found to be the binding site for thalidomide, which is effective in the management of ENL.

We found that stimulation of PBMCs with *M. leprae* did not affect CRBN protein production nor its gene expression. However, CRBN gene expression and protein production were

significantly increased in PBMCs of BCG-vaccinated individuals in response to *M. leprae* stimulation compared to unstimulated cells. This increased *CRBN* gene expression in response to *M. leprae* could be due to previous exposure to mycobacteria (BCG).

M. leprae stimulation with CRBN blockade significantly increased *NF-kB* production and its gene expression compared to PBMCs without CRBN blockade. CRBN-knockdown THP-1 cells show enhanced *NF-κB* activation leading to up-regulation of *NF-κB*-dependent gene expression and increased pro-inflammatory cytokine levels in response to TLR4 stimulation.[31] Previously our group has shown that *M. leprae* activates TLR4, and TLR4 protein expression was upregulated on macrophages derived from healthy volunteers after incubation with *M. leprae*.[32] Therefore, it could be postulated that incubation of PBMCs with *M. leprae* after CRBN blockade enhanced the TLR4 signaling pathway, which in turn upregulates *NF-kB*.

We found that *NF-kB* was significantly upregulated in unstimulated PBMCs from BCG-vaccinated participants, while it was almost abolished in the BCG-unvaccinated individuals. Several studies have suggested that BCG vaccination induces either antigen-independent memory T cells by a process called heterologous immunity or through inducing trained innate immunity.[33–35] Therefore, we suggest that the higher production of NF-kB protein and its gene expression in unstimulated PBMCs of BCG-vaccinated individuals compared to BCG-naïve cells could be due to the non-specific protection of BCG vaccination.[36] The mechanism through which BCG induces trained immunity (nonspecific protections) has not been fully elucidated, but epigenetic reprogramming, metabolic reprogramming, and long-term protection mediated by hematopoietic stem cells have been suggested mechanisms.[36]

We also investigated *PARK2* gene expression and parkin production by incubating PBMCs with or without CRBN blockade. We found that *PARK2* gene expression and parkin levels were significantly decreased in cells incubated with *M. leprae* compared to unstimulated PBMCs. Incubation of PBMCs cells with *M. leprae* after CRBN blockade reverses the downregulation of *PARK2* gene expression and parkin protein production. In a subgroup analysis, *PARK2* gene expression and parkin protein were decreased in the PBMCs of unvaccinated individuals following incubation with *M. leprae* compared to the vaccinated group. This lower production in the unvaccinated following incubation with *M. leprae* compared to vaccinated individuals suggests that BCG-naïve individuals are more susceptible to *M. leprae* infection compared to vaccinated individuals. Previous studies have reported that the upregulation of parkin plays an important role *in vivo* against a broad range of intracellular bacterial infections, which supports our hypothesis.[29,37] The downregulation of the parkin and *PARK2* gene expression by *M. leprae* may be mediated by CRBN. To our knowledge, this is the first report to show that *M. leprae* downregulates parkin and *PARK2* gene expression *in vitro*. Our findings provide a new insight, making *PARK2* gene expression and parkin putative targets for the diagnosis of subclinical *M. leprae* infection. The inhibitory effect of *M. leprae* on *PARK2* and parkin is eliminated by blocking CRBN. This is an interesting new insight into the modulation of the transcriptional response of *PARK2* to *M. leprae* and possible mechanisms of CRBN-mediated infection control.[15,38]

Although this study was conducted in the UK, the volunteers were from 14 countries. Some volunteers were born in leprosy-endemic countries. However, we did not compare the CRBN gene expression of PBMCs in response to *M. leprae* stimulation from leprosy-endemic countries with non-endemic countries for two reasons. Firstly, the sample size from each country is too small to draw a conclusion. Secondly, being born in an endemic country does not necessarily confirm exposure to mycobacterium. However, we used BCG vaccination status as a proxy indicator and compared the response between BCG-vaccinated and BCG-unvaccinated

individuals. Future studies should include clinical samples and blood samples from household contacts to further establish our first-ever findings of CRBN expression in PBMC samples in response to *M. leprae* stimulation.

Author contributions

Conceptualization: Edessa Negera and Stephen L. Walker; Data curation: Edessa Negera; Formal analysis: Edessa Negera; Funding acquisition: Stephen L. Walker and Edessa Negera; Investigation: Edessa Negera, Diana N. J. Lockwood, Stephen L. Walker and Barbara de Barros; Project administration: Edessa Negera, Stephen L. Walker; Resources: Edessa Negera and Barbara de Barros; Software: Edessa Negera, Supervision: Diana N. J. Lockwood and Stephen L. Walker; Validation: Edessa Negera; Visualization: Edessa Negera; Writing – original draft: Edessa Negera; Writing – review & editing: Edessa Negera, Diana N. J. Lockwood, Stephen L. Walker and Barbara de Barros.

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