SCIENTIFIC REPORTS

OPEN

SUBJECT AREAS:
BACTERIAL PATHOGENESIS
MOLECULAR BIOLOGY

Received
4 October 2013

Accepted
1 September 2014

Published
31 October 2014

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Calmodulin-like protein from *M. tuberculosis* H37Rv is required during infection

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M. tuberculosis constitutes very sophisticated signaling systems that convert the environment signals into appropriate cellular response and helps the bacilli to overcome the onslaught of host defence mechanisms. Although mycobacterial two-component systems and STPKs have gained lot of attention as virulence factors, mycobacterial calcium signaling has not been very well studied. Calcium signaling has been the primary mechanism in eukaryotes for regulation of kinases, however in prokaryotes auto-phosphorylation of number of kinases has been reported. We have previously reported a small calmodulin-like-protein (CAMLP) from _M. tuberculosis_ regulating enzymes of heterogeneous origin. To understand its role in both viability and virulence, we have assessed the effect of reduced expression of CAMLP coding gene Rv1211 on _M. tb_ growth _in vitro_ and _ex vivo_. Further, we have also studied the expression profile of Rv1211 in various conditions simulating host microenvironments. Our results highlight the possible role of CAMLP in growth and survival of _M. tb_ during infection.

Mycobacterium tuberculosis (M. tb), the causative agent of tuberculosis, is a complex organism known to highly regulate its metabolic and physiological pathways during infection in the human host. Its ability to latently survive within the host for decades with a lifetime risk of re-activation makes it one of the most difficult pathogens to handle. *M. tb* possesses the capacity to sense and convert the extracellular environmental signals into intracellular transcriptional changes, thereby allowing it to sustain the harsh conditions during infection. Many groups have dissected the transcriptome of *M. tb* during various stages of infection in order to understand the role of virulence factors and regulatory proteins involved in mycobacterial adaptation in the host[1]. Mycobacterial signal transduction systems are thought to play critical role in this adapataion processCalcium signaling is one of the signaling mechanisms that has been intensively studied in eukaryotes, wherein Ca^{2+} ion has been shown to act as an effector of stimulus-response coupling of various physiological processes of a cell. Calmodulins are a family of proteins that can transduce a calcium mediated signal into a cellular response and thus play central role in calcium signalling. In prokaryotes, however, the presence of calcium binding proteins and their role in the regulation of cellular processes is poorly understood. Calmodulin-like protein (CAMLP) from prokaryotic origin was first described in *Bacillus subtilis*[2] *and Escheria coli*[3]. Subsequently presence of CAMLP was also shown in extracts of five major mycobacterial strains *M. smegmatis*, *M. phlei*, *M. bovis* BCG, and *M. tuberculosis* H37Ra and H37Rv[4–7]. The size of the mycobacterial protein varies from 55 to 75 amino acids. Although, most CAMLPs show sequential similarity to eukaryotic calmodulins in terms of presence of signature calcium binding sites called EF hands, however sequential diversity has been observed in calcium binding sites of many prokaryotic proteins. Our study aims at determining the possible role of *M. tb* Calmodulin-like protein (CAMLP) during TB infection cycle.

The *M. tb* genome has a single *orf* encoding a CAMLP that has been shown to be highly conserved across all the mycobacterial strains. It displays 100% similarity with its homologue in *M. tb* H37Ra and *M. bovis subsp. bovis* strain AF2122/97, whereas 99% similarity in *M. ulcerans* Agy99 and 98% similarity in *M. avium*. It is noteworthy that in the non-pathogenic *M. smegmatis*, the first 20 amino acids from the N terminal are absent. The CAMLP coding gene Rv1211 is located between the genes tagA (DNA-3-methyladenine glycosylase I) and Rv1212c (putative glycosyl transferase) in *M. tb H37Rv*.

We have previously shown that Rv1211 of *M. tb H37Rv* codes for a small, 75 amino acids long protein similar in function to the eukaryotic calmodulin, thus given the name Calmodulin-like protein. We have cloned and over-expressed the calmodulin-like protein (CAMLP) of *M. tb* H37Rv in *E. coli*. CAMLP has been shown to stimulate

the activity of Bovine brain Phosphodiesterase and plant NAD Kinase, which are the two classical examples of downstream targets of eukaryotic calmodulin. Like in eukaryotes the stimulation is sensitive to trifluoperazine, a known calmodulin antagonist[8]. The gene Rv1211 has been found to be TraSH essential[9] and amongst the top 100 most expressed genes of *M. tb* in log phase[10]. The present study focuses on determining the essentiality of CAMLP during growth and survival of *M. tb* in the human host.

Results

The Rv1211 antisense expression plasmid reduces expression of Rv1211 gene in *M. tb*. If *M. tuberculosis* CAMLP is essential for its survival, its absence or scarcity would lead to perturbation in growth of the bacilli. We attempted to decrease the production of CAMLP in *M. tb* by introducing a Rv1211 antisense expression vector in *M. tuberculosis* to develop the strain *M. tb*-Rv1211AS to be used to assess the consequences of decreased production of CAMLP on growth and survival of *M. tuberculosis*. Thus after electroporation of the Rv1211 antisense expression plasmid (pJHR2) into *M. tb* H37Rv, it was very important to analyze the impact of the antisense construct on the expression of CAMLP in *M. tuberculosis*. Transcript levels of Rv1211 in acetamide induced and uninduced antisense transformants of *M. tuberculosis* and wild type *M. tuberculosis* were determined by quantitative real-time PCR (qRT-PCR). The expression levels of Rv1211 were determined by the comparative CT method after normalizing with a 16S rRNA control. As can be seen in figure 1, both uninduced and induced *M. tb*-Rv1211AS showed reduction in Rv1211 expression levels by 0.30 fold (64%) and 0.37 fold (67%) respectively, when compared to the wild type strain. This is so because the Rv1211 antisense was cloned in the vector pJFR19, under the control of amidase promoter, which is an acetamide controlled expression system in *M. smegmatis*. However, in *M. tb*, amidase promoter is known to constitutively express. Thus even in the absence of acetamide, pJHR2 is expected to show leaky expression of Rv1211 antisense in *M. tb*. However, to obtain optimum induction, expression levels of Rv1211 were also checked after inducing *M. tb*-Rv1211AS with 0.2% acetamide as given in the materials and methods. The recombinant was further used for *in vitro* and *ex vivo* growth analysis.

Reduced expression of Rv1211 significantly affects growth of *M. tb* in broth cultures during late log phase. Since expression level of Rv1211 was found to be reduced by 67% in acetamide induced *M. tb* Rv1211AS, as detected by qRT PCR, the strain was further used for *in vitro* growth analysis The influence of the reduction of the amount of CAMLP protein on the *in vitro* growth rates of *M. tuberculosis* was determined by comparing growth of *M. tb*-Rv1211AS with *M. tb H37Rv (WT)*, *M. tb-pJFR19* (EV) and *M.tb-Rv1211S* in the presence of acetamide in MB7H9 broth, by measuring A_{600nm} (Figure 2A) and by CFU assay (Figure 2B) at different time points. Results display no significant difference observed between growth rates of *M. tb H37Rv (WT)*, *M. tb-pJFR19* (EV) and *M.tb-Rv1211S*. However, *M. tb*-Rv1211AS displayed slower growth rate from day nine onwards highlighting the requirement of Rv1211 protein for normal growth of bacilli. The maximum difference in growth rate was observed on day 15, with *M. tb*-Rv1211AS displaying 3.5 fold and 2.3 fold less number of CFUs when compared to *M. tb H37Rv (WT)* and *M. tb-pJFR19* (EV), respectively.

Reduced expression of Rv1211 in *M. tb* influences establishment of macrophage infection by bacilli. Success of *M. tb* as an intracellular pathogen lies in its ability to multiply and survive within host macrophages. We wanted to evaluate the impact of Rv1211 expression on the growth and survival of *M. tb* within macrophages. For this purpose we used the THP-1 cell line and monocyte derived macrophages (MDMs) infection model. Acetamide induced cultures of *M. tb H37Rv (WT)*, *M. tb-pJFR19* (EV), *M.tb-Rv1211S* and *M.tb-Rv1211AS* were grown till log phase and used to infect the human macrophage cell line THP-1. Before setting up infection, cells from bacilli starter culture were serially diluted and plated on 7H11 agar to evaluate the exact number of bacilli for infection The infection was set up at an MOI of 10 : 1 and was allowed to proceed for 4 hours, after which supernatant was replaced with fresh RPMI 1640 supplemented with 10% FCS. Intracellular growth and survival of all the strains was monitored by lysing THP-1 cells on days 0 (immediately after 4h infection), 1, 3, 5 and 7 and plating the lysate to enumerate intracellular CFUs. Infection of MDMs was done in a similar way with *M. tuberculosis H37Rv (WT)* and *M.tb-Rv1211AS*. However, in this case, MDMs were lysed on days 0 (immediately after 4h infection), 1, 2 and 3 to enumerate intracellular CFU. Results clearly display the difference in intracellular growth rate of *M.tb-Rv1211AS* when compared to that of *M. tb H37Rv (WT)*, *M. tb-pJFR19* (EV) and *M.tb-Rv1211S* in both the models. Number of bacilli in the case of *M. tuberculosis* H37Rv increased 9-fold by 7 days post infection as compared to a 5-fold increase for *M.tb-Rv1211AS* at the same time point in THP-1 infection model (Figure 3). In case of MDMs *M.tb-Rv1211AS*

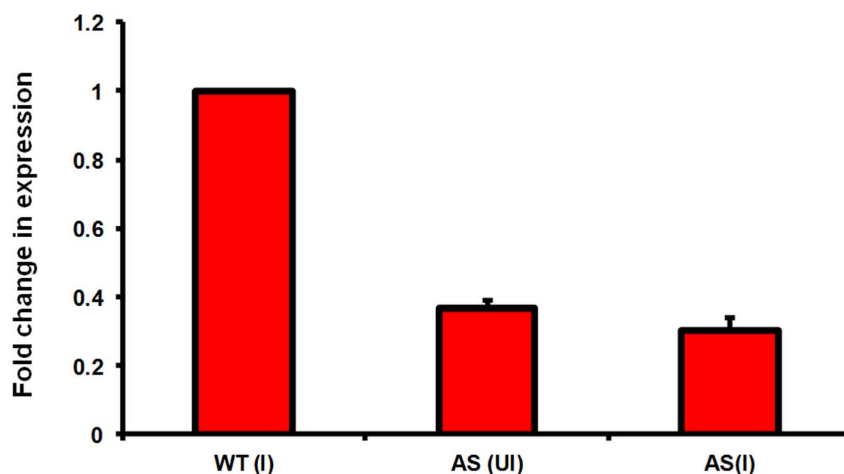

Figure 1 | Expression level of Rv1211 in *M. tuberculosis* Rv1211AS. Expression levels of Rv1211 was determined in wild type *M. tb* H37Rv and uninduced (UI) and acetamide induced (I) *M. tuberculosis* Rv1211AS by qRT PCR. Graph depicts fold change in expression of Rv1211 in *M. tb* Rv1211AS (induced and uninduced) relative to Rv1211 expression in wild type *M. tb* H37Rv. Values represent mean ± SD of duplicates from two independent experiments.

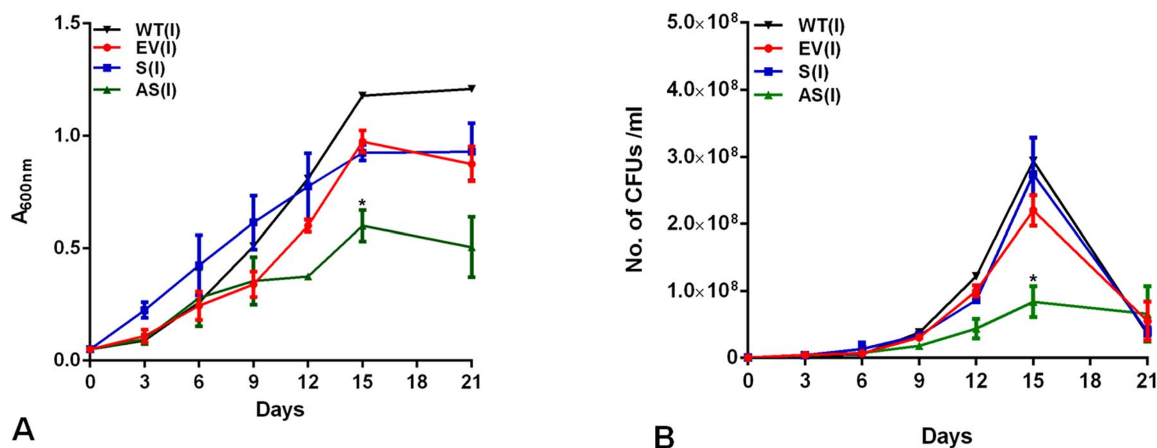

Figure 2 | Effect of altered expression of Rv1211 on growth kinetics of *M. tb in vitro*. Cultures were induced with 0.2% acetamide and growth was monitored by measuring A_{600nm} (A) and by CFU assay (B). Graph depicts growth kinetics of *M. tb H37Rv (WT)*, *M. tuberculosis-pJFR19* (EV), *M.tb-Rv1211S* and *M.tb-Rv1211AS* for period of 21 days. Data was considered significant (*) if $p < 0.05$. Values represent mean ± SD of duplicates from two independent experiments.

showed 1.1 fold increase in number of CFUs as compared to a 2-fold increase in *M. tb* H37Rv CFUs by 3 days post infection (Figure 4). Given these observations, we concluded that Rv1211 plays an important role *in vivo*.

Expression of Rv1211 under different conditions. Mycobacterium is known to have remarkable metabolic flexibility. The physiological conditions present during infection act as signals that induce regulation of several metabolic pathways. Since *M. tb-Rv1211AS* showed intramacrophage growth defect, we hypothesized that Rv1211 might be involved in the adaptation of mycobacterium to harsh host environment and would thus be differentially expressed under those conditions. To obtain insight into the environment where Rv1211 may be functionally active, we determined the expression profile of Rv1211 under various stresses like hypoxia, starvation, presence of nitric oxide and intra-macrophage. Expression under normal conditions in broth was taken as control and was used as a baseline for calculation of relative expression using real-time quantitative PCR (qRT-PCR). In accordance with the growth defect in macrophages, Rv1211 expression was observed to increase by 2.5 fold in intracellular bacilli. This confirmed the requirement of the gene within host cells. When Rv1211 gene expression was analyzed under various *in vitro* stress conditions, a different expression pattern was observed. RNA samples obtained from hypoxic cultures showed an increase in Rv1211 expression by 7.2 fold. However, presence of nitric oxide and nutrient limitation had a negative effect on expression of Rv1211 displaying 0.34 and 0.94 fold expression, respectively (Table 1). Correlation of the Rv1211 expression pattern with the mycobacterial global expression data available, points towards the possible function of the gene as discussed below.

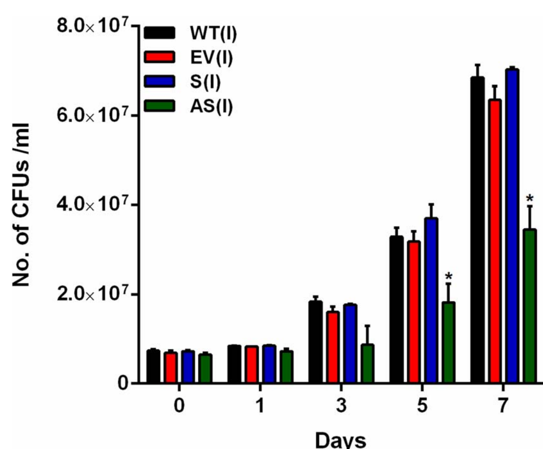

Figure 3 | Effect of altered expression of Rv1211 on growth of *M. tb ex vivo*. THP-1 cells were infected with induced cultures of *M. tb H37Rv (WT)*, *M. tb-pJFR19* (EV), *M.tb-Rv1211S* and *M.tb-Rv1211AS* separately. Intracellular growth and survival was monitored by enumerating CFUs as given in the materials and methods. Graph depicts intracellular growth kinetics of *M. tb H37Rv (WT)*, *M. tbs-pJFR19* (EV), *M.tb-Rv1211S* and *M.tb-Rv1211AS* over a period of 7 days post infection. Data was considered significant (*) if $p < 0.05$. Values represent mean ± SD of duplicates from two independent experiments.

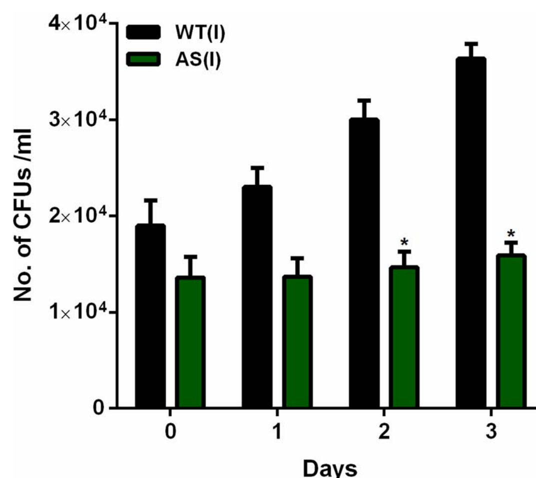

Figure 4 | Effect of reduced expression of Rv1211 on growth of *M. tb ex vivo*. MDMs were infected with both *M. tb* H37Rv and *M. tb-Rv1211AS* separately. Intracellular growth and survival was monitored by enumerating CFUs as given in the materials and methods. Graph depicts intracellular growth kinetics of *M. tb* H37Rv and *M. tb-Rv1211AS* over a period of 3 days post infection. Data was considered significant (*) if $p < 0.05$. Values represent mean ± SD of duplicates from two independent experiments.

Table 1 | Expression analysis of Rv1211 under different growth conditions

Growth conditions	Fold difference
Broth	1
Macrophage	2.5
Hypoxia	7.2
Starvation	0.35
DETA NO (50 μM)	0.94

Discussion

Calmodulins have established role in activation of number of enzymes involved in various cellular processes. Amongst eukaryotes enzymes like cyclic nucleotides phosphodiesterase (PDE)[12], plasma membrane ATPases[13], myosin light chain kinase[14] and NAD kinase[15] have been shown to be calmodulin-dependent. In prokaryotes calcium mediated signaling has been shown to regulate various processes like cell structure, motility, cell division, gene expression and cell differentiation processes such as sporulation, heterocyst formation and fruiting body development[16]. Among the prokaryotic CAMLP dependent enzymes are CaM Kinase of *M. smegmatis*[17] and adenylate cyclase of *Bordetella pertussis*[18]. Our previous work has already established the presence of a Calmodulin like protein encoded by the gene Rv1211 in *M. tb* H37Rv[8].

Antisense technique has been used for functional analysis of a number mycobacterial genes like *ahpC* from *M. bovis*[19], *dnaA* from *M. smegmatis*[20], FAP-P from *M. avium* subsp. *paratuberculosis*[21] or *pknF* from *M. tuberculosis*[22].In the present study we used the Mycobacterium integration proficient vector pJFR19 having the amidase promoter, to construct Rv1211 sense and antisense expression vector and assess the importance of the CAMLP for the growth of *M. tb in vitro* and *in vivo.*

In order to understand the biology of *M. tuberculosis* it is important to know which genes are active during normal growth and their possible interrelation in a global genome-wide sense. A number of regulatory proteins have been shown specifically expressed during mycobacterial infection and thus aid in its virulence[23]. The fact that Rv1211 is among the top 100 genes being expressed in actively growing *M. tb*[10] and is also *in vivo* essential[9] points towards its importance in *M. tb* life cycle. In the present study, we assessed the effect of altered expression of Rv1211 on growth rate of *M. tb* H37Rv in broth cultures. Our results clearly demonstrate a slower rate of replication of *M. tuberculosis* Rv1211AS in broth cultures during the late log phase as compared to its wild type, empty vector and over expression counterparts.

Many studies have dissected the transcriptional profile of *M. tb* during infection which mirrors the physiological states of the intracellular bacilli[9,24–27]. It has been repeatedly shown that genes involved in fatty acid metabolism like that isocitrate lyase and glycine dehydrogenase are up regulated during growth in macrophages. In addition, genes involved in modeling of the cell surface, intermediary metabolism and signal transduction are also up regulated. The attenuated intra-macrophage phenotype displayed by *M.tb-Rv1211AS* sheds light on involvement of this gene in these pathways. Moreover the observed increase in the intracellular expression level of Rv1211 further suggests its requirement in establishing infection.

TB infection is generally associated with a number of stresses encountered by the bacilli. The continued identification of genes regulating various aspects of persistent infection has enhanced our knowledge of the complex and highly dynamic interaction between the pathogen and host. Our results suggest up regulation of Rv1211 expression during growth of the pathogen in macrophage and under hypoxia thus supporting the notion that these environmental conditions induce its expression. Progression of *M. tuberculosis* towards latency requires realignment of its metabolism to assure survival. It has been demonstrated by a number of studies that hypoxia induces a DosR mediated regulon of approximately 50 genes that help the bacilli to survive during dormancy. The response basically increases synthesis of triglycerides as evident by up regulation of tgs1 gene. β-oxidation of fatty acids acts as major source of carbon is whereas dramatic reduction in generation of pyruvate from glycolysis has been observed[28]. Gradual decrease in oxygen and nutrients is also accompanied by up regulation of the enzymes involved in glyoxylate cycle[29]. The glyoxylate shunt allows the bacteria to avoid the carbon dioxide generating steps of the Krebs cycle, enabling them to shunt carbons from fats to carbohydrate synthesis. Lack of oxygen also alters the respiratory chain in *M. tuberculosis* where nitrate acts as an electron acceptor[30]. This is evident by the up regulation of gene coding nitrate reductase and NDH-2 during anaerobic conditions. In addition genes encoding for sigma factors and two component systems have been shown to play essential roles during the *M. tuberculosis* persistent infection stage[31]. Although many studies have shown transcriptional profile of *M. tubercilosis* in response to hypoxic conditions to be overlapping with its transcriptional profile on exposure to NO[32], our results suggest downregulation in expression of the gene Rv1211 when the bacilli were exposed to stresses like NO and starvation. It is interesting to see that putative transcriptional regulators and proteins thought to be involved in cell envelope and energy metabolism are suppressed, suggesting a downshift of the metabolism in response to RNI (10). On the other hand microarray profiling of starved *M. tb* cultures shows down regulation of aerobic respiration, translation, cell division and lipid biosynthesis[33].

In this context our results strongly point towards the regulatory role of Rv1211 coded CAMLP in *M. tb*. We hypothesize that CAMLP might be regulating activation of various transcriptional regulators or sigma factors via phophorylations and dephosphorylations of kinases. Although the requirement of calcium by mycobacterial kinases has not been fully studied, its possibility cannot be ruled out. Differential expression of Rv1211 under various stress conditions strengthens the hypothesis of its possible involvement in the regulation of latency. It will not be wrong to speculate that adaptation of *M. tb* to host conditions thus involves the regulation of expression of CAMLP to some extent. Our future efforts will focus on studies required to unravel the exact CAMLP-mediated mechanism in *M. tb.*

Methods

Ethics Statement. The use of Human PBMCs in the current study has been approved by the Institutional Ethics Committee of Sri Venkateswara College, University of Delhi, New Delhi. All study participants gave their written informed consent.

Bacterial strains, plasmids and culture conditions. *M. tb* H37Rv was grown in Middlebrook 7H9 medium (Difco) with 0.2% glycerol and 0.05% Tween-80. The medium was supplemented with 10% albumin dextrose complex (ADC) before inoculation. Media for growth of *M. tb* H37Rv containing the plasmids pJHR1 and pJHR2 were supplemented with 100 μg/ml of Hygromycin. *E. coli* DH5α containing recombinant plasmids was grown in Luria–Bertani broth (LB), supplemented with 50 μg/ml of Ampicillin (for pHR1 and pHR2) and 100 μg/ml Hygromycin (for pJHR1 and pJHR2). Plasmid pGEM-T (Promega) was used as cloning vector for manipulation in *E. coli.* Plasmid pJFR19,an *Mycobacterium* integration proficient vector containing the gene that confers Hygromycin resistance as a selectable marker and the amidase promoter of *M. smegmatis* for expression of integrated genes, was used to alter Rv1211 expression in *M. tb*. The macrophage cell line THP-1 (ATCC) was maintained in RPMI 1640 medium (GIBCO) with 10% FCS (Supplementary table 1). Exponentially growing cultures of *M. tuberculosis* in 7H9-ADC were exposed to nitric oxide donor, DETA-NO (Alexis). DETA-NO was prepared fresh and used at a final concentration of 50 μM for 16 h[11]. For hypoxia, 10 ml of cultures were grown standing in 15 ml sealed tubes for ~2 weeks and attainment of hypoxic state was monitored by disappearance of methylene blue in a parallel culture tube maintained under same conditions.

DNA manipulations. PCR was performed with the PCR reagents from Fermentas. Oligonucleotide primers were purchased from The Center for Genomic Applications. Restriction enzymes were obtained from New England Biolabs. DNA gel extractions were carried out using QIAquick® Gel Extraction kit (Qiagen). Ligation reactions were carried out with the T4-DNA-Ligase from New England Biolabs. Transformation of *E. coli* was performed according to the method described in the QIAexpressionist (Qiagen protocols). *M. tb* H37Rv was transformed by

electroporation using Bio-rad electroporator. Plasmids were isolated from *E. coli* with the QIAGEN® Plasmid Maxi kit (Qiagen).

Construction of *M. tb* H37Rv derivatives carrying Rv1211 antisense and sense expression plasmid. The CAMLP coding gene Rv1211 was amplified from genomic DNA of *M. tuberculosis* H37Rv by PCR using the primers sets HR1.1 (forward) and HR1.2 (reverse) for sense and HR2.1 (forward) and HR2.2 (reverse) for antisense. PCR amplification was carried out by using Taq DNA polymerase and the following protocol: denaturation at 94°C for 10 min, followed by 30 cycles of 94°C for 30 sec, 68°C for 30 sec and 72°C for 30 sec, with a final extension step at 72°C for 10 min. The PCR primers used were provided with restriction sites for the restriction enzymes XbaI (HR1.2 and HR2.1) and NdeI (HR1.1 and HR2.2) to allow insertion of the amplified DNA into the XbaI/NdeI digested vector pJFR19 (Supplementary table 2). The amplicon was cloned in pGEMT-easy vector by using TA cloning. The recombinant vectors were designated as pHR1 (sense) and pHR2 (antisense). The fragments were extracted from pHR1 and pHR2 by restriction digestion with enzymes NdeI and XbaI and sub-cloned in pJFR19 under the transcriptional control of the amidase promoter. Sequencing of the insert from the recombinant plasmids designated as pJHR1 (sense) and pJHR2 (antisense) confirmed the orientation of the inserts and the absence of mismatches. The recombinant constructs were introduced into *M. tb* H37Rv via electroporation by using a cell porator (Bio-Rad) to obtain strains expressing the antisense and sense mRNA of Rv1211. The strains were designated as *M. tb-Rv1211S* (sense) and *M.tb-Rv1211AS* (antisense). Empty vector strain i.e. *M. tb* containing pJFR19 was also used as control[34].

Characterization of *M.tb-Rv1211S* and *M.tb-Rv1211AS* by PCR. Genomic DNA isolation: *M. tb* cells from 50 ml culture of $A_{600nm} \sim 1$ were centrifuged at 5000 rpm for 20 mins at RT and pellet was resuspended in 7 ml of TE. Suspended cells were vortexed for 3 minutes followed by incubation at 100°C for 30 mins. Sample was immediately snap chilled at −20°C for 30 mins and brought to RT. 500 µl of 20 mg/ml lysozyme was added, mixed properly and allowed to incubate at 37°C for 2 hrs. Subsequently, 70 µl of 10 mg/ml proteinase K was added followed by addition of 700 µl of 10% SDS. Sample was mixed gently and incubated at 37°C for 30 mins. Equal volume of chloroform:isoamylalcohol was added and sample was incubated at room temperature for 5 mins. Sample was then centrifuged at 12000 rpm, 4°C for 7 mins and aqueous layer was transferred into a fresh tube. Equal volume of isopropanol was added and incubated at −20°C for 1 hr. Sample was then centrifuged at 14000 rpm, 4°C for 20 mins. Supernatant was discarded and pellet was washed with 70% ethanol. Pellet was air dried at 37°C and dissolved in 120 µl of TE and concentration was checked by spectrophotometer. Genomic DNA isolated from *M. tb H37Rv (WT)*, *M. tb*-Rv1211S and *M.tb*-Rv1211AS was used as templates DNA for separate PCR amplifications using pJFR19 specific primers JR1.1 and JR1.2 (supplementary table 2) with the following protocol: denaturation at 94°C for 10 min, followed by 25 cycles of 94°C for 30 sec, 68°C for 30 sec and 72°C for 30 sec, with a final extension step at 72°C for 10 min. Amplicons were analysed on 1.2% agarose gels.

RNA isolation, cDNA synthesis and real time PCR. RNA was isolated by using a protocol adapted from *Mark Yoder's protocol for RNA extraction, JHU.* Briefly, cell pellets were suspended in 1 ml Trizol reagent (Invitrogen) and transferred to 2 ml screw cap tubes containing 0.5 ml of 0.1 mm diameter zirconia/silica beads (BioSpec Products). Cells were disrupted in a bead beater with four 15 s pulses. Cell debris was separated by a 3 min centrifugation at 14000 rpm, 4°C. The supernatant was transferred to 1.5 ml tube (Eppendorf) and incubated at RT for 5 mins. 200 ul of chloroform was added, inverted rapidly for 15 s, and incubated 3 min at RT. Samples were centrifuged at 12000 g, 4°C for 15 min and the aqueous phase was added to equal volume of isopropanol. Samples were incubated 15 min at RT and centrifuged for 20 min at 12000 rpm, 4°C. The RNA pellets were washed with 1 ml 70% ethanol, centrifuged 10 min at 7500 g, 4°C and air-dried. Pellets were resuspended in 86 µl of DEPC treated water. 10 ml of DNase I 10X buffer and 8 units of DNase I (NEB) were added, and the samples were incubated for 60 min at 37°C. Samples were again subjected to chloroform extraction, isopropanol precipitation and 70% ethanol wash. Finally pellets were resuspended in 50 µl of DEPC treated water. RNA purity and concentration was checked by using nano drop spectrophotometer. Total RNA isolated from each culture was used for gene specific (Rv1211) cDNA synthesis. 1 µg of total RNA was used for cDNA sythesis by using Fermentas RevertAid H Minus First Strand cDNA Synthesis Kit in 20 µl reaction mix with the following protocol: 42°C for 60 mins followed by termination of the reaction at 70°C for 5 mins. cDNA obtained was then diluted 1 : 10 and 1 : 100 and 5 µl of each dilution along with neat sample was separately used for real time analysis by using Qiagen Quantifast SYBR Green PCR dye in a 20 µl reaction mix. Expression of 16 s gene was used for normalization.

Growth experiments with *M.tb-Rv1211AS* in broth cultures. *The M. tb H37Rv (WT)*, *M. tb-pJFR19* (EV), *M.tb-Rv1211S* and *M.tb-Rv1211AS* cultures were grown in 10 ml of complete Middlebrook 7H9 to $A_{600nm} \sim 0.8$ supplemented with 0.2% acetamide in order to get primary culture for each strain. Uninduced controls (without acetamide) were also kept for all strains. 100 µg/ml of hygromycin was added to the media for recombinants. Comparison of the growth rates of recombinants and wild types was carried out by inoculating secondary cultures using the above primary cultures to obtain an initial $A_{600nm} \sim 0.05$ and measuring the A_{600nm} for each culture on days 0, 3, 6, 9, 12, 15 and 21 post inoculation.

Cell culture experiments with *M.tb-Rv1211AS*. *THP-1 infection model.* Effect of altered expression of Rv1211 in *M. tuberculosis* on intracellular growth kinetics of *M. tuberculosis* was checked using THP-1 cell infection model. THP-1 cells were infected with *M. tb H37Rv (WT)*, *M. tbs-pJFR19* (EV), *M.tb-Rv1211S* and *M.tb-Rv1211AS* with M.O.I of 1 : 10. The infected cells were cultured in RPMI1640 for 7 days at 37°C, 5% CO_2. Bacterial growth and survival was monitored by lysing macrophages on days 0, 1, 3, 5, and 7, using lysis buffer (Normal saline + 0.05% Triton X-100) and vortexing. The released bacteria were plated in triplicates on 7H11 agar plates after serial dilution and CFU were enumerated after 3-week incubation at 37°C. Hygromycin at concentration of 100 µg/ml was added in 7H11 agar plates for plating of recombinants. Macrophage viability was monitored by trypan blue exclusion at each time point.

Peripheral blood mononuclear cells model. Peripheral blood mononuclear cells (PBMCs) were isolated from 10 ml human blood collected from a healthy donor. Blood sample was diluted in 40 ml of 0.15 M PBS. PBMCs were isolated by centrifugation with Ficoll-Hypaque (Histopaque 1077, Sigma) at 1800 rpm for 30 minutes at room temperature. PBMCs were washed twice with PBS and resuspended in serum free RPMI 1640 medium containing 25 mM HEPES (*N*-2-hydroxyethylpiperazine- *N*9-2-ethanesulfonic acid) (Gibco Laboratories, Grand Island, N.Y.) supplemented with 2 mM L-glutamine. Cells were dispensed in two separate tissue culture petriplates and incubated for 2 h at 37°C under humidified 5% CO_2 for panning. After 2 hrs, the non-adherent cells were aspirated and complete RPMI 1640 with 10% heat inactivated FCS was dispensed in the petri-plates containing the adhered monocytes. Monocytes were allowed to differentiate into macrophages by incubating for 5 to 6 days and replacing media twice in between. Finally human monocyte dervived macrophages (MDMs) were seeded at density of 1×10^4 per well in a 96 well plate. For infection freshly cultured *M. tb* H37Rv and *M.tb-Rv1211AS* were brought to a density of 1×10^6 bacilli/ml. Cells were pelleted and resuspended in equal volume of RPMI 1640. 100 µl of the above suspension was added in each well containing adhered macrophages to make an MOI of 10 : 1. Infection protocol followed was same as given for THP-1 model. MDMs viability was checked by using MTT assay.

1. Voskuil, M. I. Mycobacterium tuberculosis gene expression during environmental conditions associated with Latency. *Tuberculosis (Edinb)* **84**, 138–43 (2004).
2. Harmon, A. C., Prasher, D. & Cormier, M. J. High Affinity Calcium Binding Proteins in *Escherichia coli*. *Biochem. Biophys. Res. Commun.* **127**, 31–36 (1985).
3. Fry, I. J., Villa, L., Kuehn, G. D. & Hageman, J. H. Calmodulin-Like Protein from *Bacillus subtilis*. *Biochem Biophys Res Commun.* **134**, 212–217 (1986).
4. Burra, S. S., Reddy, P. H., Falah, S. M., Venkitasubramanian, T. A. & Murthy, P. S. Calmodulin-like protein and the phospholipids of *Mycobacterium smegmatis*. *FEMS Microbiol. Lett.* **64**,189–194 (1991).
5. Burra, S. S., Reddy, P. H. & Murthy, P. S. Effect of some antitubercular drugs on the calmodulin content of *Mycobacterium tuberculosis*. *Indian. J. Clin. Biochem.* **10**, 126–128 (1995).
6. Falah, A. M. S., Bhatnagar, R., Bhatnagar, N., Singh, Y., Sidhu, G. S., Murthy, P. S. & Venkitasubramanian, T. A. On the presence of calmodulin like protein in mycobacteria. *FEMS Microbiol. Lett.* **56**, 89–93 (1988).
7. Reddy, P. H., Burra, S. S. & Murthy, P. S. Correlation between calmodulin like protein, phospholipids, and growth in glucose-grown *Mycobacterium phlei*. *Can. J. Microbiol.* **38**, 339–342 (1992).
8. Koul, S., Somayajulu, A., Advani, M. J. & Reddy, P. H. A novel calcium binding protein in Mycobacterium tuberculosis--potential target for trifluoperazine. *Indian J. Exp. Biol.* **47**, 480–8 (2009).
9. Sassetti, C. M., Boyd, D. H. & Rubin, E. J. Genes required for mycobacterial growth defined by high density mutagenesis. *Mol. Microbiol.* **48**, 77–84 (2003).
10. Fu, L. M. & Fu-Liu, C. S. The gene expression data of *Mycobacterium tuberculosis* based on Affymetrix gene chips provide insight into regulatory and hypothetical genes. *BMC Microbio.* **7**, 37 (2007).
11. Ohno, H. *et al.* The effects of reactive nitrogen intermediates on gene expression in *Mycobacterium tuberculosis*. *Cellular Microbiol.* **5**, 637–648 (2003).
12. Das, S. B. *et al.* Calmodulin-dependent cyclic nucleotide phosphodiesterase (PDE1) splice variants from bovine cardiac muscle. *Gene* **396**, 283–92 (2007).
13. Boursiac, Y. & Harper, J. F. The origin and function of calmodulin regulated Ca^{2+} pumps in plants. *J Bioenerg Biomembr.* **39**, 409–14 (2007).
14. Raina, H., Zacharia, J., Li, M. & Wier, W. G. Activation by Ca2+/calmodulin of an exogenous myosin light chain kinase in mouse arteries. *J. Physiol.* **587**, 2599–612 (2009).
15. Turner, W. L., Waller, J. C., Vanderbeld, B. & Snedden, W. A. Cloning and characterization of two NAD kinases from Arabidopsis. identification of a calmodulin binding isoform. *Plant Physiol.* **135**, 1243–55 (2004).
16. Dominguez, D. C. Calcium signalling in bacteria. *Mol. Microbiol.* **54**, 291–297 (2004).
17. Sharma, S., Giri, S. & Khuller, G. K. Ca2+/calmodulin dependent protein kinase from Mycobacterium smegmatis ATCC 607. *Mol. Cell. Biochem.* **183**, 183–91 (1998).
18. Wolff, J., Cook, G. H., Goldhammer, A. R. & Berkowitz, S. A. Calmodulin activates prokaryotic adenylate cyclase. *Proc. Nati. Acad. Sci. USA.* **77**, 3841–844 (1980).
19. Wilson, T., de, Lisle, G. W., Marcinkeviciene, J. A., Blanchard, J. S. & Collins, D. M. Antisense RNA to ahpC, an oxidative stress defence gene involved in isoniazid

resistance, indicates that AhpC of *Mycobacterium bovis* has virulence properties. *Microbiol.* **144**, 2687–2695 (1998).
20. Greendyke, R., Rajagopalan, M., Parish, T. & Madiraju, M. V. Conditional expression of Mycobacterium smegmatis dnaA, an essential DNA replication gene. *Microbiol.* **148**, 3887–3900 (2002).
21. Secott, T. E., Lin, T. L. & Wu, C. C. *Mycobacterium avium* subsp. paratuberculosis fibronectin attachment protein facilitates M-cell targeting and invasion through a fibronectin bridge with host integrins. *Infect Immun* **72**, 3724–3732 (2004).
22. Deol, P. *et al.* Role of *Mycobacterium tuberculosis* Ser/Thr kinase PknF: implications in glucose transport and cell division. *J Bacteriol* **187**, 3415–3420 (2005).
23. Smith, I. *Mycobacterium tuberculosis* pathogenesis and molecular determinants of virulence. *Clin. Microbiol. Rev.* **16**, 463–496 (2003).
24. Rohde, K. H., Veiga, D. F., Caldwell, S., Balázsi, G. & Russell, D. G. Linking the transcriptional profiles and the physiological states of Mycobacterium tuberculosis during an extended intracellular infection. *PLoS Pathog* **8**, (2012).
25. Fontán, P., Aris, V., Ghanny, S., Soteropoulos, P. & Smith, I. Global transcriptional profile of Mycobacterium tuberculosis during THP-1 human macrophage infection. *Infect. Immun.* **76**, 717–25 (2008).
26. Rohde, K. H., Abramovitch, R. B. & Russell, D. G. *Mycobacterium tuberculosis* invasion of macrophages: linking bacterial gene expression to environmental cues. *Cell Host Microbe.* **2**, 352–64 (2007).
27. Waddell, S. J. & Butcher, P. D. Microarray analysis of whole genome expression of intracellular *Mycobacterium tuberculosis*. *Curr. Mol. Med.* **7**, 287–96 (2007).
28. Zahrt, T. C. Molecular mechanisms regulating persistent *Mycobacterium tuberculosis* infection. *Microbes. Infect.* **5**, 159–167 (2003).
29. Wayne, L. G. & Lin, K. Glyoxylate metabolism and adaptation of *Mycobacterium tuberculosis* to survival under anaerobic conditions. *Infect. Immun.* **37**, 1042–1049 (1982).
30. Sohaskey, C. D. Regulation of nitrate reductase activity in *Mycobacterium tuberculosis* by oxygen and nitric oxide. *Microbiol.* **151**, 3803–3810 (2005).
31. Tyagi, J. S. & Sharma, D. Signal transduction systems of mycobacteria with special reference to *M. tuberculosis. Current Science* **86** (2004).
32. Voskuil, M. I. *et al*. Inhibition of respiration by nitric oxide induces a *Mycobacterium tuberculosis* dormancy program. *J. Exp. Med.* **198**, 705–713 (2003).
33. Betts, J. C., Lukey, P. T., Robb, L. C., McAdam, R. A. & Duncan, K. Evaluation of a nutrient starvation model of *Mycobacterium tuberculosis* persistence by gene and protein expression profiling. *Mol. Microbiol.* **43**, 717–31.
34. Malhotra, V., Okon, B. P. & Clark-Curtiss, J. E. *Mycobacterium tuberculosis* protein kinase K enables growth adaptation through translation control. *J Bacteriol.* **194** (16), 4184–96 (2012).

Acknowledgments

We acknowledge Prof. Anil K. Tyagi, Dept. of Biochemistry, UDSC, New Delhi, INDIA, for critical reading of the manuscript and for his valuable suggestions. Experiments with M. tuberculosis (H37Rv) have been carried out in DBT supported BSL-3 Tuberculosis Aerosol Challenge Facility (TACF) at ICGEB, New Delhi, whose technical support is highly acknowledged. We are grateful to Dr. Vandana Malhotra for the kind gift of strain M. tb-pJFR19, to be used as a control in the present study. Financial support from Defence Research & Development Organization, New Delhi, is highly acknowledged. This work was supported, in part, by National Institutes of Health Grants AI48417 (to M. R.)

Author contributions

M.A., M.R. and P.H.R. conceived and designed the experiments. M.A. performed the experiments. M.A., P.H.R. and MR analyzed the data. M.A. and P.H.R. wrote the manuscript with inputs from M.R.

Additional information

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Advani, M.J., Rajagopalan, M. & Reddy, P.H. Calmodulin-like protein from *M. tuberculosis* H37Rv is required during infection. *Sci. Rep.* **4**, 6861; DOI:10.1038/srep06861 (2014).

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