KefB inhibits phagosomal acidification but its role is unrelated to *M. tuberculosis* survival in host

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*kefB* is annotated as a potassium/proton antiporter in *M. tuberculosis*. There have been divergent reports on the involvement of KefB in phagosomal maturation in *M. bovis* BCG and no investigation has been carried out on its role in *M. tuberculosis*, the pathogenic species responsible for causing tuberculosis. This study was taken up to ascertain the involvement of KefB in the growth of *M. tuberculosis* and its role in phagosomal maturation and survival of the pathogen in guinea pigs. Our findings show that *kefB* mutant of *M. tuberculosis* (*Mtb*Δ*kefB*) was impaired i) for growth in high concentrations of potassium and ii) in arresting phagosomal acidification. However, the disruption of *kefB* had no adverse effect on the survival of *M. tuberculosis* in macrophages as well as in guinea pigs suggesting that the role of KefB in phagosomal acidification is unrelated to the survival of the pathogen in the host.

Phagocytosis of the invading microorganisms represents the first line of defense by macrophages triggering the signaling cascade of immune responses for subsequent killing of the ingested microbe. An intricate mechanism follows the ingestion of microbe, which involves formation of the phagosomes. These early phagosomes with pH ~ 6.5 are marked by the presence of Rab5 and early endosomal autoantigen 1 (EEA1) and are relatively poor in proteases, however, their fusion with various endosomal compartments leads to the formation of late phagosomes marked by the presence of Rab7, Rab9, lysobisphosphatidic acid, mannose-6-phosphate receptor and a low pH of ~5.5. Consequently, the fusion of late phagosomes and lysosomes [which is marked by the presence of lysosome-associated membrane proteins (LAMPs)] triggers the release of several hydrolytic enzymes responsible for killing the pathogen. However, intracellular pathogens such as *Mycobacterium tuberculosis* are endowed with various strategies that help them in subverting the phagosomal maturation pathways and survive in the macrophages in spite of the host assault. These strategies prevent acidification of *M. tuberculosis* containing phagosomes beyond pH ~ 6.5, restrict phagolysosomal fusion and provide the pathogen with an environment that is conducive for its survival in the host. Several components of *M. tuberculosis* have been demonstrated for their involvement in arresting phagosomal maturation. Lipid products of *M. tuberculosis* such as lipoarabinomannan (LAM) and phosphatidylinositol mannoside (PIM) are necessary to accomplish inhibition of phagolysosomal fusion. This involves binding of mannose-capped LAM to mannose receptors on macrophages for the incorporation of the former into macrophage cell membranes and thereby affecting the host signaling platform resulting in the altered function of protein kinases and cytokine production that is necessary for vesicle fusion and phagosomal maturation. Additionally, glycosylated LAM and PIM by their intercalation into the host membranes have been demonstrated to inhibit the acquisition of EEA-1, syntaxin 6 and cathepsin D, the molecules which are required for the normal membrane fusion involved in the phagosome trafficking. *M. tuberculosis* PtpA, a secreted protein tyrosine phosphatase contributes towards the inhibition of phagosome-lysosome fusion by dephosphorylating and inactivating the host vacuolar protein sorting 33B (VPS33B) (a member of the class C VPS complex that regulates membrane fusion within the endocytic pathway). PtpA also binds to subunit H of macrophage V-ATPase and blocks its activity required for phagosome acidification. Another *M. tuberculosis* protein namely Nucleoside Diphosphate kinase (Ndk) exhibits GAP (GTPase activating protein) activity against Rab5 and Rab7 leading to their inactivation by depleting the γ-phosphate from GTP bound Rab proteins required for phagolysosomal maturation. Another *M. tuberculosis* protein SecA2 (an export system protein) has been proposed for its involvement in the phagosomal maturation based on the increased acidification of phagosomes containing secA2 mutant of *M. tuberculosis* in comparison to the phagosomes containing the parental strain. *M. tuberculosis* SapM, a secretory phosphatase,
has also been shown to be essential for phagosomal maturation by virtue of its ability to dephosphorylate PI3P that is required by the phagosomes for docking of Rab effector proteins important for phagolyosomal fusion16–20.

*kefB* has been annotated as potassium/proton antiporter in *M. tuberculosis*. However, there has been no study to ascertain whether *kefB* has any role in the growth of *M. tuberculosis* as a function of potassium concentration. In a transposon mutagenesis based strategy to identify phagosomal acidification defective mutants of *M. bovis* BCG, *kefB* was found to prevent acidification of phagosomes as *kefB* mutants of *M. bovis* BCG were observed to accumulate in the acidified phagosomes21. However, in another study, by employing a targeted *kefB* mutant of *M. bovis* BCG, it was demonstrated that the *kefB* mutant could arrest the phagosomal maturation similar to the parental *M. bovis* BCG strain22. Moreover, this *kefB* mutant displayed enhanced survival in comparison to the parental *M. bovis* BCG when grown in host macrophages22. Thus, there have been divergent observations about the role of KefB in the phagosomal maturation arrest in *M. bovis* BCG. More importantly, in *M. tuberculosis*, the pathogenic species responsible for causing tuberculosis, the role of *kefB* has not been investigated. Hence, in this study, we aimed to ascertain the role of KefB in the growth, phagosomal maturation and pathogenesis of *M. tuberculosis* by employing a *kefB* mutant of the pathogen.

**Results**

**Construction and characterization of *kefB* mutant of *M. tuberculosis***. To evaluate the role of KefB in the physiology and pathogenesis of *M. tuberculosis*, we constructed a *kefB* mutant of *M. tuberculosis* (*Mtb*Δ*kefB*) as described in the methods. Disruption of *kefB* was confirmed by three approaches (Fig. 1A). Firstly, the *kefB* gene was amplified from *M. tuberculosis* and *Mtb*Δ*kefB* genomic DNA by employing the primers KefB-F-NdeI and KefB-R-NdeI. While a product of 1.1 kb was amplified from *Mtb*Δ*kefB* (Fig. 1B), it was demonstrated that the *kefB* mutant could arrest the phagosomal maturation similar to the parental *M. bovis* BCG strain22. Moreover, this *kefB* mutant displayed enhanced survival in comparison to the parental *M. bovis* BCG when grown in host macrophages22. Thirdly, by employing primers KefB-up and Hyg-up which yielded a product of 961 bp (Fig. 1C and 1D). Thirdly and more importantly, the amplification products were subjected to DNA sequencing that further confirmed the site specific recombination as well as disruption of *kefB* in *Mtb*Δ*kefB*. A complemented strain *Mtb*Δ*kefB* Comp was generated by the introduction of plasmid pVR1-pro*kefB* into the electro-competent cells of *Mtb*Δ*kefB*. For confirming the genetic complementation of *kefB* gene, pVR1-pro*kefB* plasmid was isolated from *Mtb*Δ*kefB*Comp cells followed by the digestion of the plasmid by Ndel restriction enzyme. The resulting 1.1 kb band observed on agarose gel during electrophoresis confirmed the presence of *kefB* gene in *Mtb*Δ*kefB*Comp (Fig. 1E).

*kefB* mutant of *M. tuberculosis* shows significant growth defect in high concentrations of potassium. KefB is annotated as a potassium efflux pump which supposedly acts as a potassium/proton antiporter effluxing out potassium ions and taking in hydrogen ions in exchange. Hence, the loss of KefB pump is expected to render *M. tuberculosis* sensitive to the levels of potassium which we assessed by growing *M. tuberculosis*, *Mtb*Δ*kefB* and *Mtb*Δ*kefB*Comp in the presence of varying concentrations of potassium and monitoring the growth of mycobacteria. As *M. tuberculosis* faces ~ 30–50 mM concentration of potassium in the phagosomes22–24, the growth kinetics was compared at 0 mM, 7 mM, 50 mM and 125 mM concentrations of potassium. In the case of complete absence of potassium, all three strains apparently exhibited a bit poor growth although in comparison to each other, there was no difference in the growth rate of any of the three strains (Fig. 2A). At 7 mM concentration of potassium, the growth of all three strains showed improvement when compared with the growth at 0 mM potassium concentration; however, the disruption of *kefB* did not appear to make any difference as the growth rate of all the three strains was comparable (Fig. 2B). These observations show that the disruption of *kefB* has no effect on the growth of *M. tuberculosis* at low potassium concentrations; however, the disruption of *kefB* in high potassium concentration arrests the phagosomal maturation arrest in *M. tuberculosis*.

![Figure 1](https://www.nature.com/scientificreports/)

**Figure 1** | Characterization of *Mtb*Δ*kefB* mutant. (A) Representation of the genomic arrangement of the disrupted *kefB* gene and the depiction of the primer pairs employed for the characterization. (B) Confirmation of *kefB* gene deletion in *M. tuberculosis* by PCR by using the gene specific primers KeFb-F-NdeI and KeFb-R-NdeI (marked by red arrows in Fig. 1A) to obtain 1.1 kb amplification in *M. tuberculosis* (lane 3) and 2.2 kb amplification in *Mtb*Δ*kefB* (lane 4). 100 bp ladder and λ HindIII were loaded in lanes 1 and 2, respectively. (C) Confirmation of *kefB* gene deletion in *M. tuberculosis* by PCR by using the primers KeFb-Dn and Hyg-Dn (marked by green arrows in Fig. 1A). Lane 1 shows a 909 bp amplification in *Mtb*Δ*kefB*, lane 2 – 100 bp ladder (D) Confirmation of *kefB* gene deletion in *M. tuberculosis* by PCR by using the primers KeFb-up and Hyg-up (marked by blue arrows in Fig. 1A). Lane 1 – 100 bp ladder and lane 2 shows a 961 bp amplification in *Mtb*Δ*kefB*. (E) Confirmation of complementation of the *M. tuberculosis* *kefB* mutant. The presence of pVR1-pro*kefB* was confirmed by restriction digestion of the plasmid isolated from the complemented strain to yield a 1.1 kb band of *kefB* gene along with its promoter (lane 3). λ HindIII and 100 bp ladder were loaded in lanes 1 and 2, respectively.
concentrations. In contrast, however, the role of KefB in the growth of *M. tuberculosis* became apparent when the strains were grown in high potassium concentrations. At 50 mM and 125 mM concentrations of potassium, the Mtb\(\Delta kefB\) grew similar to the parental strain until ~ mid-logarithmic phase after which the mutant exhibited an impaired growth as it transitioned to the stationary phase at a much lower \(A_{600 \text{ nm}}\) when compared with *M. tuberculosis* or Mtb\(\Delta kefB\)Comp (Fig. 2C and 2D).

**Disruption of kefB reduces the ability of *M. tuberculosis* to arrest phagosomal maturation.** As no studies have been carried out on the involvement of KefB in arresting phagosomal maturation in *M. tuberculosis*, we employed FITC labeled *M. tuberculosis*, Mtb\(\Delta kefB\) and Mtb\(\Delta kefB\)Comp and studied the localization of the pathogen in the acidic compartments by using LysoTracker Red dye. The latex beads, used as control for the experiment, underwent ~87% colocalization with the LysoTracker rich compartments. *M. tuberculosis* exhibited only 22.8% colocalization with the LysoTracker labeled acidified compartments, however, the mutant strain with disruption of *kefB* exhibited a substantially higher (61.5%) colocalization with the acidified compartments (Fig. 3). Mtb\(\Delta kefB\)Comp, the complemented strain, exhibited 16% colocalization with the LysoTracker labeled acidified compartments which was not significantly different from the corresponding value observed in the case of *M. tuberculosis* (Fig. 3). These observations demonstrate the role of KefB in the phagosomal maturation. Additionally, we analyzed the colocalization of FITC labeled *M. tuberculosis*, Mtb\(\Delta kefB\) and Mtb\(\Delta kefB\)Comp with the early endosomes by employing antibodies against Rab5, which is an early endosomal marker. It was observed that *M. tuberculosis* and Mtb\(\Delta kefB\)Comp accumulated in the Rab5 containing early endosomes resulting in an effective arrest of phagosome-lysosome fusion while Mtb\(\Delta kefB\) and latex beads exhibited very poor colocalization with Rab5 indicating their inability to arrest the phagosome maturation (Fig. S1).

**kefB mutant of *M. tuberculosis* exhibits no adverse effect on its ability to survive in macrophages.** To assess the ability of *M. tuberculosis*, Mtb\(\Delta kefB\) and Mtb\(\Delta kefB\)Comp to survive in macrophages, RAW 264.7 macrophages were separately infected with all three strains with an MOI of 5:1 and the growth was monitored at various time points. We observed that *M. tuberculosis* and Mtb\(\Delta kefB\)Comp grew normally inside the macrophages until 6 days post-infection in a comparable manner. Mtb\(\Delta kefB\) also exhibited similar growth pattern initially for 2 days post-infection, however, thereafter the mutant strain displayed an enhanced growth when compared with the parental strain and at the end of 6 days post-infection, the intracelular CFU of Mtb\(\Delta kefB\) was ~2 fold higher when compared with *M. tuberculosis* and Mtb\(\Delta kefB\)Comp (Fig. 4).

**Disruption of kefB exhibits no effect on the growth of *M. tuberculosis* in guinea pig model of infection.** The importance of *kefB* in the growth and pathogenesis of *M. tuberculosis* in the host was determined by employing guinea pig model of experimental tuberculosis. Guinea pigs were infected by aerosol route of infection with 10–30 bacilli of *M. tuberculosis*, Mtb\(\Delta kefB\) or Mtb\(\Delta kefB\)Comp using the aerosol route of infection and euthanized at 5 weeks and 10 weeks post-infection. We observed no significant difference in the pulmonary bacillary load of all the three strains at 5 weeks post-infection which corresponded to 5.03 log CFU, 5.04 log CFU and 5.07 log CFU in the case of infection with *M. tuberculosis*, Mtb\(\Delta kefB\) or Mtb\(\Delta kefB\)Comp, respectively. Similarly, the splenic bacillary load amongst the guinea pigs infected with these strains was also comparable with 4.84 log CFU, 4.88 log CFU and 4.80 log CFU in the case of infection with *M. tuberculosis*, Mtb\(\Delta kefB\) or Mtb\(\Delta kefB\)Comp, respectively (Fig. 5A). On extending the time period to 10 weeks...
also, the bacillary load in the organs of the animals infected with \textit{M. tuberculosis} or \textit{Mtb\text{\textsubscript{DkefB}}} or \textit{Mtb\text{\textsubscript{DkefB}Comp}} was comparable. The bacillary load in the lungs of the guinea pigs infected with \textit{M. tuberculosis}, \textit{Mtb\text{\textsubscript{DkefB}}} or \textit{Mtb\text{\textsubscript{DkefB}Comp}} was 5.13 log\textsubscript{10}CFU, 4.97 log\textsubscript{10}CFU and 5.06 log\textsubscript{10}CFU, respectively, while the bacillary load in the spleens was found to be 5.16 log\textsubscript{10}CFU, 5.43 log\textsubscript{10}CFU and 5.02 log\textsubscript{10}CFU, respectively (Fig. 5B). These observations show that KefB does not play a vital role in the pathogenesis of \textit{M. tuberculosis} in guinea pig model of experimental tuberculosis.

Guinea pigs infected with \textit{M. tuberculosis} or \textit{Mtb\text{\textsubscript{DkefB}}} exhibit comparable pathology and survival time. The organs of the guinea pigs infected with \textit{M. tuberculosis}, \textit{Mtb\text{\textsubscript{DkefB}}} or \textit{Mtb\text{\textsubscript{DkefB}Comp}} were analyzed for gross pathological as well as for histopathological differences. At 5 weeks post-infection, the gross pathological changes in the organs of guinea pigs infected with all the three strains were comparable. The lungs of these animals displayed moderate involvement with occasional large tubercles whereas liver and spleen displayed numerous small sized tubercles (Fig. 6A). This was further substantiated by the histopathological analyses (Fig. 6B). The lungs of the infected animals from all groups exhibited comparable granulomatous inflammation encompassing a large proportion of the total lung area with a severe loss of the lung parenchyma while the liver tissues displayed effacement of a large proportion of the hepatic parenchyma along with granulomatous infiltration. At 10 weeks post-infection, the lungs, liver and spleen of the guinea pigs infected with \textit{M. tuberculosis}, \textit{Mtb\text{\textsubscript{DkefB}}} or \textit{Mtb\text{\textsubscript{DkefB}Comp}} exhibited extensive pathological changes which were more severe than those observed at 5 weeks. However, comparable pathology was observed in animals infected with all three strains with no significant differences in the gross pathological scores between the organs (Fig. 7A). Both lungs and spleens of the infected animals displayed inflammation and heavy involvement marked by the presence of numerous large tubercles (Fig. 7A). Scattered areas of necrosis were also observed both in the liver and spleen of the infected animals. Moreover, the extent of histopathological changes was much more pronounced than that observed at 5 weeks post-infection, however, a comparative analysis amongst the animals from different groups revealed that the pathological changes in the organs of the animals infected with various \textit{M. tuberculosis} strains were comparable as no significant differences could be discerned (Fig. 7B). The lung sections exhibited severe lung pathology characterized by multiple coalescing granulomas and widespread lymphocytic infiltration resulting in complete loss of pulmonary micro-architecture. Similarly, the liver sections also

**Figure 3 | Influence of kefB disruption on the ability of \textit{M. tuberculosis} to arrest phagosomal acidification.** (A) RAW264.7 macrophages were infected with FITC labeled \textit{M. tuberculosis}, \textit{Mtb\text{\textsubscript{DkefB}}}, \textit{Mtb\text{\textsubscript{DkefB}Comp}} and latex beads (green) separately. The cells were then subjected to LysoTracker Red staining followed by observation under confocal microscope. Representative fluorescent images depict that disruption of kefB leads to the accumulation of \textit{Mtb\text{\textsubscript{DkefB}}} in acidified organelles (overlap of green and red images appears yellow as shown by arrows) while the \textit{M. tuberculosis} and \textit{Mtb\text{\textsubscript{DkefB}Comp}} were found in non-acidified organelles. Latex beads employed as a positive control display colocalization with the LysoTracker rich compartments. The scale bars depict 5 µm. (B) Bar diagram represents the percentage of phagosomes containing \textit{M. tuberculosis}, \textit{Mtb\text{\textsubscript{DkefB}}}, \textit{Mtb\text{\textsubscript{DkefB}Comp}} and latex beads that colocalized with LysoTracker Red. Data is the mean (±SE) of 3 independent experiments carried out in triplicates (***p < 0.001, One- way ANOVA).
displayed extensive pathological changes with large areas covered by multi-focal granulomas (Fig. 7B). Together, our findings on bacillary load and pathological changes demonstrate that the disruption of kefB did not influence the pathogenesis of M. tuberculosis in guinea pigs.

When the survival of guinea pigs infected with M. tuberculosis, Mtb\(\Delta\)kefB or Mtb\(\Delta\)kefBComp strains was monitored, we found that there was no difference in the median survival time of the animals infected with any of the three strains (Fig. 8). We observed that the median survival time of M. tuberculosis infected guinea pigs was 130.5 days (Fig. 8). The guinea pigs infected with Mtb\(\Delta\)kefB also exhibited a comparable median survival time of 144 days (Fig. 8). A median survival time of 117.5 days as observed in the case of guinea pigs infected with Mtb\(\Delta\)kefBComp confirmed that the disruption of kefB does not influence the disease causing ability and pathogenesis of M. tuberculosis in guinea pigs (Fig. 8).

**Discussion**

*M. tuberculosis* is a formidable pathogen which successfully exploits the host systems for its own survival. Arresting the phagosomal acidification and maturation is one of the important features of *M. tuberculosis* that turns phagosomes into a niche for the survival and replication of the pathogen. Multiple mycobacterial factors have been reported for their involvement in subverting phagolysosomal fusion in *M. tuberculosis*. Due to divergent observations about the involvement of kefB in arresting phagosomal maturation in *M. bovis* BCG and lack of studies on its function in the pathogenic *M. tuberculosis*, we aimed to ascertain the influence of KefB on the growth of *M. tuberculosis* in guinea pigs.

First, to evaluate the influence of KefB on the growth of *M. tuberculosis*, we compared the ability of M. tuberculosis with Mtb\(\Delta\)kefB as well as the complemented strain Mtb\(\Delta\)kefBComp to survive in broth culture with various potassium concentrations ranging from 0 to 125 mM. While no difference in the growth pattern amongst the three strains was observed at 0 and 7 mM potassium, at higher potassium concentrations (50 mM and 125 mM) the mutant exhibited impaired growth by transiting to stationary phase at much lower \(A_{600}\) nm when compared with the parental or the complemented strain, which supports the proposed role of KefB as a potassium efflux pump. At higher potassium concentrations, the presence and function of potassium efflux pump would be necessary to reduce the increasing intracellular potassium concentration resulting from the influx of potassium in order for it to prevent the cell from potassium toxicity. Thus, for the first time, we show that KefB is important for the growth of *M. tuberculosis* at high potassium concentrations.

Further, the role of kefB in the phagosomal maturation arrest was studied by employing *M. tuberculosis* along with the kefB mutant and the complemented strain in colocalization experiments by using LysoTracker Red. When macrophages were infected with *M. tuberculosis*, as expected, the bacilli exhibited very less colocalization with the LysoTracker Red dye thus confirming that the pathogen significantly inhibited the phagosomal acidification and resided majorly in non-acidified phagosomes with only ~22% of the bacilli in the acidified phagosomes. However, the mutant strain was unable to prevent the phagosomal acidification to the same magnitude as the parental strain as was evident from the observation that 62% of the mutant bacteria resided in the acidified compartments confirming thereby the involvement of KefB in preventing phagosomal acidification. The Mtb\(\Delta\)kefBComp strain exhibited similar results as observed in the case of *M. tuberculosis* with only ~16% of the bacilli residing in acidified phagosomes. The most likely mechanism for KefB to mediate phagosomal acidification arrest could stem from the antipporter nature of this pump wherein potassium can be effluxed out into the phagosomal lumen and protons can be taken inside the bacterial cytoplasm for preventing the acidification of phagosomes. The most likely mechanism for KefB to mediate phagosomal acidification arrest could stem from the antipporter nature of this pump wherein potassium can be effluxed out into the phagosomal lumen and protons can be taken inside the bacterial cytoplasm for preventing the acidification of phagosomes.
of *M. tuberculosis* Rv2693c and *mmpL9* did not show any attenuation of growth in macrophages in spite of their trafficking to late endosomal compartments. In another study, *M. tuberculosis* mutants that were incapable of phagosomal acidification arrest were identified and two of these mutants (in the genes Rv1522c and Rv2930) displayed normal intracellular growth and behaved similar to the wild type *M. tuberculosis*. In another study by Stewart et al., it was observed that a few BCG mutants exhibited normal survival and growth in macrophages in spite of losing their ability to inhibit phagosomal maturation. Hence, the acidification of phagosomes does not necessarily seal the fate of the pathogen; it could escape death in spite of acidification which could possibly mean that the mutation in such cases although related to acidification of phagosomes does not necessarily influence the survival of the pathogen.

Further, when *M. tuberculosis, MtbΔkefB* or *MtbΔkefBComp* were evaluated for their growth and pathogenesis in guinea pigs, we observed that guinea pigs infected with all three strains separately exhibited comparable bacillary load in the lungs and spleens and also there was no difference in the pathological changes. The guinea pig model of low-dose, air-borne tuberculosis infection with virulent *M. tuberculosis* is the most widely employed model for the elucidation of events in the pulmonary tuberculosis pathogenesis. Infection of guinea pigs with a low dose of <10 CFU of virulent *M. tuberculosis* results in the dissemination of the pathogen from lungs to pulmonary lymph nodes within 10 to 12 days post-infection via hematogenous spread and appears in spleen ~3 weeks post-infection, after which secondary pulmonary granulomas are formed by reseeding of the bacilli in the lungs by ~4 weeks post-infection. The

Figure 6 | Gross pathological and histopathological changes in the organs of infected guinea pigs at 5 weeks post-infection. (A) The figure depicts representative photographs of gross pathological lesions and graphical depiction of gross scores of lungs, liver and spleen of guinea pigs infected with *M. tuberculosis*, *MtbΔkefB* or *MtbΔkefBComp* and euthanized at 5 weeks post-infection. The organs of guinea pigs infected with all the strains exhibited comparable pathology. No significant differences were observed in the gross pathological scores for the lungs, liver and spleen of guinea pigs infected with any of the strains. Each data point represents the score of an individual animal and the bars depict medians (± interquartile ranges) for each group. (B) The figure depicts representative 40× photomicrographs of hematoxylin and eosin (H&E) stained 5 μm sections of lung and liver of guinea pigs euthanized at 5 weeks post-infection. The histopathological changes observed in the lung and liver of guinea pigs infected with *MtbΔkefB* were similar when compared with the changes observed in *M. tuberculosis* or *MtbΔkefBComp* infected animals exhibiting numerous foci of granulomatous infiltration. The scale bars depict 200 μm.
comparable bacillary load in the organs of the guinea pigs infected with either *M. tuberculosis* or MtbΔkefB indicates that KefB may not be indispensable for the growth and pathogenesis of *M. tuberculosis* in guinea pigs. Our studies in RAW 264.7 macrophages also support these observations. In conclusion, for the first time, we show that *M. tuberculosis* KefB, the proposed potassium efflux pump, is important for maintaining the normal growth pattern of *M. tuberculosis* in high potassium concentrations. We also demonstrate the involvement of KefB in preventing the acidification of phagosomes. However, the lack of kefB in *M. tuberculosis* has no adverse effect on its ability to survive in macrophages and guinea pigs suggesting that the role of KefB in the acidification of phagosomes is unrelated to its survival in host.

**Methods**

**Bacterial strains and growth conditions.** *Escherichia coli* strains XL-1 Blue and HB101 were used for cloning and were grown in Luria Bertani (LB) broth or on LB agar. Mycobacterial strains were grown in Middlebrook (MB) 7H9 broth supplemented with 10% ADC, 0.2% glycerol and 0.2% tween 80 with constant shaking at 200 rpm or on MB7H11 agar supplemented with 10% OADC and 0.2% glycerol at 37°C. Kanamycin and chloramphenicol were used at a concentration of 25 μg/ml and 30 μg/ml, respectively. Hygromycin was employed at a concentration of 50 μg/ml for mycobacteria or at 150 μg/ml for *E. coli*.

**Disruption of kefB (Rv3236c) and genetic complementation of the mutant.** Primers KeB-AI-F (5’ gagtatcggtaacgccgccggtgcgtatgtgtg3’) and KeB-AI-R (5’ gaattctctagagaatcggcgacagcggaatc3’) were designed to amplify Amplicon I (695 bp), comprising of 100 bp 5’ proximal region of kefB and 595 bp sequence immediately upstream to kefB, while the primers KeB-AII-F (5’ gaattcctagagaatcggcgacagcggaatc3’) and KeB-AII-R

Figure 7 | Gross pathological and histopathological changes in the organs of infected guinea pigs at 10 weeks post-infection. (A) The figure depicts representative photographs of gross pathological lesions and graphical depiction of gross scores of lungs, liver and spleen of guinea pigs infected with *M. tuberculosis*, MtbΔkefB or MtbΔkefBComp and euthanized at 10 weeks post-infection. Heavy involvement and extensive pathology was observed in the organs of guinea pigs infected with any of the strains. No significant differences were observed in the gross pathological scores for lungs, liver and spleen of guinea pigs infected with any of the strains. Each data point represents the score of an individual animal and the bars depict medians (±interquartile ranges) for each group. (B) The figure depicts representative 40× photomicrographs of hematoxylin and eosin (H&E) stained 5 μm sections of lung and liver of guinea pigs euthanized at 10 weeks post-infection. No difference was observed in the histopathological changes in the lung and liver of guinea pigs infected with MtbΔkefB when compared with the histopathological changes observed in *M. tuberculosis* or MtbΔkefBComp infected animals. The scale bars depict 200 μm.
(5’ gatactcactgctgatgagtgcggagggc) were employed for the amplification of Amplicon II (991 bp), comprising of 96 bp of 3’ distal region of kefB and 595 bp sequence immediately downstream to kefB. The amplicons I and II were PCR amplified and cloned into the vector pYUB858 flanking the hygromycin resistance cassette at KpnI/Xbal and Xhol/Spel, respectively, to generate pYUBkefB. A 3.4 kb fragment (kefB-F-5yg) was excised from pYUBkefB by employing KpnI/Spel and the resulting linear Allelic Exchange Substrate (AES) was electroporated into M. tuberculosis as described earlier29–31 to generate the kefB mutant of M. tuberculosis (MtBkefB). For the confirmation of kefB disruption, KeF-F-Ndel (5’ ggttactgtagtggctttcggggtgg3’), KeF-R-Ndel (5’ ggttactgtagtgctagactttg3’), KeF-Dm (5’ ttggctgaggcaacctacc3’), Hyg-Dm (5’ aaracgtgcctgcaaccaac3’), KeF-up (5’ ggtgctc tggattagcatgccgcac3’) and Hyg-up (5’ ggcacataaagagttggg3’) primers were employed.

For the genetic complementation of MtBkefB, the kefB gene and its promoter were cloned into pVR14 as follows. A 500 bp region comprising the promoter region of kefB gene was PCR amplified by using the primers Pre-KeF-F-Xbal (5’ gggatctagattgagttgctgg3’) and Pre-KeF-R-Sphl (5’ ggttactgtagtgctagactttg3’) and then cloned into the vector pVR1 at the promoter cloning sites Xbal/Sphl resulting in the plasmid pVR1-pro. The kefB gene was then PCR amplified by using the primers KeF-F-Ndel and KeF-R-Ndel and the resulting amplicon was cloned into pVR1-pro at Ndel restriction site downstream to the cloned promoter. The resulting plasmid pVR1-prokefB was subjected to sequencing and was introduced into MtBkefB mutant, after confirming the sequence of the promoter region as well as kefB gene, by electroporation to generate the complemented strain MtBkefBComp.

Growth kinetics under various conditions. M. tuberculosis, MtBkefB and MtBkefBComp strains were grown in minimal media (MM) with defined potassium concentrations and the growth was monitored daily by measuring the absorbance at 600 nm. MM was prepared by adding 0.5% asparagine (wt/vol), 2% glycerol, 0.5 mg of MgSO4.7H2O liter−1, 0.1 mg of MnSO4.7H2O liter−1, and 40 mg of CaCl2.9H2O liter−1 in double distilled water and desired concentrations of KH2PO4.

Infection of RAW 264.7 macrophages and bacterial enumeration. M. tuberculosis, MtBkefB and MtBkefBComp strains were grown in MB7H9 media containing 0.2% tween 20, 10% ADC and appropriate antibiotics. Exponentially growing cells were harvested at ~4,000 × g for 10 minutes and washed twice with MB7H9 media. Resulting bacterial cells were resuspended in 10 ml of RPMI media containing 10% FCS to which ~6 gram of 0.5 mm glass beads were added following vortexing for 15 minutes. The suspension was centrifuged at 50 × g in order to remove any remaining bacterial clumps. The CFU of the resulting homogeneous single cell suspension of bacteria was estimated by measuring the absorbance of this suspension at 600 nm (A600, nm) of 0.5 corresponds to 3 × 106 M. tuberculosis CFU/ml under our culturing conditions.

RAW 264.7 cells were grown in RPMI media containing 10% FCS and antibiotic-antimycotic mix. The macrophage cells were scrapped by using a sterile scraper and were counted by using trypan blue. This suspension of cells was infected with M. tuberculosis at an MOI of 5:1 (bacteria : macrophages) in RPMI media containing 10% FCS at 37°C for 2 hours with a constant shaking at 100 rpm. The cells were then harvested by centrifugation to ensure the removal of any non-phagocytosed extracellular bacteria and washed once with RPMI media. The remaining extracellular bacilli, if any, were killed by the addition of 200 μg/ml amikacin at 37°C for 1 hour with a constant shaking at 100 rpm. Finally, two rounds of washing with RPMI media were carried out to get rid of the remaining extracellular bacteria. 104 infected cells were then seeded in each of the 96 well microtiter plates in a final volume of 20 μl. The plates were then kept at 37°C in the presence of 5% CO2. On day 0, 2, 5 and 6, the infected macrophage monolayers were lysed with 200 μl of 0.025% SDS to release intracellular mycobacteria, which were then enumerated by plating appropriate dilutions on MB7H11 agar. Colonies were counted after 4 weeks of incubation at 37°C and CFU/ml was calculated.

Study of phagosomal maturation by using confocal microscopy. M. tuberculosis strains were labeled with Fluorescein isothiocyanate (FITC) (Sigma, MO, USA). Briefly, the M. tuberculosis cultures were grown to A600 nm of 0.5. The culture was harvested, washed twice with 0.5 M sodium bicarbonate buffer (pH 9.5) and resuspended in the same buffer supplemented with 100 μg/ml FITC followed by an overnight incubation at 4°C. Thereafter, the bacteria were pelleted, washed twice with PBS (pH 7.4) and single cell suspension was made as described above. Commercially available fluorescent latex beads (Diameter - 1.0 μm, Sigma, MO, USA) were used several times to control. Macrophages (purity of povo > 95%) coated glass coverslips within a 12-well plate at a density of 5 × 106 macrophages per well and infected separately with 2.5 × 104 FITC labeled mycobacteria (ratio of 5:1, bacteria : macrophages). After a 4 h incubation, the cells were washed twice with fresh RPMI media and treated with 200 μg/ml amikacin for 2 h at 37°C to remove extracellular bacteria. Subsequently, the cells were incubated with 50 nM LysoTracker Red DND-99 (Invitrogen Life Technologies, CA, USA) in RPMI (supplemented with 10% FBS) for 1 h. After this, the cells were washed once with fresh RPMI media (supplemented with 10% FBS) and fixed with 4% paraformaldehyde in PBS. Coverslips were mounted by using ProLong® Gold antifade reagent (Invitrogen Life Technologies, CA, USA) and analyzed by using Leica TCS SP5 confocal laser scanning microscope (Leica Microsystems, Mannheim, Germany). Subsequently, the fraction of FITC labeled mycobacteria that colocalized with LysoTracker Red was determined by analyzing ~100 phagosomes.

For Rab5 staining, the protocol employed was similar as described above until amikacin treatment. Subsequently, cells were fixed with 4% paraformaldehyde in PBS for 16 hours. Blocking was carried out with 2% FBS for 1 hour at room temperature on a rocker followed by incubation with 5 μg/ml Anti-Rab5 antibodies (Biovision Research Products, CA, USA, raised in rabbit) for 3 hours at room temperature on a rocker. Cells were washed twice with PBS and further incubated with Texas Red® AffiniPure Goat Anti-Rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., PA, USA) at 1:1500 dilution for 1 h at room temperature on a rocker. Cells were washed with PBS and the coverslips were mounted as described above.

In vivo guinea pig experiments. Pathogen-free out-bred female guinea pigs of the and M. tuberculosis strain were obtained from the Duncan-Hartley strain in the weight range of 250–350 grams were obtained from the Disease Free Small Animal House Facility, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar, India. To study the influence of kefB disruption on the growth and pathogenesis of M. tuberculosis, guinea pigs were infected by the aerosol route with 10 to 30 bacilli of either M. tuberculosis, MtBkefB or MtBkefBComp. Animals (n = 7) were euthanized at 5 weeks and 10 weeks post-infection by CO2 asphyxiation. After dissecting the animals, lungs, liver and spleen were scored for the infected animals were aseptically removed for bacterial enumeration. The specific load in the lungs or spleen of infected guinea pigs, one-way ANOVA with the Tukey post test was employed. For comparison of the gross pathological scores of various

Statistical analysis. For comparing the growth of M. tuberculosis strains under i) various in vitro conditions and ii) in RAW 264.7 macrophages, two-way analysis of variance (ANOVA) with the Bonferroni multiple comparison test was employed. For the phagosomal maturation in RAW 264.7 macrophages and comparison of bacular load in the lungs or spleen of infected guinea pigs, one-way ANOVA with the Tukey post test was employed. For comparison of the gross pathological scores of various

Figure 8 Influence of disruption of kefB gene of M. tuberculosis on the survival of guinea pigs post-infection. Guinea pigs (8 per group) were aerogenically infected with 10–30 bacilli of either M. tuberculosis, MtBkefB or MtBkefBComp and monitored for survival post-infection. The median survival time (MST) for animals in each infected group is stated in brackets.
groups, the nonparametric Kruskal-Wallis test was employed. Differences were considered significant when $P < 0.05$. For the statistical analysis and generation of graphs, Prism 5 software (version 5.01; GraphPad Software Inc., CA) was used.

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Author contributions
G.K. and A.K.T. conceived and designed the experiments. G.K., V.R. and P.S. performed the experiments. G.K. analyzed the data. G.K. and A.K.T. wrote the manuscript. A.K.T. provided overall supervision throughout the study.

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