Apurinic/Apyrimidinic Endonucleases of *Mycobacterium tuberculosis* Protect against DNA Damage but Are Dispensable for the Growth of the Pathogen in Guinea Pigs

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Abstract

In host cells, *Mycobacterium tuberculosis* encounters an array of reactive molecules capable of damaging its genome. Non-bulky DNA lesions are the most common damages produced on the exposure of the pathogen to reactive species and base excision repair (BER) pathway is involved in the repair of such damage. During BER, apurinic/apyrimidinic (AP) endonucleases repair the abasic sites that are generated after spontaneous DNA base loss or by the action of DNA glycosylases, which if left unrepaird lead to inhibition of replication and transcription. However, the role of AP endonucleases in imparting protection against DNA damage and in the growth and pathogenesis of *M. tuberculosis* has not yet been elucidated. To demonstrate the biological significance of these enzymes in *M. tuberculosis*, it would be desirable to disrupt the relevant genes and evaluate the resulting mutants for their ability to grow in the host and cause disease. In this study, we have generated *M. tuberculosis* mutants of the base excision repair (BER) system, disrupted in either one (*Mtb*Δ*end* or *MtbdΔxthA*) or the two AP endonucleases (*MtbdΔendΔxthA*). We demonstrate that these genes are crucial for bacteria to withstand alkylation and oxidative stress *in vitro*. In addition, the mutant disrupted in both the AP endonucleases (*MtbdΔendΔxthA*) exhibited a significant reduction in its ability to survive inside human macrophages. However, infection of guinea pigs with either *MtbdΔend* or *MtbdΔxthA* or *MtbdΔendΔxthA* resulted in the similar bacillary load and pathological damage in the organs as observed in the case of infection with wild-type *M. tuberculosis*. The implications of these observations are discussed.

Introduction

Despite the potentially intimidating environment of the macrophage, *M. tuberculosis* persists in the host as a result of several defense mechanisms [1,2,3,4,5,6]. The repair of damaged DNA has been considered as an important mechanism for the survival of *M. tuberculosis* in the host [7]. The damages, that frequently occur to DNA as a consequence of reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) produced by the macrophages, include the base modifications, generation of abasic sites and DNA strand breaks. The DNA damaged in such a manner represents the most common substrate for the Base excision repair (BER) pathway [8]. BER pathway is initiated by DNA glycosylases, a highly specialized class of enzymes, that specifically recognize and excise modified bases in DNA by hydrolyzing the N-glycosidic bond between the base and the sugar [9]. This step leads to the creation of abasic (also known as apurinic/apyrimidinic or AP) sites [9]. Abasic sites can also arise in DNA spontaneously [10]. The accumulation of AP sites in DNA is detrimental as they daunt essential processes such as replication and transcription [10]. For this reason, class II AP endonucleases are considered important enzymes that cleave the phosphodiester backbone on the 5’ end of the AP site leaving a 3’-hydroxyl group. DNA repair is completed by the actions of a DNA polymerase that fills in new base and DNA ligase that finally seals the gap [11,12].

AP endonucleases have been classified into two families, the exonuclease III (ExoIII or Xth) and endonuclease IV (EndoIV or Nfo) families, based on their homology to the two *Escherichia coli* enzymes. In *E. coli*, XthA, which is the major AP endonuclease, represents 90% of the cellular AP endonuclease activity, while Nfo accounts for the remaining 10% activity [13]. In addition to AP endonuclease and 3’→5’ exonuclease activities, the *E. coli* AP endonucleases also exhibit additional 3’ phosphatase and 3’ phosphodiesterase activities that are responsible for removing a multitude of blocking groups, including 3’ phosphate and 3’ phosphoglycolate, that are present at single-stranded breaks in DNA, induced by oxidative agents [14,15]. *Saccharomyces cerevisiae* also possesses two AP endonucleases, the Apn1 and Apn2 proteins that represent the EndoIV and the ExoIII family, respectively [16]. However, the major AP endonuclease in this organism is

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Apn1, that exhibits a strong AP endonuclease activity in yeast cells while the Apn2 protein, is a weak AP endonuclease that exhibits strong 3'→5' exonuclease and 3' phosphodiesterase activities [16,17,18,19]. The human AP endonucleases, Ape1 and Ape2, are both members of the ExoIII family where Ape1 is the major human AP endonuclease. The EndoIV homologs are not known to be present in humans [20]. Although neither of the two AP endonuclease genes is universal, all species encode at least one of these genes, suggesting that AP endonuclease activity is required for all species [21].

E. coli mutant deficient in both the AP endonuclease genes (nfo and xth) had been demonstrated to display increased lethality to ionizing radiation and chemical oxidants implicating Nfo and Xth in the repair of such damages [22]. AP endonucleases have been evaluated for their importance in the pathogenesis of several bacteria such as Salmonella typhimurium, Neisseria meningitides and Brucella abortus [23,24,25]. To determine whether the repair of oxidatively damaged DNA is involved in the growth and pathogenesis of S. typhimurium, mutants of the AP endonuclease genes were generated that were deficient in one (Asth, Ayfo) or both (Asth/nfo) the AP endonucleases [23]. S. typhimurium Asth and Asth/nfo were significantly impaired for survival in RAW 264.7 murine macrophages and in C57BL/6 primary murine macrophages activated with IFN-γ [23]. In addition, Asth/nfo was 12-fold attenuated when compared with the wild type in the murine typhoid fever model [23]. Two AP endonuclease paralogues namely Nape and Nexo (both belonging to the Xth family) have been identified and characterized in the human pathogen N. meningitides [24]. By employing the mutants of these genes (Dnfo, Dnpe and Dnexo/nape), it has been demonstrated that these enzymes are necessary for the survival of N. meningitidis under oxidative stress [24]. In addition, the Dnfo and Dnape were recovered from the bloodstream of infected infant rats at significantly lower levels than the wild-type strain, the most significant reduction being observed in the case of double mutant (Dnexo/nape) demonstrating that both Nape and Nexo are required for the full virulence of N. meningitidis [24]. The Brucella abortus possesses two XthA homologs (xthA-1 and xthA-2) but have no homolog of nfo [25]. The B. abortus xthA-1 mutant exhibited increased sensitivity to oxidative and alkylate stress when compared with the parental strain [25]. However, the mutant and the parental strains displayed equivalent spleen colonization profiles in BALB/c mice through 8 weeks post-infection and equivalent intracellular survival and replication profiles in the macrophages from C57BL/6 mice [25]. These authors suggested that residual AP endonuclease activity provided by XthA-2 may be responsible for the lack of attenuation of the xthA-1 mutant in the murine model [25].

M. tuberculosis, is amongst the few bacteria to possess homologues of all known BER genes [21,26]. The sequencing of M. tuberculosis genome revealed the presence of E. coli AP endonuclease homologs- XthA and Nfo namely, Exonuclease III (XthA) and Endonuclease IV (End) that are encoded by the genes xthA (Rx0427c) and end (Rx0670), respectively [27]. The biological importance of xthA in M. tuberculosis is highlighted by the fact that no variations in xthA have been observed in clinical strains [28]. Furthermore, a second AP endonuclease gene, encoding the endonuclease IV (End), is present in M. tuberculosis [27]. We have previously carried out biochemical characterization of the annotated M. tuberculosis AP endonucleases, namely Endonuclease IV (End) and Exonuclease III (XthA) and demonstrated that these proteins are functional AP endonucleases [29]. Given the importance of AP endonucleases in other bacteria, these proteins appear to be good targets to design anti-tubercular molecules.

Besides, in a study by Sassetti and Rubin, these AP endonucleases have been implicated in the in vivo growth of the pathogen in mouse model of infection when the spleen colonization profiles of mice infected intravenously with an M. tuberculosis transposon mutant library were evaluated [30]. However, as suggested by the authors, this study had several limitations [30]. For example, the study employed a library of transposon mutants in M. tuberculosis to intravenously infect C57BL/6j mice, hence, the effect of single gene mutation in M. tuberculosis was not evaluated in isolation [30]. The phenotypes of individual AP endonuclease mutants observed in this study could have been influenced by the presence of fully virulent bacteria that predominated the pool which could have led to an accentuation of mutant phenotypes caused by competition. Besides, this study employed intravenous route of infection rather than the aerosol route which is the common route of infection for M. tuberculosis. Moreover, the animal model employed for these experiments may also have influenced the results of the identification of in vivo essential genes as the disease in mice differs markedly from human illness. Hence, though End and XthA seem to be important drug targets, these proteins need to be validated for their in vivo essentiality in a relevant model of experimental tuberculosis before their addition to the drug development pipeline. In the present study, we have deleted the AP endonuclease gene(s) – end or/and xthA from the M. tuberculosis genome to evaluate their biological significance to this pathogen and describe the phenotypes of these mutants in the context of their importance in defense against DNA damage, intracellular growth in macrophages and in the pathogenesis of M. tuberculosis by employing the guinea pig model of experimental tuberculosis.

Materials and Methods

Bacterial strains and growth conditions

The details of bacterial strains and plasmids used in this study are listed in Table 1. Escherichia coli strains XL-1 Blue (Stratagene, Heidelberg, Germany) and HB101 (Life Technologies, CA, USA) were used for cloning and were grown in Luria-Bertani (LB) broth or on LB agar. Mycobacterial strains were grown on Middlebrook (MB) 7H11 agar supplemented with 10% OADC (oleic-acid albumin dextrose catalase) and 0.2% glycerol or in MB7H9 broth supplemented with 10% ADC (albumin dextrose catalase), 0.2% glycerol and 0.05% Tween 80 at 37°C with shaking at 200 rpm. For the generation of mutants, M. tuberculosis H37Rv transformed with pJV53 was employed, as described previously [31]. Kanamycin and chloramphenicol were used at concentrations of 25 μg/ml and 30 μg/ml, respectively. Hygromycin was used at a concentration of 50 μg/ml for mycobacteria or at 150 μg/ml for E.coli.

Disruption of AP endonuclease genes and genetic complementation of the mutants

Oligonucleotides (Table 2) were designed to amplify (i) a ~700 bp ampiclon (amplicon I) comprising of ~200 bp of the 5' proximal region of the gene (end or xthA) in addition to a ~500 bp region immediate upstream to it, and (ii) a ~700 bp ampiclon (amplicon II) comprising of ~200 bp of the 3' distal region of the gene (end or xthA) in addition to a ~500 bp region immediate downstream to it. The amplicons I and II, specific for end, were PCR amplified by employing the oligonucleotides End-F1/End-R1 and End-F2/End-R2, respectively and cloned into the vector pYUB854 flanking the hygromycin cassette at Xhol/SpeI and KpnI/ XbaI, respectively, for the generation of pYUBAend. For the generation of pYUBAxthA the amplicons I and II, specific for xthA, were PCR amplified by employing the oligonucleotides XthA-F1/
Table 1. Bacterial strains, plasmids and cell line employed in this study.

| Strains/Plasmids/Cell line | Description | Reference |
|---------------------------|-------------|-----------|
| **Strains**               |             |           |
| E.coli XL-1 Blue          | endA1 gynA96(nalR) thi-1 recA1 relA1 lac glnV44 F’[::Tn10 proAB+ lacF Δ(lacZM15) hsdR17(λc m’l)] | Stratagene, Heidelberg, Germany |
| E.coli HB101              | F−(gpt-proA) 62 leuB6 glnV44 ora-14 galK2 lacY1 (mcrC-mrr) rpsL20 (Str) yafS5 mtl-1 recA13 | Life Technologies, CA, USA |
| MtbbWT*                   | M.tuberculosis expressing recombineering proteins gp60 and gp61/wild-type strain/parental strain | [36] |
| MtbbΔend*                 | M.tuberculosis H37Rv end mutant | This Study |
| MtbbΔxthA*                | M.tuberculosis H37Rv xthA mutant | This Study |
| MtbbΔendΔxthA*            | M.tuberculosis H37Rv end xthA double mutant | This Study |
| MtbbΔendComp*             | MtbbΔend complemented with end | This Study |
| MtbbΔxthAComp*            | MtbbΔxthA complemented with xthA | This Study |
| **Plasmids**              |             |           |
| pYUB854                   | Cloning vector with hygromycin resistance gene cassette flanked with two multiple cloning sites | [57] |
| pJV53                     | Mycobacterium - E.coli shuttle vector encoding recombineering proteins gp60 and gp61 | [31] |
| pVR1                      | A derivative of pDS5 containing chloramphenicol resistance gene under M.tuberculosis ribosomal RNA promoter | [32] |
| pYUBΔend                  | pYUB854 with end·hyg | This Study |
| pYUBΔxthA                 | pYUB854 with xthA·hyg | This Study |
| pYUBΔCATxthA              | pYUB854 with xthA·CAT·trm | This Study |
| pIT338.proend             | pLITMUS38 carrying the end gene with native promoter | This Study |
| pIT338.proxthA            | pLITMUS38 carrying the xthA gene with native promoter | This Study |
| pVRend                    | pVR carrying the end gene with native promoter | This Study |
| pVRxthA                   | pVR carrying the xthA gene with native promoter | This Study |
| **Cell line**             |             |           |
| THP-1                     | The human acute monocytic leukemia cell line | NCCS, Pune, India |

* After generation of the mutants, all the strains were grown in the absence of kanamycin resulting in the loss of plasmid pJV53. The loss of plasmid pJV53 was confirmed by PCR analysis.

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XthA-R1 and XthA-F2/XthA-R2, respectively and cloned into the vector pYUB854 flanking the hygromycin cassette at KpnI/XhoI and XhoI/SpeI, respectively. The linear allelic exchange substrates (AES), end·hyg and xthA·hyg were excised from pYUBΔend and pYUBΔxthA, respectively, by using the restriction enzymes KpnI/SpeI and electroporated into wild-type M.tuberculosis carrying pJV53 separately, to generate the end (MtbbΔend) and xthA (MtbbΔxthA) mutants of M.tuberculosis [31]. For the generation of a double mutant of M.tuberculosis, xthA was disrupted in MtbbΔend. Briefly, a hygromycin resistance cassette in pYUBΔxthA was removed by using XhoI and XhoI and was replaced with the chloramphenicol resistance gene expression under the M.tuberculosis ribosomal RNA promoter to generate pYUBΔCAT·xthA. This vector was digested with KpnI/SpeI and the resulting AES (ΔxthA:CAT·trm) was electroporated into MtbbΔend to generate the double mutant of M.tuberculosis (MtbbΔendΔxthA). For the generation of mutants by employing recombineering method, wild-type M.tuberculosis carrying pJV53 (for the generation of MtbbΔend and MtbbΔxthA) and MtbbΔend (for the generation of MtbbΔendΔxthA) were cultured separately in MB7H9, 4% OADC, 0.05% Tween 80 and 25 μg/ml kanamycin at 37°C with shaking at 200 rpm, sub-cultured in MB7H9, 0.05% Tween 80, 25 μg/ml kanamycin and 0.2% succinate, grown to mid-log phase and induced by the addition of 0.2% acetamide. After growth for 48 h at 37°C, electrocompetent cells were prepared and transformed with AES.

To facilitate genetic complementation, a 1233 bp PCR product (proend) encompassing end gene (759 bp) along with its promoter region (474 bp) was PCR amplified by using M.tuberculosis H37Rv genomic DNA as template by employing the oligonucleotides EndF3/EndR3. The above PCR product was cloned at EcoRV site in pLITMUS38 plasmid and the clone was confirmed by DNA sequencing. proend was excised out from pIT338.proend by using BglII, end-repaired by Klenow polymerase and cloned in pVR1 plasmid (digested by using Mbol/Xbol and end-repaired by Klenow polymerase) [32]. The resulting plasmid pVR·end, was electroporated into MtbbΔend to generate the complemented strain, MtbbΔend Comp. For the complementation of MtbbΔxthA mutant, two separate PCR products were amplified- a 876 bp region of xthA gene was PCR amplified by employing oligonucleotides XthA-F3/XthA-R3 and a 441 bp promoter region of xthA (immediately upstream to Rv0429c) was PCR amplified by employing oligonucleotides XthA-F4/XthA-R4 by the overlap extension method as described previously [33]. The resulting PCR product, proxthA (1317 bp) was digested with EcoRV and cloned at EcoRV site in pLITMUS38 plasmid and the clone was
confirmed by DNA sequencing. proxxA was excised out from plh38:proxA by using EsECR and cloned in pVR1 plasmid (digested by using Mbol/XbaI and end-repaired by Klenow polymerase). The resulting plasmid pVRxthA, was electroporated into MbtΔxthA to generate the complemented strain, MbtΔxthA-Comp.

**Sensitivity to DNA damaging agents**

To evaluate the ability of the AP endonuclease mutants to withstand DNA damage, the wild-type and the mutant strains were subjected to different concentrations of DNA damaging agents, such as methyl methane sulfonate (MMS), hydrogen peroxide (H2O2) and mitomycin C (MMC) for 24 h and the effect of the deletion of the gene(s) on the survival of *M. tuberculosis* was evaluated by CFU enumeration. Briefly, early log phase cultures of the deletion of the gene(s) on the survival of *M. tuberculosis* were diluted to &times; 105 cells per well in 24-well tissue culture plates and differentiated to macrophages by using 30 nM phorbol 12-myristate 13-acetate (PMA) (Sigma, MO, USA) for 16 h at 37°C in 5% CO2. Cells were washed with RPMI medium and rested for 2 h before infection in 1 ml RPMI medium supplemented with 10% FBS. The monolayers were infected with wild-type *M. tuberculosis*, MbtΔend, MbtΔxthA and MbtΔendΔxthA separately, at a multiplicity of infection of 1 bacterium per 5 THP-1 macrophages for 4 h at 37°C in triplicates, following which the monolayers were washed twice with the medium. Subsequently, extracellular bacteria were removed by treatment with 200 μg/ml amikacin for 2 h at 37°C. On days 0 (6 h), 2, 4 and 6, the infected macrophage monolayers (three wells per strain) were lysed with 1 ml of 0.025% SDS (Sigma, MO, USA) to release intracellular mycobacteria, which were then enumerated by plating serial dilutions on MB7H11 agar. Colonies were counted after 4 weeks of incubation at 37°C and the data were expressed as CFU/ml. The extent of THP-1 infection by *M. tuberculosis* strains was calculated by employing the formula ω100 × (Number of intracellular bacteria obtained on Day 0 (i.e., 6 h post infection) per well/Number of THP-1 macrophages seeded per well).

**In vivo guinea pig experiments**

Pathogen-free out-bred female guinea pigs of the Duncan-Hartley strain in the weight range of 250 to 350 g were obtained from the Disease Free Small Animal House Facility, Chaudhary Charan Singh Haryana Agricultural University, Hisar, India. The animals were maintained in a biosafety level 3 facility and routinely cared for according to the guidelines of the CPCSEA (Committee for the Purpose of Control and Supervision on Experiments on Animals), India. To study the influence of end or/ and xthA disruption on the growth and pathogenesis of *M. tuberculosis*, guinea pigs were infected by the aerosol route with 10 to 30 bacilli of either wild-type *M. tuberculosis*, MbtΔend, MbtΔxthA and MbtΔendΔxthA, Animals (n = 5) were euthanized at 4 weeks and 10 weeks post-infection by CO2 asphyxiation. After dissecting the animals, lungs, liver and spleen were scored for gross pathological damage such as the extent of involvement of the organ, number and size of tubercles, areas of inflammation and damage due to necrosis. The gross pathological scores were graded from 1–4 based on the modified Mitchison scoring system [37,38]. Left caudal lung lobe and caudal segment of spleen from the infected animals were aseptically removed for bacterial enumeration. The specific segments of lung and spleen from the infected animals were aseptically removed for histopathological evaluation, the right lung and a portion of left dorsal lobe of liver from the infected animals were removed and fixed in 10% buffered formalin. 5 μm thick sections from the formalin fixed, paraffin embedded tissues were stained with haematoxylin and eosin (H&E). The tissues were coded and the coded samples were analysed by a certified pathologist having no knowledge of the experimental groups.
Ethics statement

Protocols for all the animal experiments included in this manuscript along with the requirement of guinea pigs were reviewed and approved by the Institutional Animal Ethics Committee of University of Delhi South Campus, New Delhi, India (Ref. No. 1/IAEC/AKT/BIOCHEM/UDSC/14.10.2011). All animals were routinely cared for according to the guidelines of the CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals). The guinea pigs were euthanized by CO2 asphyxiation and all efforts were made to minimize animal suffering.

Statistical analysis

For comparing the i) sensitivity of \( M.\text{tuberculosis} \) strains against DNA damaging agents and ii) growth of mycobacterial strains in THP-1 cells, two-way analysis of variance (ANOVA) with the Bonferroni multiple comparison test was employed. For the comparison of bacillary 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 groups, the nonparametric Kruskal-Wallis test followed by the Mann-Whitney \( U \) 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.

Results

Disruption of the AP endonuclease gene(s) in \( M.\text{tuberculosis} \) and characterization of the mutants

To investigate the importance of AP endonucleases in \( M.\text{tuberculosis} \), we employed recombineering method to generate three deletion mutants of \( M.\text{tuberculosis} \) lacking end (Mtb\( \Delta \text{end} \), xthA (Mtb\( \Delta \text{xthA} \)) and both the genes (Mtb\( \Delta \text{end} \Delta \text{xthA} \)) (Fig. 1A) [31]. The frequency of mutant isolation was observed to range between 63% and 88% and was calculated by using the formula—\( 100 \times \text{Number of legitimate recombinants confirmed by PCR}/\text{Number of transformants screened} \). The mutations were confirmed by three approaches. i) We confirmed all the deletion mutations by PCR analysis. Primers employed for the PCR confirmation were designed 100 bp external to the linear AES and 100 folds (0.63%) when compared with the parental strain \( M.\text{tuberculosis} \). However, when the concentration of MMS was further increased, the differences between the single gene mutants and double gene mutant became apparent. At a concentration range of 0.2 to 10 mM MMS, 0.2 to 10 mM H\( \text{O}_2 \) and 0.1 to 10 \( \mu \text{M} \) MMC against wild-type \( M.\text{tuberculosis} \). Based on the results from these studies, a concentration range of 0.5 to 2.0 mM in the case of MMS, 0.5 to 2.0 \( \mu \text{M} \) in the case of H\( \text{O}_2 \) and 1 to 10 \( \mu \text{M} \) in the case of MMC was employed for the assay. The influence of disruption of AP endonuclease(s) on the ability of \( M.\text{tuberculosis} \) to withstand alkylation stress was measured by exposure to methylethyl sulfonate (MMS) (Figure 2A). At a concentration of 0.5 mM, MMS displayed no effect on the survival of any of the \( M.\text{tuberculosis} \) strains. However, when the concentration of MMS was further increased, the differences between the single gene mutants and double gene mutant became apparent. At a concentration of 1 mM MMS, the survival of \( M.\text{tuberculosis} \) was significantly reduced (62.49%) strains was comparable, suggesting that the function of any of these enzymes can be compensated by the other under these experimental conditions. However, a clear indication of the importance of these enzymes became apparent when the sensitivity of Mtb\( \Delta \text{end} \Delta \text{xthA} \) towards MMS was evaluated. The double gene mutant exhibited a significantly enhanced sensitivity to the toxic effect of MMS at a concentration of 1 mM as demonstrated by a significantly reduced survival (35%), when compared with the wild-type strain, indicating thereby that these AP endonucleases play important role in protecting the pathogen against the alkylation damage (Figure 2A). Similarly, at a concentration of 2 mM MMS, while the survival of the parental (60.63%), Mtb\( \Delta \text{end} \) (54.39%) and Mtb\( \Delta \text{xthA} \) (62.49%) strains was comparable, the survival of Mtb\( \Delta \text{end} \Delta \text{xthA} \) significantly reduced by 100 folds (0.63%) when compared with the parental strain (Figure 2A). These results signify an important role of AP endonucleases in withstanding alkylation stress in \( M.\text{tuberculosis} \).

The influence of disruption of AP endonuclease(s) on the ability of \( M.\text{tuberculosis} \) to withstand oxidative stress was measured by exposure to hydrogen peroxide (H\( \text{O}_2 \)) (Figure 2B). At a concentration of 0.5 mM H\( \text{O}_2 \), the survival of MtbWT and Mtb\( \Delta \text{xthA} \) was 79.64% and 92.91%, respectively, which was not
significantly different from each other. However, the survival of Mtb\textit{Dend} (33.84%) was significantly reduced in comparison to the parental strain. These observations indicate that at this concentration of H$_2$O$_2$, End protects bacteria from oxidative stress whereas XthA does not. However, at this concentration of H$_2$O$_2$, the highest sensitivity to peroxide damage was exhibited by the double gene mutant i.e. Mtb\textit{DendDxthA} which exhibited a significant reduction in its survival (34.57%) in comparison to the parental strain indicating the importance of AP endonucleases in protecting the pathogen against oxidative damage (Figure 2B).

Similarly, at a concentration of 1 mM H$_2$O$_2$, it was observed that the survival of Mtb\textit{WT} (70.66%) and Mtb\textit{DxthA} (75.27%) was not significantly different from each other, while the survival of Mtb\textit{Dend} (39.08%) was significantly reduced in comparison to the wild-type strain. The double mutant was maximally sensitive at this concentration with a significantly reduced survival of 6.85% in comparison to the wild-type strain. At a concentration of 2 mM H$_2$O$_2$, all the strains exhibited a markedly reduced survival with no statistical differences in their sensitivity to peroxide stress. Taken together, these observations point out that while both the AP endonucleases are important in protecting \textit{M.tuberculosis} from the damage caused by oxidative radicals, their contribution may not necessarily be comparable and End appears to be playing a more important role than XthA because of its higher capacity to withstand oxidative damage.

The AP endonuclease mutants were also examined for their sensitivity against another DNA damaging agent, mitomycin C (MMC) along with the wild-type strain (Figure 2C). The survival of significantly different from each other. However, the survival of Mtb\textit{Dend} (33.84%) was significantly reduced in comparison to the parental strain. These observations indicate that at this concentration of H$_2$O$_2$, End protects bacteria from oxidative stress whereas XthA does not. However, at this concentration of H$_2$O$_2$, the highest sensitivity to peroxide damage was exhibited by the double gene mutant i.e. Mtb\textit{DendDxthA} which exhibited a significant reduction in its survival (34.57%) in comparison to the parental strain indicating the importance of AP endonucleases in protecting the pathogen against oxidative damage (Figure 2B).

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MtbΔend, MtbΔxthA and MtbΔendΔxthA was similar when compared with MtbWT at all the concentrations of the stress agent employed in the assay (Figure 2C). The similar sensitivity of MtbWT, MtbΔend, MtbΔxthA and MtbΔendΔxthA against MMC suggest that End and XthA do not contribute to the repair of DNA damages produced in response to the DNA cross linker MMC.

MtbΔendΔxthA exhibits attenuated growth in human THP-1 macrophages

Intracellular growth of MtbWT, MtbΔend, MtbΔxthA and MtbΔendΔxthA was compared in the THP-1 human macrophage cell line. The extent of THP-1 infection by M. tuberculosis strains by employing a multiplicity of infection of 1 bacterium per 5 macrophages was 15–20%. Wild-type M. tuberculosis grew normally in THP-1 macrophages up to 6 days post-infection. During this time period, the growth of MtbWT, MtbΔend and MtbΔxthA was comparable. Initially, up to 2 days post-infection, no significant difference was observed in the growth of double gene mutant (MtbΔendΔxthA) when compared with the wild-type strain. However, thereafter, MtbΔendΔxthA exhibited a significant attenuation in its growth with a 2 fold and 4 fold reduction in CFU at 4 and 6 days post-infection, respectively, in comparison to wild-type M. tuberculosis (Fig. 3). These observations with the double gene mutant show that End and XthA are important for intracellular survival of M. tuberculosis. However, the fact that MtbΔend, MtbΔxthA and MtbWT exhibited a comparable intracellular growth suggests that the loss of a singular activity i.e. either End or XthA can be compensated by the presence of the other enzyme.

Disruption of AP endonuclease(s) has no affect on the growth of M. tuberculosis in guinea pig model of infection

In order to elucidate the importance of AP endonucleases in the growth of M. tuberculosis in the host, we have employed guinea pig model of experimental tuberculosis. Guinea pigs were infected with 10–30 bacilli of MtbWT, MtbΔend, MtbΔxthA, MtbΔexthAComp, MtbΔxthA, MtbΔxthAComp or MtbΔendΔxthA by using aerosol route of infection and euthanized at 4 weeks (Fig. 4A) and 10 weeks (Fig. 4B) post-infection. At 4 weeks post-infection, the bacillary load in the lungs of the guinea pigs infected with any of the M. tuberculosis strains was comparable (6.5 to 7.2 log\textsubscript{10} CFU). Similarly, the spleens of the guinea pigs infected with various M. tuberculosis strains, exhibited a comparable bacillary load (5.7 to 6.4 log\textsubscript{10} CFU) (Fig. 4A). Similarly, at 10 weeks post-infection also, the bacillary load in the lungs of animals infected with any of the M. tuberculosis strains was comparable and corresponded to a range of 6.2 to 7.2 log\textsubscript{10} CFU, while the bacillary load in the spleens of these animals corresponded to a range of 5.8 to 7.1 log\textsubscript{10} CFU (Fig. 4B). This data indicates that the absence of AP endonucleases does not influence the growth of M. tuberculosis in the guinea pig infection model.
Infection of guinea pigs with *M. tuberculosis* or AP endonuclease mutants results in comparable pathological damage

The gross pathological damage in the organs of the guinea pigs infected with *M. tuberculosis* or AP endonuclease mutants was comparable at 4 weeks post-infection. The lungs of the guinea pigs infected with any of the *M. tuberculosis* strains displayed moderate involvement with occasional large tubercles. Hepatic and splenic tissues of guinea pigs infected with the parental, mutant or the complemented strains exhibited numerous small sized tubercles. No significant differences were observed in the gross pathological scores for lungs, liver or spleen of the animals infected with the parental, mutant or the complemented strains (data not shown). Moreover, commensurate to the gross pathological findings, the lung and liver of the animals infected with *M. tuberculosis* strains exhibited moderate involvement with occasional large tubercles. However, the pathological damage in the animals from all groups was comparable. A comparison of gross pathological damage and gross pathological scores for the organs of animals infected with *M. tuberculosis* or AP endonuclease mutants shows that the absence of AP endonucleases does not influence the growth of *M. tuberculosis* in guinea pigs.

**Discussion**

In the host, *Mycobacterium tuberculosis* is exposed to an environment that is rich in reactive oxygen and nitrogen intermediates capable of damaging the genome of the pathogen [39,40]. It has been thought that the BER pathway, which repairs the cytotoxic and mutagenic AP sites in DNA, may play a major role in maintaining the integrity of DNA in mycobacteria [26,41,42]. However, the experimental evidence gathered by us in this study suggests that though under *in vitro* conditions, the AP endonucle-
This tempted us to evaluate the role of these AP endonucleases in the repair of DNA damage induced in response to alkylation stress. However, a simultaneous disruption of both the AP endonucleases in *M. tuberculosis* (MtbΔendΔxthA) significantly reduces the ability of the pathogen to withstand alkylation stress when compared with wild-type *M. tuberculosis*, thereby indicating the importance of AP endonucleases in protecting the pathogen against alkylation damage. In the previous studies, it has been demonstrated that the AP endonuclease mutants of *E. coli* and *B. abortus* exhibit a significantly enhanced sensitivity to MMS when compared with the parental strain, indicating the involvement of AP endonucleases in the repair of MMS induced DNA lesions [22,25].

H$_2$O$_2$ generates nicks and breaks in DNA with blocked 3’ termini apart from giving rise to abasic sites [43,44]. Our results on the role of these AP endonucleases against oxidative damage by employing H$_2$O$_2$ demonstrate that while both the AP endonucleases make an important contribution in the repair of DNA damage in *M. tuberculosis*, End plays a more important role than XthA in protecting this pathogen against oxidative assault.

The intra- and interstrand cross-linking of DNA resulting from the damage caused by MMC is repaired by nucleotide excision repair pathways rather than BER in bacteria [45]. However, based on microarray and quantitative RT-PCR analysis Rand *et al*. reported that addition of MMC to *M. tuberculosis* induces the expression of *xthA* indicating a plausible role of XthA in the repair of DNA damage induced in response to MMC [46]. This tempted us to evaluate the role of these AP endonucleases in the repair of MMC induced DNA damage. However, the experimental evidence gathered in our study does not support any apparent role of XihA or End in the repair of such damage to DNA.

Our studies demonstrate that the disruption of any one of the AP endonucleases in *M. tuberculosis* (MtbΔend or MtbΔxthA) did not affect the growth of the pathogen in THP-1 macrophages when compared with the parental strain. However, disruption of both the AP endonucleases in *M. tuberculosis* (MtbΔendΔxthA) significantly reduced the growth of the pathogen in THP-1 cell line. These observations may be attributable to the inability of *M. tuberculosis* to repair the DNA damage inflicted upon by ROI and RNI produced by the THP-1 macrophages, in the absence of both the AP endonucleases. However, in the guinea pig infection model, we observed that disruption of either one or both the AP endonucleases did not affect the growth of *M. tuberculosis* apparently indicating that these AP endonucleases may not be indispensable for the growth and pathogenesis of *M. tuberculosis*. Our observations were substantiated by gross pathological and histopathological damage.

The difference in the influence of mutations *in vitro* and in the host, we think, may be related to the fact that the autonomous *in vitro* response of the bacterium in the macrophages may be limited. However, *in vivo*, the outcome may depend upon the host-pathogen interaction resulting in the induction of gene expression that is vital for the bacterium to survive in the face of assault mounted by the host. In this context, it is important to note that *M. tuberculosis* has ada operon responsible for what is called the Ada response, which is an adaptive response to alkylation damage [47,48]. This ada operon encodes a composite protein of AdaA and AlkA and a separate AdaB/Ogt protein [48]. By employing an *M. tuberculosis* mutant lacking the *ada* operon and the parental strain, Durbach *et al*. demonstrated that the mutant grew normally.
and was hypersensitive to the alkylation damage in vitro but displayed no attenuation in vivo in murine model of infection [49]. Such apparent differences in the function of the proteins responsible for Ada response, or AP endonucleases such as End or XthA in vitro and in vivo in M. tuberculosis, may be attributable to either variations in the DNA damage inflicted upon the pathogen during its growth in vitro and in the host or due to redundancy in such activities evolutionarily developed by the pathogen to safeguard its genome. The phenotype of MtbΔpolAΔxthA also resembles the phenotype of a recA mutant of M. bovis BCG, that has also been demonstrated to exhibit hypersensitivity to DNA damage in vitro but displayed no growth impairment in mice model of infection [50].

Based on these observations, it appears possible that M. tuberculosis may also possess repair pathways or proteins that overlap with AP endonucleases in order for it to protect its DNA from damage during its survival in the host.

We demonstrate that M. tuberculosis AP endonucleases are important in protecting the pathogen against alkylation and oxidative damage in vitro as well as for its growth in human macrophage cell line. However, M. tuberculosis may induce overlapping repair pathways and proteins to protect its DNA from damage during its survival in the host that make the role of these AP endonucleases in vivo dispensable.

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Author Contributions

Conceived and designed the experiments: RVP PVR AKT. Performed the experiments: RVP PVR. Analyzed the data: RVP PVR. Contributed reagents/materials/analysis tools: AKT. Wrote the paper: RVP AKT.

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