Modern clinical Mtb strains leverage the type I IFN pathway for a pro-inflammatory response in the host

Deepthi Shankaran[1,2#], Prabhakar A[1, 2#], Sheetal Gandotra[1,2], Vivek Rao[1,2*]

1-CSIR- Institute of genomics and Integrative Biology, New Delhi-110025, India.

2- Academy of Scientific and Innovative Research (AcSIR), ACSIR- Human Resource Development Centre, (CSIR-HRDC) Campus, Uttar Pradesh- 201 002, India

#- Equal contribution

*Corresponding author: Tel: +91 11 29879229, E-mail: vivek.rao@igib.res.in

Running title: Type I IFN induction is Mtb strain dependent

Key words: Mycobacterium tuberculosis/ clinical lineages/ Type I IFN/ macrophage

ABSTRACT:

Host phagocytes are adept at responding to infections by innate immune mechanisms and metabolic alterations designed to restrict the invading pathogen. However, this very plasticity of the response provides an ideal platform for pathogen mediated manipulation. Clinical lineages of *Mycobacterium tuberculosis* have evolved distinct and numerous ways of achieving optimal infection. We demonstrate an early, active and prominent induction of the macrophage type I IFN response by the modern lineages (L3 and L4) in contrast to the protracted and diminished capacity of the ancient Mtb lineage L1. We reveal a strong correlation between the localization of modern Mtb to acidified phagosomes and their ability to induce strong type I IFN in macrophages. Further we provide evidence for a positive regulatory loop between this augmented IFN response and increased expression of CH25H resulting in early and enhanced IL6 expression in macrophages infected with modern Mtb strains highlighting yet another unique ability of Mtb to manipulate host cell physiology to foster a proinflammatory response.

Abbreviations: IFN- Interferon, BAF A- bafilomycin A, MDM- monocyte derived macrophages, Mtb- *Mycobacterium tuberculosis*, BCG- *Bacille Calmette Guerin*, CH25H- cholesterol 25-hydroxylase, lyso^{+}- lysotracker positive, L1, L3- Lineage 1,3.

Significance Statement:

Co-evolution with humans has enabled development of novel adaptive mechanisms for survival in host specific environments in the human TB pathogen-Mtb. We present one such mechanism of modern Mtb strains harnessing the type I IFN immune axis to regulate the host pro-inflammatory response. Our results definitively demonstrate the use of host intracellular endosomal transit as a mechanism by these strains specifically to ensure a strong type I IFN response in macrophages. We also demonstrate the ability of Mtb to regulate macrophage cholesterol metabolism in order to fine tune the host innate responses. These findings lay the foundation of the future development of a host axis directed intervention strategy against this pathogen.

Introduction:

A long-standing association with the human population has been critical to the development of specific immune modulatory mechanisms by *Mycobacterium tuberculosis* (Mtb) for efficient survival within the host. With the advent of high throughput genome analysis, Mtb strains have been differentiated into 7 distinct groups that can be classified into phylogenetically ancient (restricted geographic penetration) and modern (widespread) lineages with substantial differences in their virulence and disease manifestation (1-3).

The initial contact of Mtb with macrophages activates the cGAS-STING-nucleic acid signaling pathway leading to type I IFN expression (4, 5). This response is mediated by host factors like ROS induced mitochondrial damage and pathogen associated factors like ESX-1 mediated phagosomal escape and mycobacterial cyclic di-nucleotides (6-10). A detrimental pathogenic effect has been associated with Mtb induced type I IFN signaling in regulating the expression of cytokines such as TNF-α, IL-12 and IL10 in macrophages (11-13). Moreover, type I IFNs have been shown to alter the IFN γ dependent activation of macrophages and consequent mycobacterial growth (14). However, several studies have also suggested a beneficial role for the mycobacteria induced type I IFN signaling in IL-12 production and antigen presentation by macrophages (15). Recent evidence also connects the type I IFN induced cholesterol 25-hydroxylase (CH25H) with the expression of pro-inflammatory cytokines like IL6 in macrophages (16-19).

57 Clinical Mtb lineages vary significantly in their inflammatory response induction and
58 immune modulatory functions (20-22). Given the duality of the outcome of type I IFN induction by
59 Mtb in macrophages, mechanisms underlying strain specific type I IFN is still unclear. We
60 demonstrate that the higher type I IFN induction by modern lineages (L3, L4 and L2) is dependent
61 on bacterial DNA presentation to the cGAS- STING signaling pathway as a result of an early and
62 increased bacterial trafficking via the endo-lysosomal pathway. Consistent with this augmented
63 IFN and enhanced expression of the IFN stimulated gene- CH25H, significantly higher levels of
64 IL6 were observed in macrophages infected with the modern Mtb strains. This study provides
65 evidence for a novel mechanism of the plasticity of modern Mtb by coupling type I IFN with
66 macrophage pro-inflammatory response. Understanding the physiological consequence of this
67 phenomenon would provide crucial insights into the successful adaptation of modern Mtb to the
68 host environment.

69 **Results:**

70 **Mtb lineages induce differential type I IFN response in macrophages.**

71 The type I IFN response of macrophages has generated interest as a pro- pathogenic response
72 induced by Mtb in macrophages (23-25). In our attempt to develop a quick high-through put
73 assay, we evaluated the ability of Mtb to induce type I IFN in the reporter cell line- THP1-Dual.
74 Detectable levels of secreted luciferase as a read out for IFN activation was observed in a
75 temporal manner in response to Mtb Erdman at a MOI of 5 with 10-15 folds activity by 24h and
76 48h (Fig.1A). Further, we also observed a distinct pattern of response in these macrophages
77 infected with clinical Mtb strains of different phylogenetic lineages. Macrophages infected with the
78 modern Mtb lineages (L3-N4, N24; L2- HN878, L4-H_{37}Rv, H37Ra, Erdman) displayed 8-20 folds
79 higher luminescence activity at 24h of infection contrasting with the lower (1.5-2.5 folds) or
80 absence of activity in macrophages infected with the ancient lineage I (N72, N73) Mtb and BCG
81 strains, respectively (Fig.1B, Fig. S1A). This higher activation potential of L3 Mtb was maintained
82 across different infective doses (Fig. 1C). While L3 Mtb strains (N4 and N24) could activate this
83 response even at a low MOI of 1 (4.6 folds) by 24h of infection, these levels were higher than the
84 average levels of IFN response induction (~3 folds) by L1 Mtb (N72 and N73) at MOI-10. Gene

expression profiles further validated the temporal and differential IFNβ expression profiles of Mtb infected macrophages; as observed in Fig. 1D, the L3 Mtb strain-N24 beginning to induce IFNβ mRNA as early as 1h with a gradual increase (~30 folds by 12h of infection) that was sustained even by 24h of infection (~16folds). In contrast, the L1 (N73) strain induced IFNβ at a significantly later time point in infection (6-12h) and at 5-7 fold levels than N24 even by 24h infection. This pattern of an attenuated IFN response by L1 Mtb was also evident in primary human MDMs and the mouse macrophage line RAW264.7 cells. Consistently L3 Mtb (N4, N24) induced significantly higher (4-10 folds) IFN response than L1 Mtb at any point in infection (Fig 1E, F).

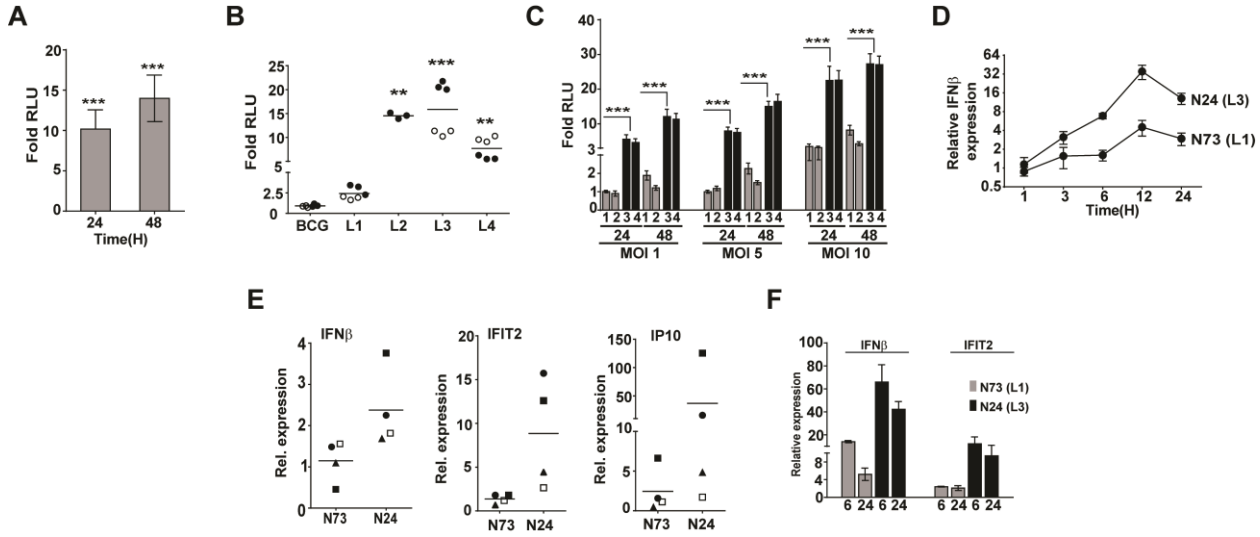


Fig.1

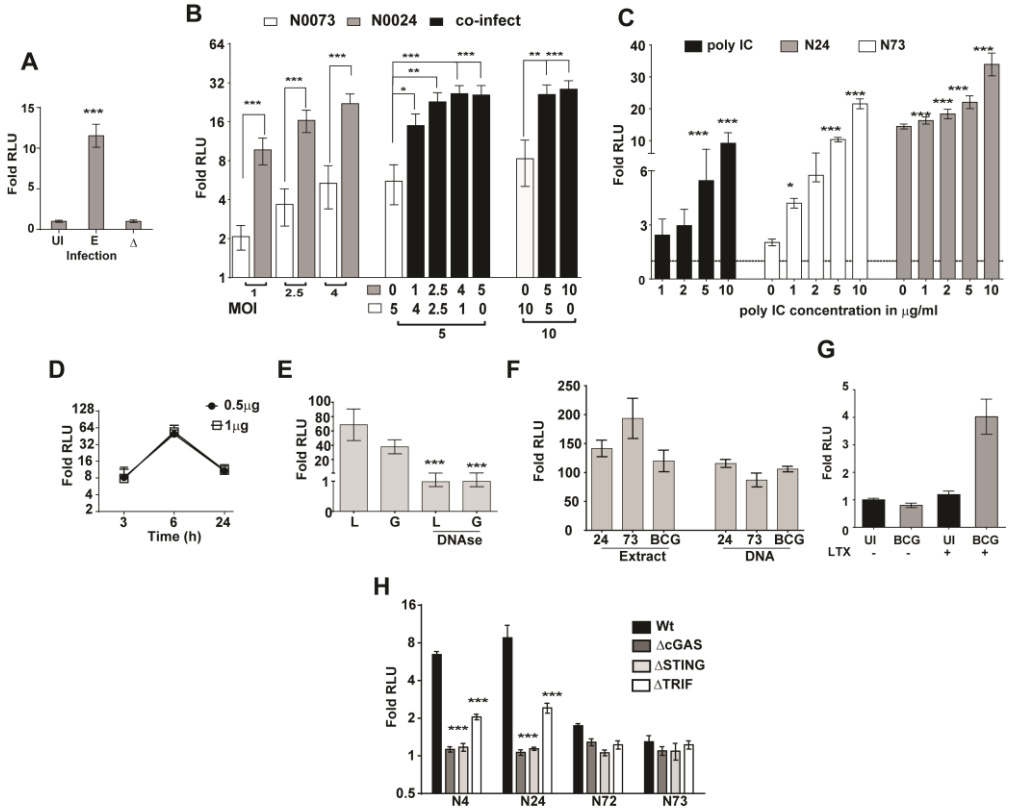
Fig.1: Modern lineages have evolved to induce a significantly higher type I IFN response

Relative luciferase activity in naïve and stimulated THP1 dual macrophages:

(A) Infected with Mtb Erdman at a MOI-5, (B) infected with Mtb strains belonging to different lineages [L1-4] at a MOI-5, 2 independent isolates of each lineage used for infection is represented as open and closed circles, (C) infected with Mtb L1 [grey bars; 1-N72, 2-N73] and L3 [black bars; 3- N4, 4-N24] at different MOIs. At the indicated time points, culture supernatants were harvested and tested for luciferase expression. Values represent average ratios ± SE of RLU in infected and control samples from triplicate wells of two independent experiments [N=2]. D-F) Relative expression in macrophages infected with N24 [L3] or N73 [L1] at a MOI-5. D) IFNβ expression at different time intervals post infection, E) IFNβ, IFIT2 and IP10 mRNA in primary MDMs from four separate individuals (symbols) and F) expression of IFNβ and IFIT2 mRNA in RAW macrophages. The average relative values ± SE (with respect to GAPDH) in infected and control samples from triplicate wells of multiple replicate experiments are represented.

107 **L1 Mtb are delayed in inducing type I IFN response in macrophages despite the presence**
108 **of an active ligand.**
109 The induced IFN response required active infection as macrophages infected with non-viable Mtb
110 failed to initiate this response (Fig. 2A). To verify if L1Mtb actively suppressed type I IFN response,
111 we employed a co-infection assay of N24 and N73 in different ratios (Fig. 2B). At all doses, N24
112 induced significantly higher response than N73, however, in mixed infections, presence of four
113 fold excess N73 (N73:N24:: 4:1) could not diminish the response induced by N24. Consequently,
114 IFN levels in mixed infections were comparable to levels in macrophages infected N24 alone even
115 at the overall higher MOI of infection of 10. Subsequently, in a trans- activation/ suppression
116 assay, again, N73 failed to suppress even the lower dose of 1µg/ml poly IC (Fig.2C). In most
117 cases, response to poly IC was clearly augmented in conditions of co-stimulation with either N24
118 ~2-2.5 folds) or 2-10 folds by N73.
119 Having ascertained that N73 did not suppress IFN induction, it was logical to assume lack/ lower
120 quantities of the active ligand as an underlying cause for the deferred IFN response by L1 Mtb. A
121 preliminary evaluation hinted at a temporal IFN response to Mtb Erdman extract (cell lysates) in
122 THP1 cells with the response increasing from ~10 folds at 3h to ~75 folds by 6h and then declining
123 to ~10 fold expression by 24h of stimulation with different doses (Fig. 2D). Even though very low
124 amounts of the extract (1.6 ng) was sufficient to induce ten-fold IFN by 6h (Fig. S2-A), the
125 complete loss of IFN only in extracts subjected DNase digestion argued for the essentiality of
126 DNA signaling in Mtb induced type I IFN in macrophages (Fig. 2E, S2-B). In fact with as low as
127 125 ng of genomic DNA from Mtb, macrophages responded with IFN expression that was again
128 abolished in cells stimulated with DNAse treated DNA. (Fig. 2E). Surprisingly, both the extracts
129 and genomic DNA from N73 as well as BCG were similar to Erdman and N24 in their IFN inducing
130 capacities (Fig. 2F), implying inaccessibility of the active ligand (DNA) to the IFN signaling
131 machinery of macrophages. The enhanced IFN response to BCG on delivery with lipofectamine
132 (3-6 fold higher than infection), lends further credence to this assumption (Fig. 2G). Additional
133 support for the essentiality of DNA signaling for IFN production by these Mtb lineages was
134 achieved in RAW 264.7 macrophages impaired in the signaling receptor/ adaptor (cGAS/ STING).

135 Wt RAW-luc (ISG) cells also portrayed a differential type I IFN response to Mtb lineages with 5-
136 10 fold higher IFN by L3 Mtb in comparison to L1 Mtb strains. Only the loss of cGAS, STING and
137 TRIF (~60% decrease) diminished the type I IFN response to L3 Mtb implicating a dominant role
138 of this signaling axis in this response against mycobacteria (Fig. 2H, S2-C).



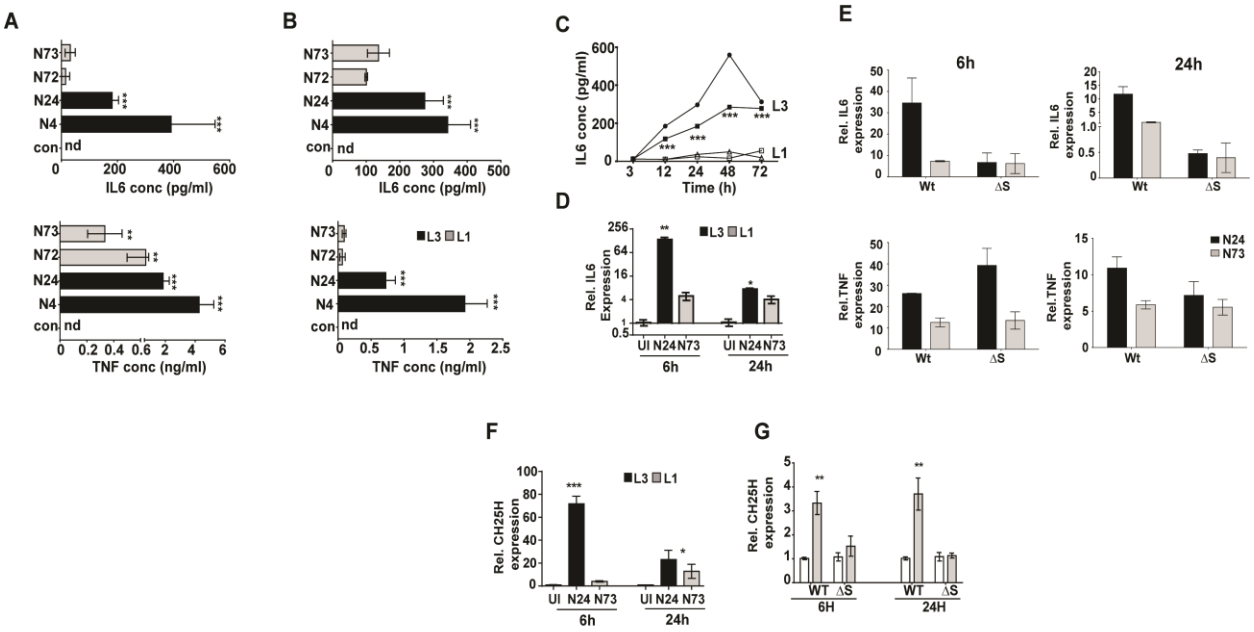
139 Fig. 2

140 **Fig.2: Strains with suppressed type I IFN response are well equipped with the active IFN inducing**
141 **ligand.**
142 Relative luciferase activity in naïve and stimulated THP1 dual macrophages:
143 (A) Infected with live or heat killed (Δ) Mtb Erdman at MOI-5, (B) with N24, N73 or in different combinations,
144 (C) stimulated with poly IC alone or in combination with Mtb at a MOI of 5, (D-G) following transfection with
145 two concentrations of crude Erdman lysates (D), crude lysate [L] or genomic DNA [G] alone or treated with
146 DNAse (E), crude lysates/ DNA of mycobacteria (F) and (G)- BCG. Value signifying the unchanged
147 expression [X=1] is indicated by a line. (H) Relative luciferase activity in Wt- ISG or mutant RAW
148 macrophages uninfected or infected with Mtb strains belonging to L3 / L1 lineages at a MOI of 5 for 24h.
149 Values represent average ratios ± SE of RLU in infected and control culture supernatants from triplicate
150 wells of three independent experiments [N=3].

151 **IFN inducing capability correlates with induction of a pro-inflammatory response via**
152 **expression of CH25H in macrophages**.

153 To test if L1 Mtb strains were attenuated in overall macrophage activation, we tested gene
154 expression and cytokine profiles of RAW264.7 macrophages infected with L1 and L3 Mtb. Both
155 TNF and IL6 patterns were strikingly similar to type I IFN, with lower levels in the case of L1 than
156 the L3 infected RAW264.7 macrophages after 24 hours (Fig. 3A). While negligible IL6 was seen
157 for the L1 Mtb strains, there was a 4-5-fold higher TNF in the N24 infected macrophages. This
158 attenuated TNF and IL6 expression was also observed in bone marrow-derived macrophages
159 from C57BL/6 mice (Fig. 3B) and in human MDMs (Fig. S3). In fact, L3 Mtb induced significantly
160 higher levels of IL6 in a temporal manner attaining peak values by 48h of infection (Fig. 3C). In
161 contrast, levels of IL6 were significantly lower in macrophages even as late as 72h following L1
162 Mtb infection.
163 The lower IFN and IL6 secretion associated with L1 Mtb suggested a common mechanism of
164 regulation in the macrophage response pathway by this mycobacterial strain. To check if the
165 regulation of IL6 expression was at the transcript level, we compared gene expression by qPCR.
166 In macrophages infected with Mtb an increase in IL6 was observed as early as 6h *p.i*. However,
167 in the N73 infected macrophages expression levels were significantly lower than in N24 infected
168 BMDMs (~ 30 fold less). In spite of a sharp decline in IL6 expression in N24 infected cells by 24h
169 of infection, expression levels were still significantly higher than in N73 infected cells (Fig. 3D).
170 Interestingly, while both strains induced IL6 by 6h, the differential higher response seen in the
171 case of N24 infection was completely dependent on the presence of STING (Fig. 3E). Clearly,
172 N73 Mtb, that failed to induce significant IL6 expression, was not affected by the presence or
173 absence of STING in the macrophages. However, N24 infection of ΔSTING macrophages induced
174 5-6 fold lower levels of IL6 than in Wt cells leading to the elimination of any difference between
175 the two Mtb strains.
176 More importantly, loss of STING did not significantly affect the TNF inducing capacity of Mtb in
177 macrophages. Recent evidence points to a positive regulatory circuit for IL6 via cGAS-STING
178 regulated expression of CH25H, responsible for the synthesis of the pro inflammatory oxysterol,
179 25 hydroxy-cholesterol (17, 19). Examination of genes involved in cholesterol biosynthesis
180 revealed a temporal but distinct pattern of expression in L3 and L1 Mtb infected BMDM (Fig. S4).

181 Most of the genes involved in the cholesterol biosynthetic pathway either up-regulated or similar
182 in L1 infected macrophages after 24h of infection. In contrast, these genes were inhibited in
183 response to infection with L3 Mtb. Surprisingly, in contrast with the overall pathway, expression
184 of CH25H was significantly upregulated in the L3 infected macrophages (60-70 fold higher than
185 the uninfected control) as opposed to the much lower expression of this gene (3-4 folds) in the L1
186 infected macrophages, by 6h of infection (Fig. 3F). This difference was however, lower at 24h,
187 wherein a sharp decline in L3 (23 folds) and an increase in L1 infected macrophages (10-12 folds)
188 was observed. Given the increased CH25H expression and requirement of type I IFN for
189 enhanced IL6 expression, we tested if STING signaling contributed to the early increase of
190 CH25H in L3 Mtb infected macrophages. The complete loss of CH25H expression in ΔSTING
191 macrophages substantiates our hypothesis of a regulatory role for type I IFN in host oxysterol
192 mediated IL6 response in modern Mtb infected macrophages (Fig. 3G).



193 Fig. 3

194 **Fig.3: IL6 response in Mtb infected macrophages primarily depends on STING mediated**
195 **CH25H expression.**

196 (A, B, C) Levels of cytokine secreted from RAW264.7 macrophages (A), BMDMs (B) at 24h, and at various
197 intervals p.i. (C) following infection with two L3 or L1 Mtb strains. Values represent average ratios ± SE *p.i*
198 in triplicate wells of two independent experiments (N=2). D, E) IL6 gene expression profiles in macrophages
199 (D-BMDMs and E- RAW -WT and ΔSTING- ΔS) following infection with N24 (L3) or N73 (L1) Mtb strains.
200 The average relative IL6 values ± SE (with respect to GAPDH) at 6h and 24h post infection in triplicate

assays of (N=2) is shown. (F, G) Expression of CH25H in BMDMs (F) and RAW macrophages (G) following infection with Mtb strains at 6h and 24h *p.i.* at MOI-5. The average relative IL6 values ± SE (with respect to GAPDH) at 6h and 24h post infection in triplicate assays of (N=2/3) is shown.

Early localization to lysosomes is important for Mtb induced type I IFN induction

The early induction of type I IFN in L3 infected macrophages advocated for molecular events that ensue immediately after infection as causative. Further, analysis of THP1 macrophages by TEM revealed the partitioning of different Mtb in distinct intracellular compartments- N24 and Erdman localized to compact double membranous vesicles at 24h in contrast to N73 that were confined in larger less defined vesicular structures with intracellular particles (Fig. 4A, S5). In support of this observation, we observed that Mtb N24 localized to lysotracker positive (lyso+) vesicles as early as 6h and at significantly higher numbers than N73 even at the later time point of 24h *p.i.* (Fig. 4B, C). This differential localization to lyso+ vesicles between N24 and N73 was also observed in RAW macrophages (Fig. 4D). In a temporal analysis of Mtb transit via the endosomal pathway, differential localization between Mtb strains arose as early as 1h of infection with more than two fold N24 in the lyso+ vesicles as compared to N73 (Fig. 4E). Despite a gradual decrease of N24 in lyso+ positive vesicles with time, consistently 2-fold higher numbers of N24 was seen throughout the experiment duration.

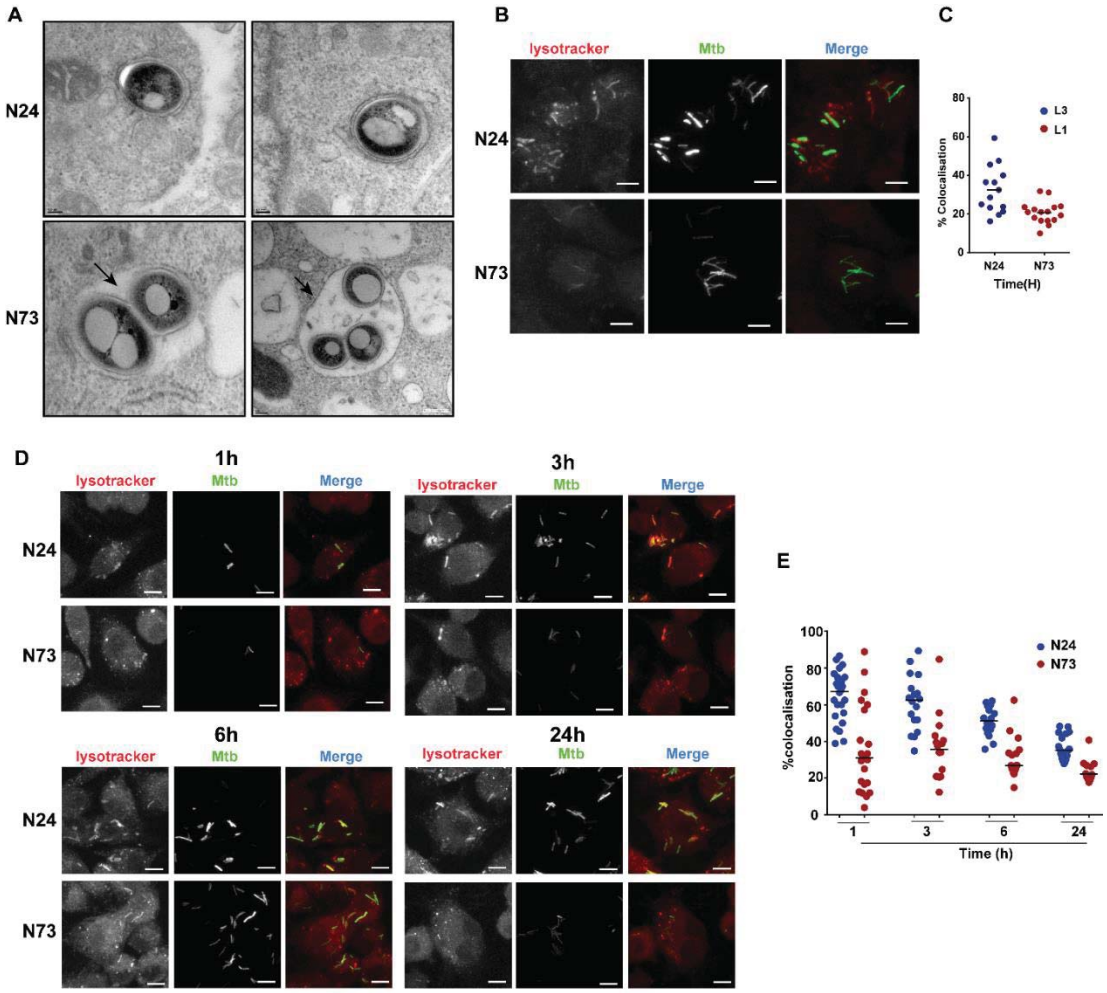


Fig. 4

Fig.4: Early and sustained localization of the high IFN inducing Mtb strains with acidified vesicles.

A) Representative TEM images (2) of Mtb N24 and N73 in THP1 macrophages at 24h post infection. While N24 is found in compact vesicles, N73 localizes in enlarged vesicles (black arrow) with cellular debris. B-E) Localization of Mtb N24 and N73 in acidified vesicles. Representative images of macrophages stained with lysotracker red and infected with GFP expressing N24/ N73 - THP1 (B) at 24h or RAW 264. 7 (D) at 1h, 3h, 6hand 24 h post infection is depicted. The extent of co- localization of Mtb with the lyso+ vesicles in THP1, 24h and RAW264.7 at 1h, 3h, 6h and 24h is shown graphically in panel C and E, respectively. Each dot represents a field of image from 2 independent experiments (> 100 infected cells).

IFN inducing capacity of Mtb relies on efficient acidification of the phagosome.

Given the surprisingly high numbers of N24 localization to lyso^{+} compartments, and comparable intracellular growth rates of N24 and N73, it was reasonable to assume an enhanced propensity of N24 to be better equipped for survival in this subcellular niche. The ability of Mtb to counter phagosomal-lysosomal fusion and acidification events is well recognized (26, 27). To understand if phagosomal acidification was important for IFN induction, we treated macrophages with an

established phagocytosis activation signal (LPS+ IFNγ) or phagosomal acidification blockers- bafilomycin A and chloroquine prior to infection with Mtb. Activation of macrophages with IFNγ and LPS enhanced the presence of Mtb in lyso^{+} vesicles (Fig. 5A). Importantly, the numbers of lyso+ N73 Mtb increased to comparable levels as N24 Mtb by 6h of infection (Fig. 5B). Consequently, a 10-fold higher IFN response was observed in N73 infected IFNγ+ LPS pretreated macrophages leading to loss of the differential IFN response between strains (Fig 5C and inset panel). While addition of IFNγ and LPS significantly boosted IFN induction even in N24 infected macrophages, on the contrary, treatment with phagosome acidification blockers like bafilomycin A or chloroquine sufficiently stunted (~5 and 2 folds, respectively) the IFN response in these cells (Fig. 5D) emphasizing the importance of phagosomal trafficking and acidification in Mtb mediated IFN induction in macrophages.

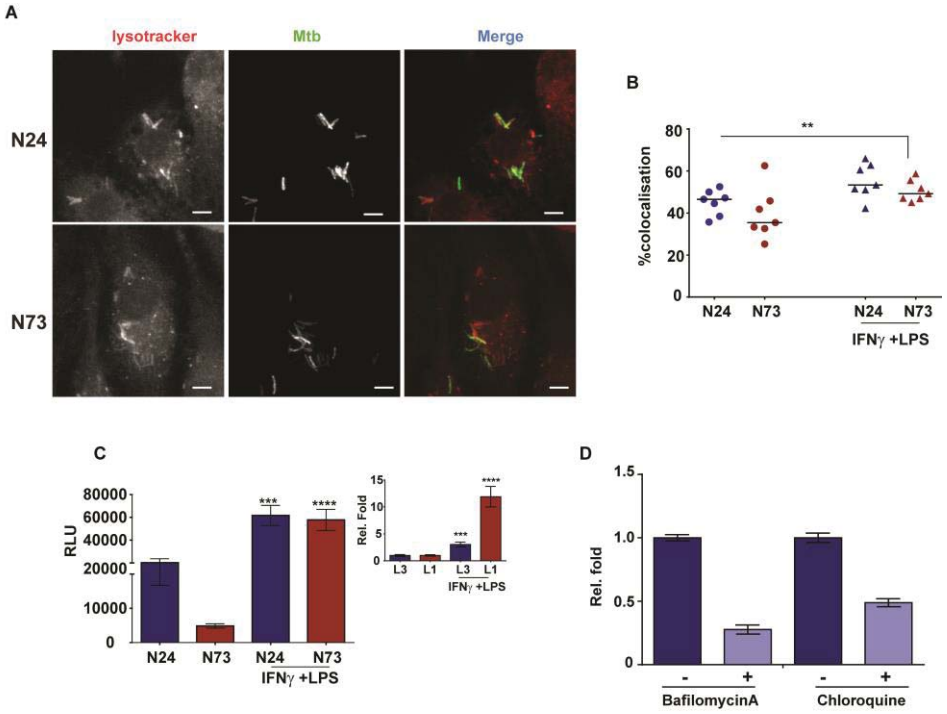


Fig. 5

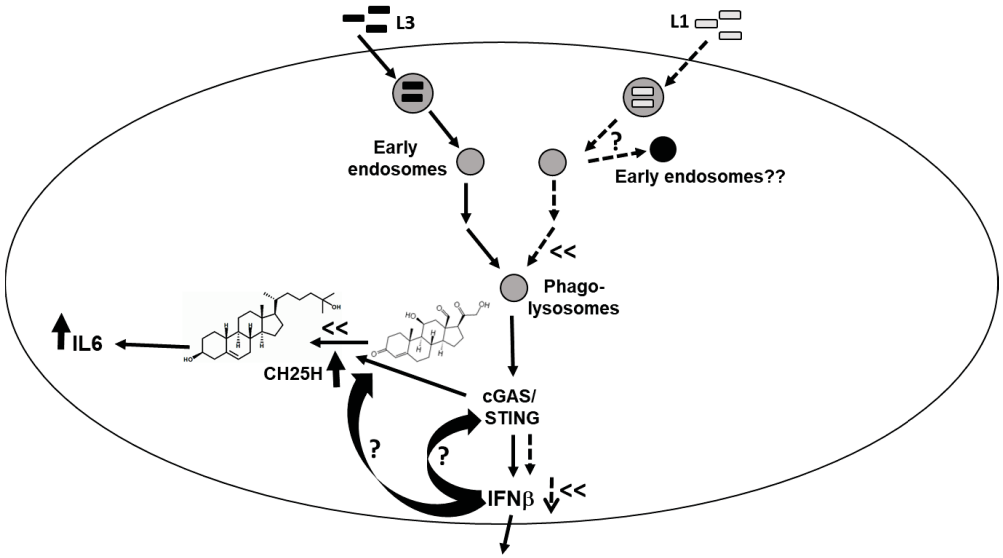
Fig.5: Phagosomal acidification is crucial for type I IFN induction by Mtb strains.

A) Localization of GFP expressing mycobacteria in lyso^{+} vesicles of IFNγ and LPS activated RAW macrophages, at 6h post infection. B) Co- localization of Mtb with the lyso+ vesicles is graphically represented. Each dot represents a field of image from a representative experiment. C, D) Relative luciferase activity in RAW macrophages left untreated or treated with IFNγ and LPS 16h prior (C) or bafilomycin A or chloroquine 1h (D) prior to infection with Mtb N24 and N73 at MOI-5 for 24h. Values

252 represent average ratios ± SE of RLU in infected and control culture supernatants from triplicate wells of
253 (N=2/3). The inset in panel C represents the fold change in RLU of the treated macrophages with respect
254 to the untreated cells.

255 **The working model**

256 Clinical Mtb strains differ in their capacities to access the host phago-lysosomal (P-L) pathway;
257 modern lineages traffic via this axis better and localize in lysosomal compartments of host
258 macrophages. In contrast, the older Mtb strains preferentially associate with the early endosomal
259 compartments and traffic less into the P-L pathway. This reduced movement hinders the access
260 of IFN inducing Mtb DNA to the nucleic acid response pathway and significantly lower type I IFN
261 response. The higher activation of type I IFN by the modern strains manifests as a substantial
262 upregulation of the pro-inflammatory CH25H and IL6 in a positive feedback loop.



263

264 **Fig.6: A putative working model of modern Mtb induced IFN and subsequent IL6**
265 **expression via the STING-CH25H pathway.**

266 **DISCUSSION**

267 The grand success of Mtb as a human pathogen stems from extreme plasticity to suit the host
268 cell niche, modulate the host response and survive multiple intracellular stresses. The immediate
269 response of host phagocytes upon contact is the activation of inflammatory responses aimed at
270 controlling the growth of the invading pathogen. Typically the type I IFN response is directed
271 against viral infections, but recent studies have highlighted the macrophage expression of type I
272 IFNβ in response to intracellular (pathogenic) and extracellular bacterial (protective) infections

273 (28-33). We observed that modern Mtb strains are proficient in inducing this response than the
274 evolutionarily older strains of Mtb. Surprisingly, we did not observe any change in IFN elicitation
275 by $H_{37}Rv$ or its avirulent derivative $H_{37}Ra$ despite significant difference in esx-1 mediated
276 secretory functions (34). Further, comparable expression of ESAT6 in our clinical Mtb strains (L1
277 and L3) (Fig. S1B) supports an esx-1 independent mechanism of IFN1 induction. Contrary to the
278 previous report of mitochondrial stress mediated IFN in Mtb infection (10), we did not observe any
279 conclusive evidence for mitochondria mediated IFN induction as the basis for differences in our
280 Mtb strains (Fig. S6 A, B).
281 As demonstrated previously, loss of STING/ cGAS completely abrogated Mtb type I IFN response
282 in macrophages implicating cellular DNA signaling as a predominant mechanism of this response.
283 Given the early induction of type I IFN and the absence of this response despite the presence of
284 active ligand, we argued that a novel mechanism that actively concealed the DNA from the cellular
285 nucleic acid signaling mechanism was responsible for the lower IFN induction by the attenuated
286 ancient Mtb strains. Interestingly, loss of TRIF also resulted in absence of IFN response in N24
287 infected macrophages suggesting a role for RNA signaling in this response. Surprisingly, we did
288 not observe loss of IFN induction by cellular extracts treated with RNAse. Recent reports have
289 identified a TRIF dependent activation of STING in the DNA induced IFN response of
290 macrophages (35); our results demonstrate a dominant role for this signaling pathway in Mtb
291 mediated type I IFN response.
292 Two separate observations supported our hypothesis that intracellular localization early in
293 infection would be primarily accountable for this differential IFN signaling by Mtb strains: Induction
294 of IFN response by 1) transfected BCG as opposed to direct infection and 2) absolute requirement
295 of naked DNA to be transfected. Bacterial lysis by the phagosome-lysosome pathway has been
296 implicated in several of the innate immune response pathways in phagocytes (36, 37).
297 Our results provide insights into the importance of transit of Mtb strains via the phagosome-
298 lysosome pathway as an important determinant of IFN induction in macrophages. While on the
299 one hand only live bacteria were able to stimulate the macrophage type I IFN response, dead Mtb
300 despite their well-documented higher rates of trafficking to acidified P-L compartments were

301 unable to induce this response in macrophages pointing at the ability of modern Mtb's unique
302 ability to utilize the host P-L pathway for active presentation of DNA (in contrast to dead bacteria
303 that fail in this presentation) to the cytosolic signaling complex. Additionally, the loss of Mtb
304 mediated IFN response in Baf A/ chloroquine treated macrophages in combination with the
305 significant increment by LPS+IFNγ pretreatment reveals an important role of phagosomal
306 acidification in type I IFN induction. A dominant role for the Mtb sulfolipids have been
307 demonstrated in modulation of the cellular phagosome maturation and acidification. Our results
308 negate any putative role for this glycolipid in the intracellular bacterial localization and IFN
309 response -1) differences in sulfolipid expression between H37Ra and H37Rv is well established
310 (38, 39), yet there was no discernable alteration of IFN expression between the two Mtb strains
311 and 2) Expression of sulfolipid in N73 also does not augment IFN expression (data not shown).
312 Our observation of a strong correlation between the STING mediated IFN induction and high IL6
313 response argued for a novel mechanism of macrophage response regulation by the modern Mtb
314 strains. Previous reports have implicated the regulatory oxysterol- CH25H in controlling IL6 gene
315 expression in host phagocytes (19, 40). Given the lower expression of genes for cholesterol
316 biosynthesis in the late stage of infection with N24 Mtb in line with the earlier report (41), it was
317 surprising that expression levels of CH25H were on the contrary increased, signifying its
318 importance in bridging type I IFN and IL6 expression in macrophages. Moreover, the complete
319 loss of CH25H expression in the STING deficient macrophages following N24 infection highlights
320 this novel regulatory loop of type I IFN- CH25H- IL6 regulation in modern Mtb infections. Overall,
321 we define a novel pathway of pro-inflammatory cytokine expression in Mtb induced macrophages
322 involving the early innate type I IFN response and active modulation of the host cell oxysterol
323 metabolism. Our results provide yet another example of the plasticity of Mtb as a human pathogen
324 by evolving multiple facets of host response manipulation.

325 **Material and Methods**

326 ***Bacterial Strains and Growth Conditions***- Mycobacterial and E.coli strains were cultured as
327 per standard protocols with the appropriate supplements and selection antibiotics as required.
328 The M6 promoter fragment was amplified as described earlier (42) and cloned upstream of GFP

329 in the mycobacterial expression vector- pMV261 and electroplated into mycobacteria. Expression
330 of ESAT6 in the culture filtrate and secreted Mtb cultures was performed by immunoblotting with
331 anti ESAT6 monoclonal antibody HYB76-8 as described earlier (3).
332 **Macrophage culture and maintenance-** All the cell lines used were tested for mycoplasma
333 contamination at regular intervals and cultured according to the manufacturer's
334 recommendations. THP1 cells were grown in HiglutaXL RPMI-1640 (Himedia laboratories, India)
335 with 10% FBS and differentiated by using PMA as described earlier (43). Human monocyte
336 derived macrophages (MDMs) were isolated from 20ml as described earlier (42). For primary
337 mouse macrophages, bone marrow cells were harvested from the hind limbs of C57Bl6 mice and
338 differentiated into macrophages in 20% L- cell supernatant for 7 days as described earlier (44).
339 **Preparation of Mtb fractions for stimulation of macrophages-** Mtb crude extracts were
340 prepared from logarithmic liquid cultures with similar bacterial densities for the different strains.
341 Cells were lysed with a mixture of 0.1 µm and 0.7 µm zirconia beads in PBS using a bead beater
342 (Biospec India). The resultant lysates were cleared off the cell debris by high speed centrifugation
343 and sterilized by filtration and used to transfect THP1 dual cells using Lipofectamine LTX
344 (Invitrogen, USA) reagent according to the recommended protocol. Genomic DNA was isolated
345 according to the manufacturer's recommendations (Nucleopore gDNA kit) and used for
346 transfection. Enzymatic treatment of lysates was performed as: 1) Proteinase K- at 55^0C for 1h,
347 2) DNase, RNase at 37^0C for 1h and thermal denaturation at 96^0C for 30min.
348 **Infection of macrophages with Mtb-** Macrophage cultures were seeded at the requisite density
349 and infected with single cell suspensions (SCS) as described earlier (43). At different time
350 intervals, the cells/ RNA/ supernatants were harvested and used for analysis. Macrophages
351 infected with fluorescent Mtb strains were used for lysosomal localization by using 100nm
352 Lysotracker–Red DND-99 (Invitrogen, USA) according to manufacturer's recommendations.
353 Macrophages transfected with mito-DsRed (kind gift from Dr. Sowmya Sinha Roy) was used for
354 analysis of mitochondrial architecture. The cells were fixed in 4% paraformaldehyde for 30 min,
355 mounted on slides with Prolong diamond anti fade (Invitrogen, USA) and subjected to microscopy
356 in the Leica TCS SP8 Confocal Microscope (Leica Microsystems, USA). For estimating

357 mitochondrial ROS by Mitosox staining, macrophages were washed thrice with PBS and stained
358 with 5μM Mitosox (Invitrogen, USA) in HBSS for 30 min at 37^0C, fixed and subjected to high
359 content microscopy in a IN Cell Analyzer 6000(GE healthcare life sciences, USA).

360 **Transmission Electron Microscopy-** THP1 cells were infected with mycobacteria at a MOI of 5
361 for 24h, fixed in 2.5% gluteraldehyde and 4% paraformaldehyde, dehydrated in graded series of
362 alcohol and embedded in Epon 812 resin. Ultrathin sections were cut and stained with uranyl
363 acetate and lead citrate and imaged by using Tecnai G2 20 twin (FEI) transmission electron
364 microscope (FEI, Thermo Fisher Scientific Inc, USA).

365 **Statistical Analysis**

366 All experiments were performed in multiple biological replicates (N=2 or 3). Statistical analysis
367 was done by using Student's t-test and corresponds to a *, **, *** for p-values<0.05, <0.01and
368 <0.001, respectively.

369 **Ethics Statement**

370 The protocol for isolation of human blood PBMCs was approved by the Institutional Human ethics
371 committee (Ref no: CSIR-IGIB/IHEC/2017-18 Dt. 08.02.2018). Blood was collected from people
372 with their informed consent as per the Institutional guidelines.

373 **References**

374 1. Comas I*, et al.* (2013) Out-of-Africa migration and Neolithic coexpansion of Mycobacterium
375 tuberculosis with modern humans. *Nat Genet* 45(10):1176-1182.
376 2. Pareek M*, et al.* (2013) Ethnicity and mycobacterial lineage as determinants of tuberculosis
377 disease phenotype. *Thorax* 68(3):221-229.
378 3. Rao V, Dhar N, & Tyagi AK (2003) Modulation of host immune responses by overexpression of
379 immunodominant antigens of Mycobacterium tuberculosis in bacille Calmette-Guerin. *Scand J*
380 *Immunol* 58(4):449-461.
381 4. Stanley SA, Johndrow JE, Manzanillo P, & Cox JS (2007) The Type I IFN response to infection with
382 Mycobacterium tuberculosis requires ESX-1-mediated secretion and contributes to pathogenesis.
383 *J Immunol* 178(5):3143-3152.
384 5. Manzanillo PS, Shiloh MU, Portnoy DA, & Cox JS (2012) Mycobacterium tuberculosis activates the
385 DNA-dependent cytosolic surveillance pathway within macrophages. *Cell host & microbe*
386 11(5):469-480.
387 6. Collins AC*, et al.* (2015) Cyclic GMP-AMP Synthase Is an Innate Immune DNA Sensor for
388 Mycobacterium tuberculosis. *Cell host & microbe* 17(6):820-828.
389 7. Pandey AK*, et al.* (2009) NOD2, RIP2 and IRF5 play a critical role in the type I interferon response
390 to Mycobacterium tuberculosis. *PLoS Pathog* 5(7):e1000500.
391 8. Redford PS*, et al.* (2014) Influenza A virus impairs control of Mycobacterium tuberculosis
392 coinfection through a type I interferon receptor-dependent pathway. *J Infect Dis* 209(2):270-274.
393 9. Dey B*, et al.* (2015) A bacterial cyclic dinucleotide activates the cytosolic surveillance pathway and
394 mediates innate resistance to tuberculosis. *Nat Med* 21(4):401-406.

395 10. Wiens KE & Ernst JD (2016) The Mechanism for Type I Interferon Induction by Mycobacterium
396 tuberculosis is Bacterial Strain-Dependent. *PLoS Pathog* 12(8):e1005809.
397 11. de Paus RA*, et al.* (2013) Inhibition of the type I immune responses of human monocytes by IFN-
398 alpha and IFN-beta. *Cytokine* 61(2):645-655.
399 12. Mayer-Barber KD*, et al.* (2014) Host-directed therapy of tuberculosis based on interleukin-1 and
400 type I interferon crosstalk. *Nature* 511(7507):99-103.
401 13. McNab FW*, et al.* (2014) Type I IFN induces IL-10 production in an IL-27-independent manner and
402 blocks responsiveness to IFN-gamma for production of IL-12 and bacterial killing in
403 Mycobacterium tuberculosis-infected macrophages. *J Immunol* 193(7):3600-3612.
404 14. Teles RM*, et al.* (2013) Type I interferon suppresses type II interferon-triggered human anti-
405 mycobacterial responses. *Science* 339(6126):1448-1453.
406 15. Giacomini E*, et al.* (2009) IFN-beta improves BCG immunogenicity by acting on DC maturation. *J*
407 *Leukoc Biol* 85(3):462-468.
408 16. Blanc M*, et al.* (2013) The transcription factor STAT-1 couples macrophage synthesis of 25-
409 hydroxycholesterol to the interferon antiviral response. *Immunity* 38(1):106-118.
410 17. Gold ES*, et al.* (2014) 25-Hydroxycholesterol acts as an amplifier of inflammatory signaling.
411 *Proceedings of the National Academy of Sciences of the United States of America* 111(29):10666-
412 10671.
413 18. Diczfalusy U*, et al.* (2009) Marked upregulation of cholesterol 25-hydroxylase expression by
414 lipopolysaccharide. *J Lipid Res* 50(11):2258-2264.
415 19. Bah SY, Dickinson P, Forster T, Kampmann B, & Ghazal P (2017) Immune oxysterols: Role in
416 mycobacterial infection and inflammation. *J Steroid Biochem Mol Biol* 169:152-163.
417 20. Portevin D, Gagneux S, Comas I, & Young D (2011) Human macrophage responses to clinical
418 isolates from the Mycobacterium tuberculosis complex discriminate between ancient and
419 modern lineages. *PLoS Pathog* 7(3):e1001307.
420 21. Weiss G & Schaible UE (2015) Macrophage defense mechanisms against intracellular bacteria.
421 *Immunol Rev* 264(1):182-203.
422 22. Manca C*, et al.* (2005) Hypervirulent M. tuberculosis W/Beijing strains upregulate type I IFNs and
423 increase expression of negative regulators of the Jak-Stat pathway. *J Interferon Cytokine Res*
424 25(11):694-701.
425 23. Liu CH, Liu H, & Ge B (2017) Innate immunity in tuberculosis: host defense vs pathogen evasion.
426 *Cell Mol Immunol*.
427 24. Petit-Jentreau L, Tailleux L, & Coombes JL (2017) Purinergic Signaling: A Common Path in the
428 Macrophage Response against Mycobacterium tuberculosis and Toxoplasma gondii. *Front Cell*
429 *Infect Microbiol* 7:347.
430 25. Dorhoi A*, et al.* (2014) Type I IFN signaling triggers immunopathology in tuberculosis-susceptible
431 mice by modulating lung phagocyte dynamics. *Eur J Immunol* 44(8):2380-2393.
432 26. Sun J*, et al.* (2010) Mycobacterial nucleoside diphosphate kinase blocks phagosome maturation
433 in murine RAW 264.7 macrophages. *PloS one* 5(1):e8769.
434 27. Vergne I*, et al.* (2004) Mycobacterium tuberculosis phagosome maturation arrest: mycobacterial
435 phosphatidylinositol analog phosphatidylinositol mannoside stimulates early endosomal fusion.
436 *Mol Biol Cell* 15(2):751-760.
437 28. Martin FJ*, et al.* (2009) Staphylococcus aureus activates type I IFN signaling in mice and humans
438 through the Xr repeated sequences of protein A. *J Clin Invest* 119(7):1931-1939.
439 29. Castiglia V*, et al.* (2016) Type I Interferon Signaling Prevents IL-1beta-Driven Lethal Systemic
440 Hyperinflammation during Invasive Bacterial Infection of Soft Tissue. *Cell host & microbe*
441 19(3):375-387.
442 30. Watanabe T*, et al.* (2010) NOD1 contributes to mouse host defense against Helicobacter pylori
443 via induction of type I IFN and activation of the ISGF3 signaling pathway. *J Clin Invest* 120(5):1645-
444 1662.
445 31. Hansen K*, et al.* (2014) Listeria monocytogenes induces IFNbeta expression through an IFI16-,
446 cGAS- and STING-dependent pathway. *Embo J* 33(15):1654-1666.

32. Abdullah Z*, et al.* (2012) RIG-I detects infection with live Listeria by sensing secreted bacterial nucleic acids. *Embo J* 31(21):4153-4164.
33. Boxx GM & Cheng G (2016) The Roles of Type I Interferon in Bacterial Infection. *Cell host & microbe* 19(6):760-769.
34. Pinto SM*, et al.* (2018) Integrated Multi-Omic Analysis of Mycobacterium tuberculosis H37Ra Redefines Virulence Attributes. *Frontiers in microbiology* 9:1314.
35. Wang X*, et al.* (2016) STING Requires the Adaptor TRIF to Trigger Innate Immune Responses to Microbial Infection. *Cell host & microbe* 20(3):329-341.
36. Herskovits AA, Auerbuch V, & Portnoy DA (2007) Bacterial ligands generated in a phagosome are targets of the cytosolic innate immune system. *PLoS Pathog* 3(3):e51.
37. Ip WK*, et al.* (2010) Phagocytosis and phagosome acidification are required for pathogen processing and MyD88-dependent responses to Staphylococcus aureus. *J Immunol* 184(12):7071-7081.
38. Gonzalo Asensio J*, et al.* (2006) The virulence-associated two-component PhoP-PhoR system controls the biosynthesis of polyketide-derived lipids in Mycobacterium tuberculosis. *The Journal of biological chemistry* 281(3):1313-1316.
39. Walters SB*, et al.* (2006) The Mycobacterium tuberculosis PhoPR two-component system regulates genes essential for virulence and complex lipid biosynthesis. *Mol Microbiol* 60(2):312-330.
40. Wilkins C & Gale M, Jr. (2013) Sterol-izing innate immunity. *Immunity* 38(1):3-5.
41. Zimmermann M*, et al.* (2017) Integration of Metabolomics and Transcriptomics Reveals a Complex Diet of Mycobacterium tuberculosis during Early Macrophage Infection. *mSystems* 2(4).
42. Arumugam P, Shankaran D, Bothra A, Gandotra S, & Rao V (2019) The MmpS6-MmpL6 Operon Is an Oxidative Stress Response System Providing Selective Advantage to Mycobacterium tuberculosis in Stress. *J Infect Dis* 219(3):459-469.
43. Bothra A*, et al.* (2018) Phospholipid homeostasis, membrane tenacity and survival of Mtb in lipid rich conditions is determined by MmpL11 function. *Scientific reports* 8(1):8317.
44. Rao V, Fujiwara N, Porcelli SA, & Glickman MS (2005) Mycobacterium tuberculosis controls host innate immune activation through cyclopropane modification of a glycolipid effector molecule. *J Exp Med* 201(4):535-543.

Acknowledgements: The authors thank CSIR (VR-BSC0123), and DBT (GAP0096) funding agency for supporting the study. The authors thank Mr. Manish Kumar and CSIR- BSC0403 for the confocal microscopy facility. The Transmission Electron microscopy (TEM) and mass spectrometry facilities are duly acknowledged. The institute BSL3 facility project (STS0016) is duly acknowledged. The student fellowships from CSIR and DBT India are acknowledged. The support provided by Academy of Scientific and Innovative research (AcSIR) is duly acknowledged.

Author Contributions: DS, PA, SG, VR were involved in conceptualizing and design of the work, the work was performed by PA and DS. VR, PA and DS were involved in the manuscript preparation and proof reading.

Supplementary figures:

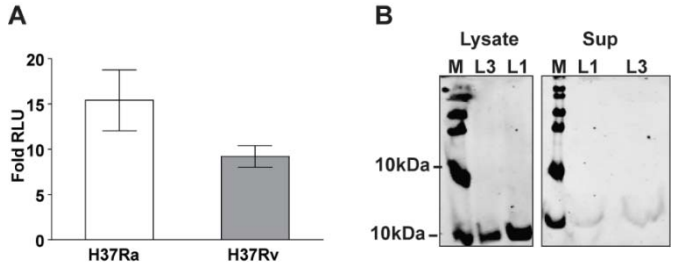


Fig.S1

A) Relative luminescence in THP1 macrophages infected with H37Ra and H37Rv at 24h *p.i.* ESAT6 expression in culture supernatant and lysates of Mtb N24 and N73.

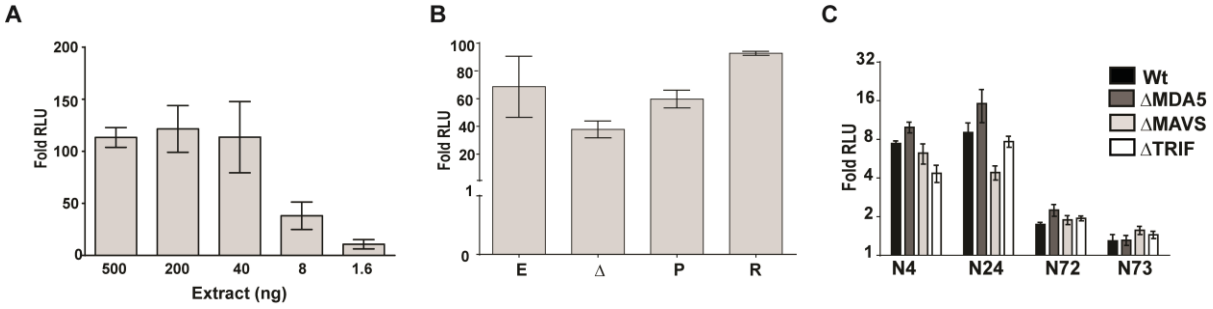


Fig.S2

A) Dose dependent IRF induction by Mtb crude extracts at 6h.
B) IRF induction by enzymatically digested 500ng of Mtb crude extracts at 6h.
C) Relative luminescence in RAW WT and KO macrophages infected with L3 and L1 Mtb at 24h post infection.

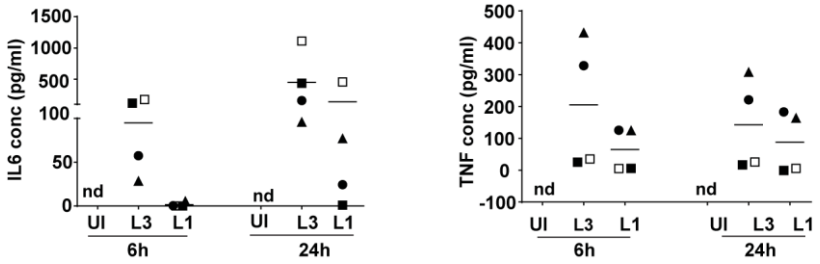


Fig. S3

IL6 and TNF secretion from human MDMs from four individuals left uninfected (UI) or following infection with L3 or L1 strains at 6h and 24h p.i. Values represent mean cytokine concentration in pg/ml in the culture supernatant of triplicate wells for each individual.

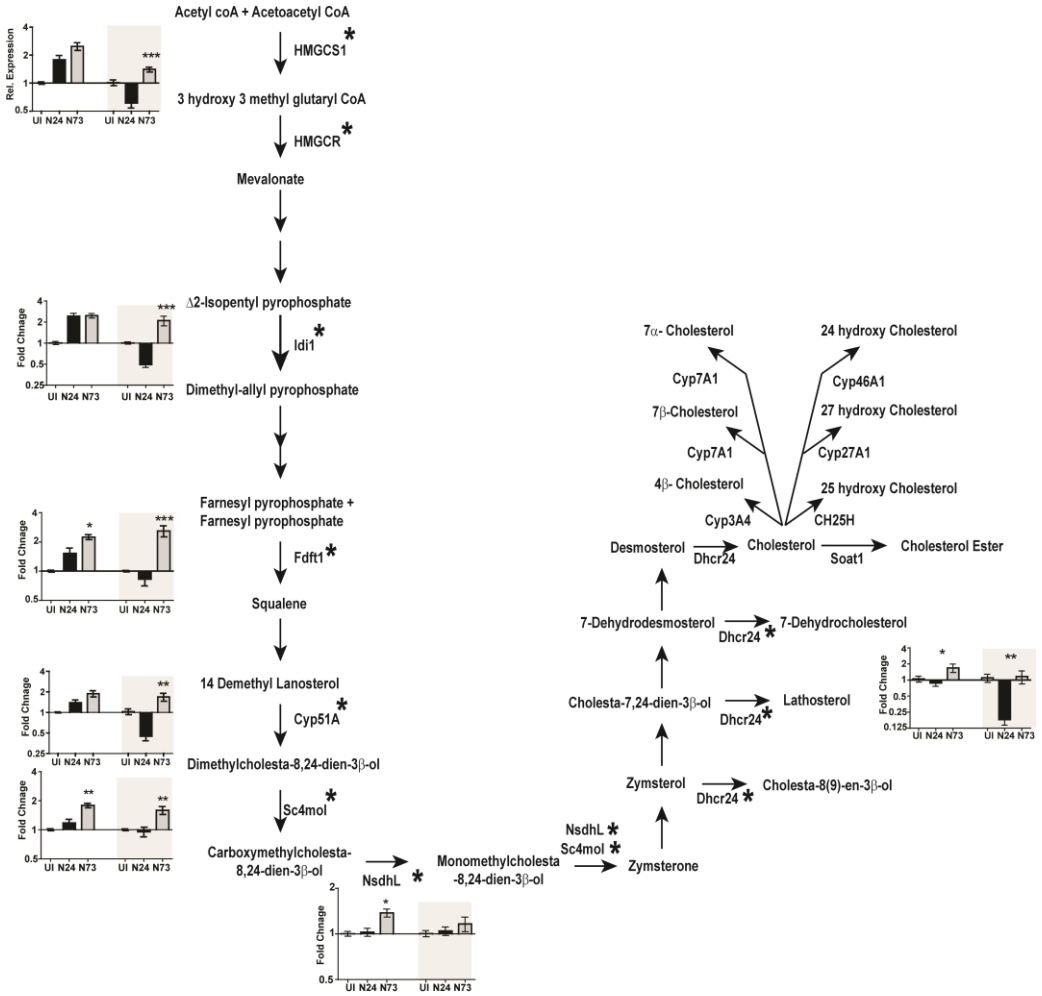


Fig.S4

Expression of genes involved in cholesterol biosynthesis in macrophages infected with N24 or N73 at 6h and 24h (shaded) estimated by qRTPCR.

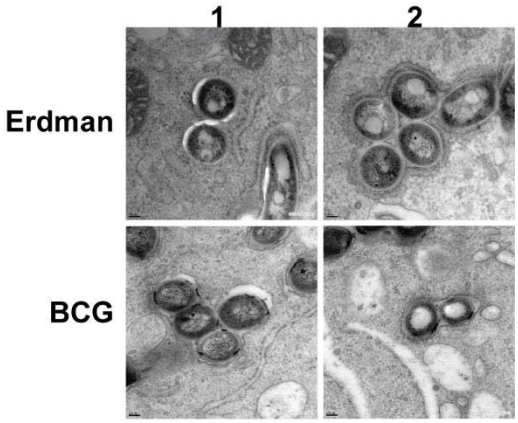
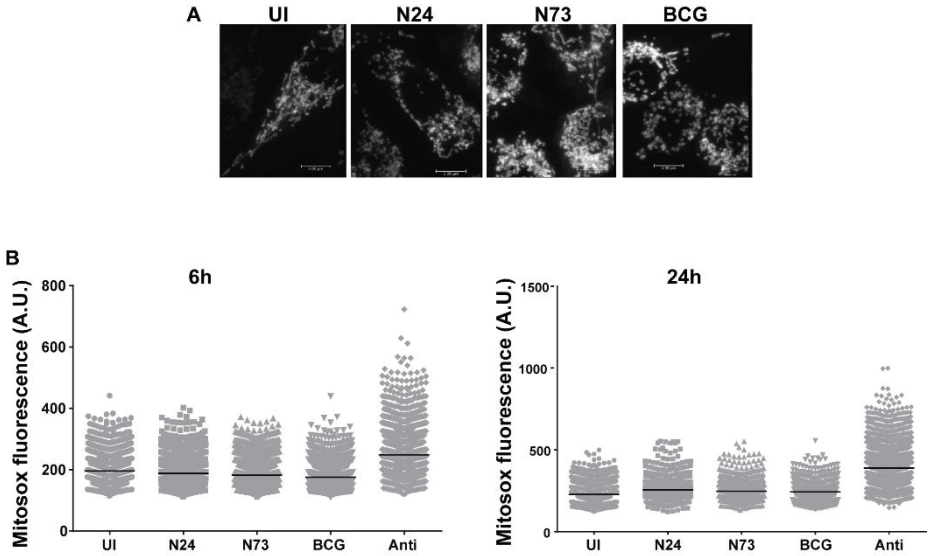


Fig.S5

Electron micrographs of THP1 macrophages infected with Mtb Erdman and BCG at 24h p.i.



517 **Fig.S6**

518 A) Mitochondrial architecture at 24h post infection with mycobacterial strains. Uninfected macrophages (UI) was
519 used as a control.

520 B) Quantitation of mitochondrial ROS by mitosox staining following infection with mycobacteria or treatment of
521 Antimycin A (+ control) at 6h and 24h.

522
523
524
525
526
527