Original Article

Mycobacterium tuberculosis strains from ancient and modern lineages induce distinct patterns of immune responses

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Abstract

Introduction: It is possible that the difference in virulence and prevalence of different strains of *Mycobacterium tuberculosis* is related to the diverse immune response they evoke in the host. Outbreak strains have been shown to subvert the innate immune response as a potential host evasion mechanism. However, the immunological outcome of the interactions of different clinical strains with different host cells is still not understood.

Methodology: Extremely Drug Resistant (XDR) Beijing, a modern lineage clinical strain and a comparator ancient lineage strain, EAI-5, were selected for the present study. The early induction of proinflammatory cytokines in human peripheral blood monocyte derived macrophages (MDM), monocyte derived dendritic cells (MDDC) and whole blood (WB) infected by selected clinical isolates and laboratory strains H37Rv and BCG were assessed.

Results: The two clinical strains exhibited distinct patterns of cytokine induction. The ancient lineage strain induced substantially higher expression of all proinflammatory cytokines like TNF-α, IL-1β, IL-12 and chemokines like MCP-1, IL-8. However, the modern lineage strain exhibited suppressed response for the same. Further, the immune responses to two strains were conserved in infected MDM, MDDC and WB i.e. showing similar patterns of response across multiple human hosts. However, the differential response pattern was not observed when bacterial sonicates were used instead of live mycobacteria.

Conclusions: The lineage specific patterns in induction of proinflammatory cytokines and chemokines by different *M. tuberculosis* strains remain similar in macrophage and dendritic cells isolated from different individuals. The present study also confirms that whole cell sonicates of different lineages of *M. tuberculosis* fail to induce such lineage specific response.

Key words: *Mycobacterium tuberculosis;* lineage; immune response.

Introduction

Mycobacterium tuberculosis (MTB) is the causative agent of human tuberculosis which is one of the top ten causes of death worldwide [1]. It is acquired, predominantly, through inhalation of droplets containing live MTB. The pathogen primarily infects lung macrophages which provide an essential niche to establish infection in the host [2]. Infecting bacilli are either killed by the host or remain 'viable but latent' inside the host macrophage for decades. In the infected individuals who develop active disease, bacilli appear to evade or subvert the host's protective cellular immune responses.

The precise pathogenesis of TB and the factors responsible for the highly variable outcome of infection remain only partly understood. MTB can infect a diverse range of host cells from macrophage, dendritic cells (DC), and epithelial cells to almost all existing cell types, which is reflected in extra-pulmonary tuberculosis. It was traditionally assumed that MTB was genetically homogenous because early studies revealed very low levels of DNA sequence variation. However, after the discovery of molecular typing methods, it is now well known that different types and subtypes of MTB do exist and that genotype might be associated with differences in virulence, antibiotic susceptibility, relapse and prevalence of disease [3–6]. Several recent studies, including ours, suggest that phylogenetic diversity of the strains is also associated with diversity in biological response of the host [7]. In our previous study, several different innate immune parameters were monitored for infection of THP-1 cells (monocytic leukemia cell line) with three drug resistance clinical isolates from different lineages of MTB and the infected cells showed lineage specific innate immune responses [8]. The ancient lineage

strain, EAI induced significantly higher expression of TNF-α, IL-1β and IL-12 as well as apoptosis compared to modern lineage strain Beijing and laboratory strain H37Rv, in *ex vivo* infection. Another group of investigators also demonstrated that ancient strains were less virulent compared to modern strains [9] and induce a higher proinflammatory response in the host [10]. In contrast, the modern Beijing/W strain was found to be significantly associated with extra-thoracic TB with rapid and severe disease [6,11]. It also led to significantly less induction of proinflammatory cytokines and apoptosis in host cells [12].

The THP-1 cell line is immortal and does not respond in the same way as primary cells. Additionally, whether the phenotypic responses induced by different lineages will differ with various genotypes of the host is not yet clearly understood. It is also not clear whether differential host responses or differences in the response to different strains of MTB lead to differential disease severity. It is crucial to examine the immune responses induced by different genotypes in different hosts in order to understand the underlying mechanisms of the virulence of this economically important pathogen. The information obtained may help in treatment and follow up.

In view of this, two strains which had shown distinctly opposite responses in our earlier study along with two laboratory strains were used to infect primary cultures of monocyte derived macrophage (MDM), monocyte derived dendritic cell (MDDC) and whole blood isolated from several individuals and various responses were evaluated in them.

Methodology

Study subject

Seven, tuberculin skin test positive healthy subjects were included (5 men and 2 women; mean age 32 years) in this study. The volunteers were BARC employees who did not have any chronic or acute medical condition at the time of the study. After the approval from the institutional medical ethics committee of the Bhabha Atomic Research Centre (BARC), heparinized blood samples were drawn from each subject after obtaining informed consent.

Mycobacterium tuberculosis strains

Two clinical isolates (EAI-5 and Beijing) used in the study were kindly provided by Department of Microbiology, Tata Memorial Hospital, Parel, Mumbai and were selected on the basis of results of our earlier study on their interaction with THP-1 cells. The

characterization of the strains was described in the earlier study [8].

Mycobacterial growth and single cell suspension

The MTB strains isolated from patients were plated on Lowenstein–Jensen (LJ) medium (HIMEDIA, Mumbai, India) and a single colony was added to Middelbrook 7H9 medium (HIMEDIA, Mumbai, India), supplemented with oleic acid/albumin/dextrose/catalase (HIMEDIA, Mumbai, India) and incubate until the culture was in mid log phase. The cells were harvested at this point and stored in glycerol at -70ºC. Before every infection study these cell stocks were grown into log phase and used. Thus the passage number was maintained at 3-5 for all the experiments. The single cell suspensions were prepared as per the standard protocol with minor modification [13]. Briefly, the cell pellets were washed, suspended in PBS containing 0.2% Tween 20 and transferred to a hard glass test tube containing around 25 glass beads (3mm diameter). After bath sonication for 10 seconds and vigorous vortexing for 5 minutes, the suspension was kept undisturbed for 30 minutes. The cell count was monitored by taking the optical density (OD) of the upper cell layer at 600nm and it was finally adjusted as required for infection experiments. The absence of clumps was confirmed by Ziehl–Neelsen Carbol Fuchsin (ZNCF) staining and the cell viability in each preparation was evaluated by a colony forming units (CFU) assay. The sonicates of all MTB strains were prepared by sonicating suspensions of 10^8 CFU per mL of dried bacilli in ice-cold PBS in a probe sonicator (Sonifier 450; Branson Ultrasonics Corp., Danbury, USA) at 40% maximum power output for 5 min. This sonicates represent all the antigens of respective strains.

Ex-vivo generation and infection of MDM and MDDC

Peripheral blood mononuclear cells (PBMC) were separated from heparinized whole blood by Histopaque-1077 (Sigma, St. Louis, USA) gradient centrifugation according to the manufacturer's protocol. To isolate monocytes, PBMCs were cultured in 24-well plates (Nuncleon, Roskilde Denmark) containing RPMI-1640 (Sigma, MO, USA) with 10% heat-inactivated foetal calf serum (FCS; Sigma-Aldrich, St. Louis, USA), 2 mM L-glutamine and 100 units of gentamicin for 2 hours at 37ºC. Non-adherent cells were removed by washing with phosphate buffered saline (PBS) containing 3% FCS. Adherent cells were incubated for 7 days at 37ºC and 5% CO_2 to obtain MDM. MDDC were generated by culturing adherent monocytes in 24-well tissue cultures plates

with 400 U/mL GM-CSF and 400 U/mL IL-4 (BD) for 5 days in RPMI-1640 with supplements as mentioned above. The purity of MDM and MDDC was assessed by flow cytometry after labelling cells with CD14 and CD1a primary antibody respectively and FITC conjugated secondary antibody. This was done only for one sample during protocol standardization and the same protocol was followed for the rest of the samples.

The cells were infected with a single cell suspension of each of the two clinical isolates (EAI-5 and Beijing), the vaccine strain (BCG) and the laboratory strain (H37Rv) for 4 hours at a multiplicity of infection (MOI) of five. The cells were washed to remove extracellular mycobacteria and were further incubated for 20 hours before RNA extraction.

RNA extraction, cDNA synthesis and real-time reverse transcriptase polymerase chain reaction (RT-PCR)

After a total of 24 hours of infection, RNA was extracted from infected macrophages and DCs using an RNA extraction kit (HIMEDIA, Mumbai, India) and digested by DNase I (Roche, Mannheim, Germany). Thereafter, cDNA was synthesized from 1µg of RNA using a cDNA synthesis kit (Fermentas life Sciences, St. Leon-Rot, Germany). Quantitative real-time RT-PCR was performed for TNF-α, IL-1β, IL-12, IL-10, MCP-1, IL-8 and β-actin using SYBR Green Master Mix (Stratagene, La Jolla, USA) with the following amplification conditions: $95^{\circ}C$ for 10 min, for 40 cycles at $95^{\circ}C$ for 15s, $60^{\circ}C$ for 30s and $72^{\circ}C$ for 30s. Melting curve analysis on the product was used to confirm specificity of the PCR and Ct values were normalized to the house-keeping gene, β-actin by $2^{-\Delta\Delta Ct}$ method. Expression levels were presented as fold induction in comparison to uninfected cells [14].

Whole Blood assay

Blood samples were collected in apyrogenic heparinized tubes (Vacutainer; Becton Dickinson, Rutherford, USA). The blood was diluted 1:1 with RPMI-1640 medium supplemented with 2mM glutamine, 100 U/mL penicillin and was then distributed in a 24 well tissue culture plate (1mL/well). Either 10^5 CFU/ml live bacteria or the sonicate of a similar number of mycobacteria was added to each well. LPS (endotoxin from *E. coli*; 026B6, Sigma, St. Louis, USA) (1µg/mL) was used as a positive control and medium only as a negative control. Plates were incubated at $37^{\circ}C$ in 5% CO_2 for 24 hr. The contents of the wells were then collected and centrifuged at 900g for 10 min. Supernatants were collected and frozen at -$70^{\circ}C$ until further use.

Cytokine estimation by ELISA (enzyme-linked immunosorbent assay)

Supernatants from whole blood cells (24 hr) were collected, centrifuged and frozen at -$70^{\circ}C$ until used. Quantification of TNF-α, IL-12, IFN-γ and IL-10 in the supernatants was carried out using commercial ELISA kits (BD OptEIA, San Diego, USA).

Statistical analysis

Correlation, Mann-Whitney U tests and Kruskal-Wallis rank tests were performed using Sigmastat software version 3.5 (Systat Software, Point Richmond, USA). A pre-defined distribution of the response tested was not assumed and so non-parametric statistical analysis was used for all parameters.
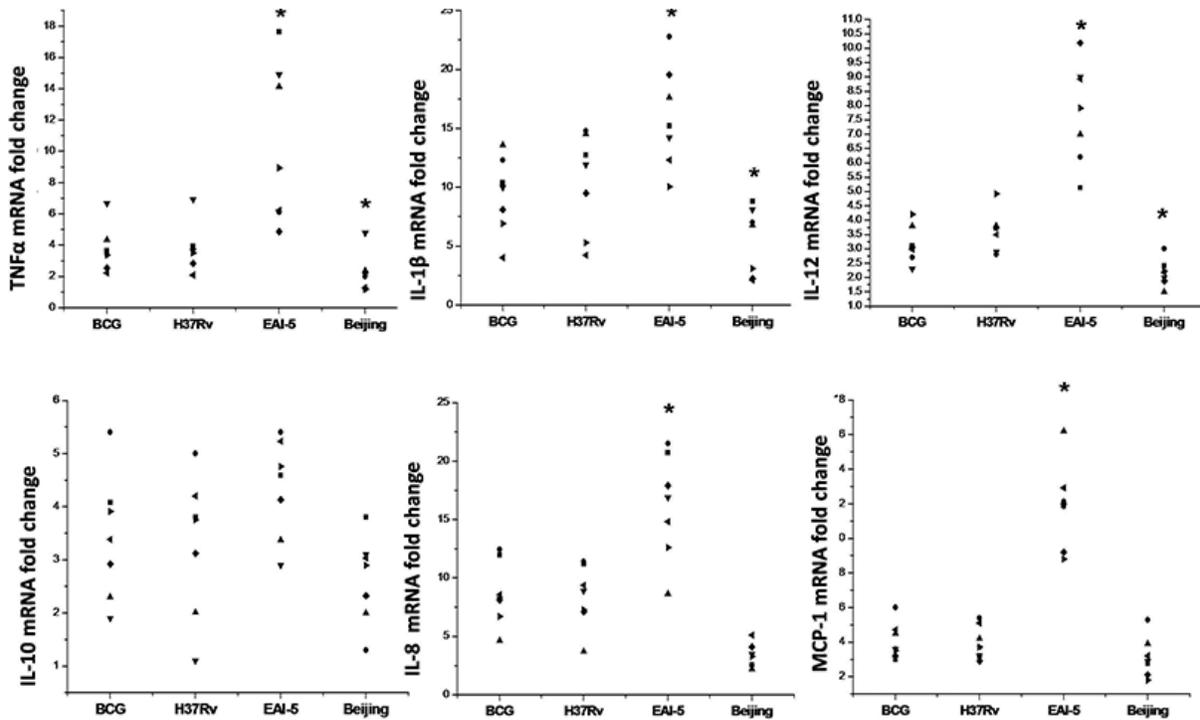
Results

Induction of cytokines by strains of EAI and Beijing genotypes in MDM and MDDC from different healthy individuals

The percentages of infected cells were measured 4 hours after infection in MDM and MDDC isolated from PBMC of a particular individual by examination under acid-fast staining. The percentage of infected macrophages and dendritic cells was 60 ± 4 and 63 ± 3 respectively at a MOI of 5. Figure 1 and Figure 2 show mRNA expression levels of different cytokines and chemokines (TNF-α, IL-1β, IL-12, IL-10, IL-8 and MCP-1) from different donors, induced after 24 hr of infection in MDM and MDDC respectively. Both the BCG and H37Rv induced similar patterns of cytokine induction. Therefore, in the next set of experiments with MDDC, only BCG was taken as standard.
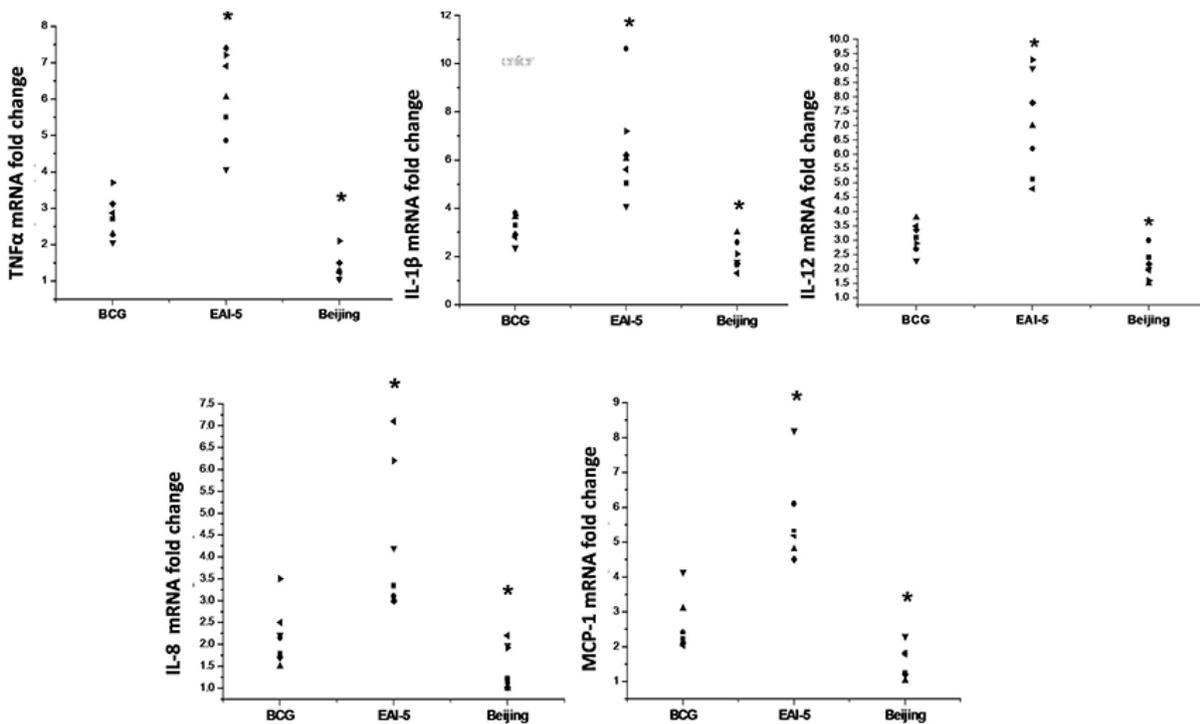
In isolated MDM and MDDC the ancient strain EAI-5 induced significantly higher levels of proinflammatory cytokines and chemokines than the other strains. The modern, Beijing, strain induced lower expression even when compared to the BCG vaccine strain or the H37Rv laboratory strain. Most strikingly, the response patterns were similar to those observed in infection studies with THP-1 cells [8]. It was observed that mRNA for proinflammatory cytokines, TNF-α, IL-1β and IL-12, were expressed at high levels preferentially by MTB-infected MDM, whereas MDDC produced comparatively lower levels of these cytokines except for IL-12. On the other hand, IL-10 gene expression was not detected in infected MDDC. There was no significant difference between the laboratory strain H37Rv and the vaccine strain BCG with respect to induction of tested cytokines.

Figure 1. Induction of cytokines and chemokines in infected monocyte-derived macrophages (MDM) by different strains of MTB.
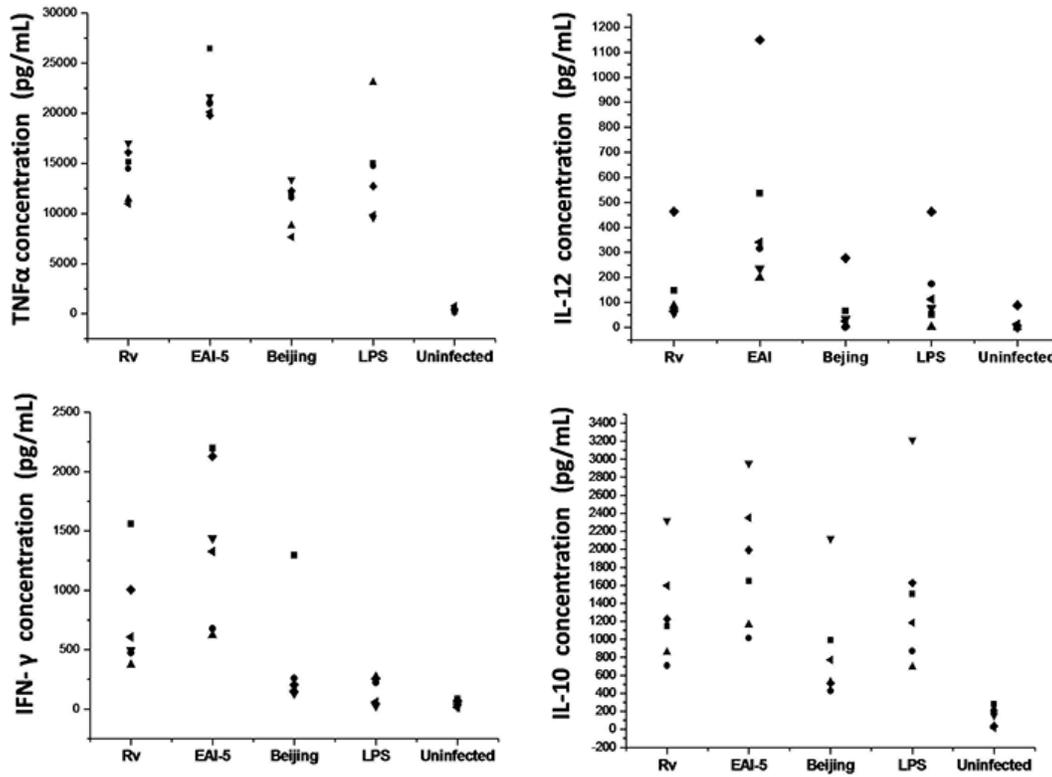

The scatter plot shows real time PCR estimated relative mRNA expression of TNF-α, IL-1β, IL-12, MCP-1 and IL-8 corrected for total mRNA using the housekeeping β-actin gene. MDM from seven independent donors was averaged. '*' indicates a significant difference compared to H37Rv where p is ≤ 0.05 using Kruskal-Wallis.

Figure 2. Induction of cytokines and chemokines in monocyte-derived dendritic cells (MDDC) by different strains of MTB after 24 hours of infection.
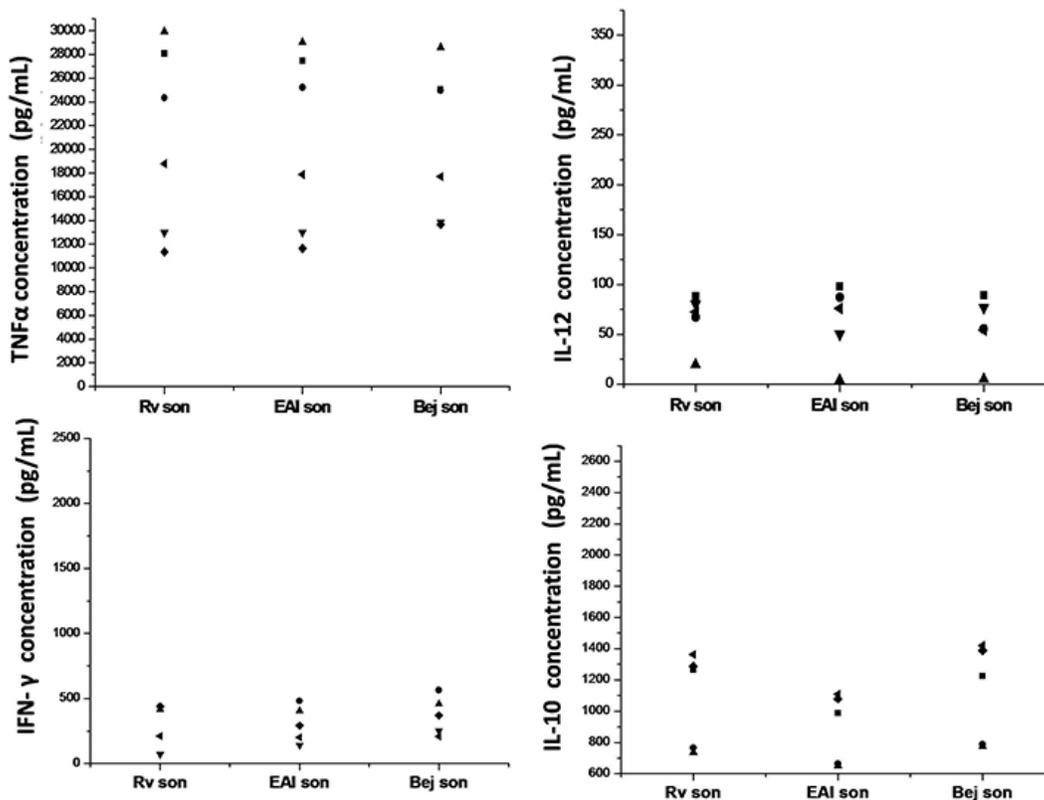

The scatter plot shows real time PCR estimated relative mRNA expression of TNF-α, IL-1β, IL-12, MCP-1 and IL-8 corrected for total mRNA using the housekeeping β-actin gene. MDDC from seven independent donors was averaged. '*' indicates a significant difference compared to H37Rv where p is ≤ 0.05 using Kruskal-Wallis.

Figure 3. Induction of cytokines in whole blood assay by live strains of MTB.

The scatter plot shows the *ex-vivo* induction of cytokines: TNF-α, IL-12, IFN-γ and IL-10 measured by ELISA in the supernatants of diluted whole blood from six healthy subjects after stimulation with 10^5 live MTB single cells. Rv is H37Rv, LPS is lipopolysaccharide.

Figure 4. Induction of cytokines in whole blood assay by whole cell sonicates of MTB strains.

The scatter plot shows the *ex-vivo* induction of cytokines: TNF-α, IL-12, IFN-γ and IL-10 measured by ELISA in the supernatants of diluted whole blood from six healthy subjects after stimulation with sonicates of 10^5 MTB cells after 24 hours are shown.

For all the seven donors a significant correlation was observed between the production of TNF-α and IL-1β (r^2 = 0.89, P < 0.0001), IL-12 and IL-1β (r^2 = 0.85, P < 0.0001) and IL-12 and TNF-α (r^2 = 0.85, P < 0.0001) (r^2 = Spearman rank correlation coefficient), shown in Supplementary Figure 1.

Cytokine response in Whole blood assay for live MTB strains and their sonicates

The concentrations of cytokines measured by ELISA in whole blood after 24 hours post infection, with different MTB strains are depicted in Figure 3. There was a clear difference in the levels of proinflammatory cytokines produced by a single host in response to infection with different strains and the relative hierarchy of low and high responses was maintained as observed in MDM and MDDC infection. The concentration of TNF-α induced by different MTB strains was in the range of 7-26 ng/mL in whole blood of different individuals, in comparison, uninfected cells showed a level of 0.185-0.78 ng/mL of TNF-α. Interleukin concentrations also varied with strain type with EAI-5 inducing significantly higher levels of IFN-γ and TNF-α compared to H37Rv, and Beijing inducing lower levels of the same. The differential response pattern was not observed when whole blood cells were co-cultured with sonicates of different isolates of MTB (Figure 4).

Discussion

The influence of bacterial and host genotype on the disease outcome is well accepted. Different strains of MTB have shown more antigenic variation than previously thought however, the major hindrance to link experimental model systems with the clinically relevant strain has been the absence of proper strain characterization. In the present study, well characterized clinical strains were used to show that the induction of the host response is associated with the strain lineage.

Macrophages and dendritic cells are the reservoir and the main effectors for the killing of MTB. These host cells first recognise the pathogen and activate an inflammatory response; which strengthens the cellular immune response against mycobacterial infection. The proinflammatory cytokines like TNF-α and IL-12 play an essential role in host defence and they dictate the containment of the intracellular pathogen and granuloma formation in TB [15,16]. In addition, the induction of proinflammatory cytokines by different MTB strains may determine its virulence [17]. TNF-α, produced by monocytes and macrophages in early infection, plays a key role in protective immunity during TB infection. Further, IL-12 production is essential to induce a protective Th1 response [18]. Furthermore, chemokines like IL-8 and MCP-1 are important in TB and recruit inflammatory cells at the site of infection [19]. As the interplay of the proinflammatory cytokines and chemokines is a hallmark of TB infection, the cytokines induced by the host after infection with different genotypes can be considered as a good marker for correlating MTB genotypes with the induced innate immune response. In view of this, in the present study, the expression of different cytokines and chemokines were assessed in MDM, MDDC and whole blood cells infected with different strains of MTB.

Several studies have reported heterogeneity in the cytokine response induced by various genotypes of MTB [5,20]. Strains of modern lineages (lineage 2, 3 and 4) were shown to induce lower levels of proinflamatory cytokines when compared with ancient lineages (lineage 1) [10]. However, there are contradictory reports available in the literature regarding the induction of proinflammatory cytokines by Beijing genotype, which belongs to a modern lineage. Lasunskaia *et al*. have demonstrated that Beijing strains from Brazil induced levels of TNF-α and IL-10 similar to H37Rv whilst MDR Beijing strains isolated in Russia, induced low TNF-α and high IL-10 in the THP-1 macrophage cell line [21]. Further, it was reported that Beijing strains induced low levels of TNF-α, IL-6, IL-10 and GRO-α production as compared to H37Rv and other genotypes of MTB in human macrophages [10]. Ancient lineage strains are known to be less virulent and genetically heterogeneous in nature [10]. It was shown that ancient lineage strains induce higher proinflammatory cytokines in human derived macrophages and higher interferon-γ in peripheral blood T cells [22]. In agreement with these observations, our earlier study also reported profound and suppressed induction of proinflammatory cytokines (TNF-α, IL1b and IL-12) by ancient (EAI) and modern (Beijing) lineages respectively, in THP-1 cells [8]. This pattern remained unchanged in the present study using MDM, MDDC and whole blood cells from different hosts. In addition, there were clear differences in the levels of proinflammatory cytokines produced by a single donor in response to different strains and a significant correlation was observed among the proinflammatory cytokines produced by a single strain across the multiple donors.

IL-10, an anti-inflammatory cytokine, suppresses TNF-α and IL-12 expression [23,24] and so explains the

inverse relationship between them. However, this relation was not always apparent in experimental conditions. Some investigators reported up regulation of this cytokine [22,25] and some showed the reverse [26,27]. In our earlier study with THP-1, we found a strain specific induction of IL-10. However, we did not observe any inverse relationship between pro and anti-inflammatory cytokines. In the current study, no differential level of IL-10 mRNA induction by different strains was observed in infected MDM. Moreover, the mRNA levels of IL-10 in infected MDDC by *M. tuberculosis* strains were undetectable. This was presumably due to the number of cells present in the assay.

It was observed in the earlier studies, that response to mycobacteria varied with the change in their microenvironment [28]. Hence, whole blood assays were carried out to evaluate whether the innate response induced by MTB strains in isolated MDM and MDDC, would change in presence of lymphocytes. In one of the earlier studies the differential host response seen in isolated MDM was not observed in peripheral blood mononuclear cells for different strains [10]. In contrast to this, Laarhoven *et al*. reported a differential cytokine response to the strains in PBMC [29]. We compared the proinflammatory cytokine responses across the isolates and there was overall a statistically significant effect of certain lineages on the cytokine levels observed in whole blood assay. Induction of a protective Th1 cytokine, IFN-γ was observed in response to EAI in whole blood assay. However, this lineage specific pattern of cytokine induction was absent when MTB cell sonicates were used instead of live bacteria. It may be speculated that lineage specific variations in the cell wall lipids of MTB are responsible for differential lineage specific immune response, although direct experimental evidence is lacking [30]. A strain specific response was not observed with sonicates of different MTB isolates, suggesting that live cells differentially modulated immune responses compared to the mixture of their components. It is possible that while growing inside the host, MTB manipulates the host environment according to its genotype.

Conclusions

In summary, we have demonstrated that different MTB strains led to lineage specific patterns of proinflammatory cytokine induction, with ancient and modern strains inducing high and low levels of these cytokines respectively. As the study involved human volunteers, due to ethical restrictions of drawing blood from single individual, limited number of MDM and MDDC were available and this restricted our study to one strain per lineage. Thus strains used in this study do not represent the global diversity of modern and ancient clinical isolates of MTB. However, the study strongly suggests that different lineages of MTB have different levels of pathogenesis. Moreover, it is evident that only live MTB strain can induce lineage specific patterns of innate responses which are independent of the type of host they infect.

Infection in cell lines and isolated primary cells has their own disadvantages as it doesn't truly represent the original infection condition. Several factors like host cell's genotype, concentration of vitamin-D, mutation in cytokine genes are also known to influence disease outcome *in vivo*. Therefore, further study of host–pathogen interaction with the selected strains of MTB should be performed in animal infection models.

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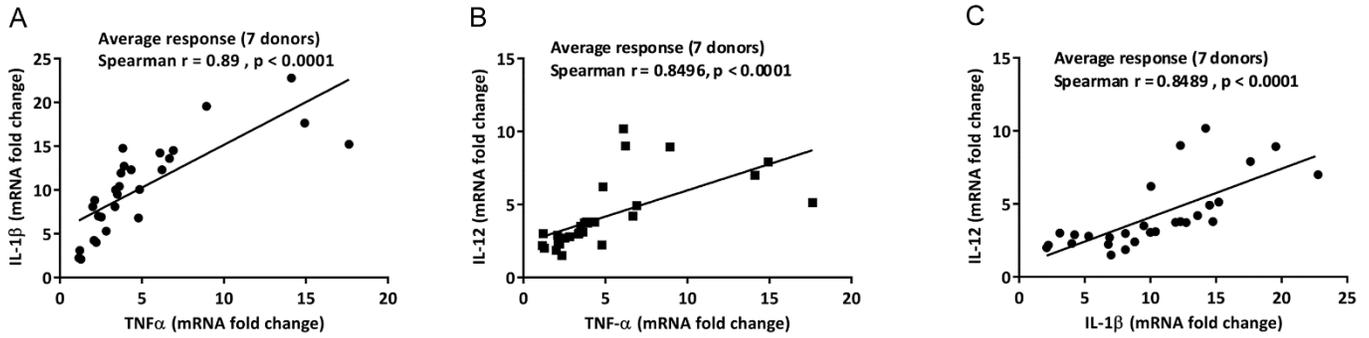Phone: +91 022 24134960 ext 211, +919821083165
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Conflict of interests: No conflict of interests is declared.

Annex – Supplementary Items

Supplementary Figure 1. Linear regression analysis showing correlation between pairs of cytokines produced in monocyte derived macrophages (MDM).

A. TNF-α and IL-1β, B. TNF-α and IL-12, C. IL-1β and IL-12. Correlation coefficients and p-values were calculated using Spearman's Rank Correlation Test.