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Reduced TNF-α and IFN-γ responses to Central Asian strain 1 and Beijing isolates of *Mycobacterium tuberculosis* in comparison with H37Rv strain

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Summary Pakistan ranks eighth in terms of tuberculosis burden worldwide, with an incidence of 181/100 000. The predominant genotypes of *Mycobacterium tuberculosis* are reported to be the Central Asian strain 1 (CAS1) and Beijing families. *Mycobacterium tuberculosis* down-regulates host pro-inflammatory cytokines, which are essential for protection against infection. There is currently little information regarding the interaction of the CAS1 genotype with host cells. We studied the growth rates of CAS1 and Beijing clinical isolates, and their ability to induce cytokines compared with the laboratory reference strain H37Rv. Host responses were studied using a THP-1 monocytic cell line model and an ex vivo whole blood assay. Growth rates of CAS1 and Beijing isolates were significantly lower ($P = 0.011$) compared with H37Rv. All clinical isolates induced significantly lower levels of TNF-α secretion ($P = 0.003$) than H37Rv in THP-1 cells and in the whole blood assay of healthy donors ($n = 8$). They also induced lower IFN-γ secretion in the whole blood assay ($P < 0.001$). A positive correlation was observed between the growth indices (GI) of H37Rv, Beijing and CAS1 strains and the TNF-α responses they induced [Pearson's correlation coefficient (R^2): 0.936, 0.775 and 0.55, respectively], and also between GI and IFN-γ production (R^2: 0.422, 0.946, 0.674). These findings suggest that reduced growth rate, together with down-modulation of pro-inflammatory cytokines, is a contributory mechanism for the predominance of the CAS genotype.

1. Introduction

Tuberculosis (TB) is a major cause of morbidity and mortality worldwide, with 8—10 million new cases and 2—3 million deaths each year.[1,2] Pakistan ranks eighth in terms of global TB burden, accounting for 44% of all TB cases in the Eastern Mediterranean Region.[3] The TB burden has become more difficult to manage because of an increase

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in multidrug-resistant (MDR) strains showing poor response to chemotherapy.[4,5] In addition, the protective efficacy of the widely used TB vaccine *Mycobacterium bovis* bacillus Calmette-Guerin (BCG) has been found to be highly variable.[6]

The control of mycobacterial infection in the host is dependent on cell-mediated immunity.[7,8] Both IFN-γ and TNF-α have been shown to play a crucial role in macrophage activation, controlling mycobacterial replication and granuloma formation in humans and mice.[9] TNF-α, in synergy with IFN-γ, influences the expression of adhesion molecules as well as chemokines and their receptors, and affects the recruitment of neutrophils, lymphocytes and monocytes/macrophages.[10,11] Virulent mycobacteria such as *M. tuberculosis* and *M. leprae* down-regulate the protective host TNF-α and IFN-γ responses, unlike the non-pathogenic species *M. bovis* and *M. smegmatis*.[12,13] Reduced pro-inflammatory responses have been associated with increased persistence/survival of *M. tuberculosis* strains[14] and have been correlated with greater virulence in animal models.[15,16] Increased survival and reduced induction of pro-inflammatory cytokines have also been linked to lower growth rates of *M. tuberculosis* strains.[14,17–19] Clinical isolate CDC1551, which induces high cytokine levels in mice, is recognized to persist for shorter periods than the Beijing strains, which are more virulent and induce lower cytokine levels.[20]

The human acute monocytic leukaemia cell line (THP-1) is a useful model for studying intracellular growth and persistence of *M. tuberculosis*.[21,22] THP-1 cells possessing both Fc and C3b receptors are valuable for in vitro experiments due to their close resemblance to human-derived macrophages, but are devoid of surface and cytoplasmic immunoglobulins.[23] The ex vivo whole blood assay model, which allows full interplay of cellular and humoral factors, has also been shown to be useful for investigating cytokine responses in TB infection.[24,25] In addition it is relatively simple and economical to use.[26,27]

We have previously shown that the CAS1 and Beijing strains, represented by a specific spacer oligonucelotide type (spoligotype) pattern determined by PCR for the direct repeat region of *M. tuberculosis*, are the predominant clade responsible for pulmonary TB in Pakistan.[28] To date, the prevalence of CAS1 strains has been reported in several countries of the Middle East and South Asia[29,30] and Beijing strains have been identified in a number of populations across the world.[31] We investigated the activation of host immune responses by CAS1 and Beijing strains using the THP-1 human monocyte model and an ex vivo whole blood assay (WBA) model. The growth rates of these strains were also determined in vitro.

2. Materials and methods

2.1. Strain selection and culture

A total of 20 clinical *M. tuberculosis* strains that had been previously spoligotyped[28] were randomly selected for this study. These included 10 CAS1 strains and 10 strains from the Beijing family. All strains selected were from patients with pulmonary TB. The strain characteristics are described in Table 1. Four of the CAS1 strains and six of the Beijing strains were MDR, as defined by resistance to at least rifampicin and isoniazid.

Mycobacterium tuberculosis H37Rv was used as a control. All strains were grown to logarithmic phase in 7H9 Middlebrook medium supplemented with 0.02% glycerol, 10% ADC Middlebrook enrichment and 0.5% Tween-80 (all from Difco Laboratories, Detroit, MI, USA). Aliquots of mycobacteria were frozen in growth medium containing 15% glycerol and stored at −70 °C. For the infection assay, aliquots of BCG, H37Rv, CAS1 and Beijing strains were freshly thawed, washed three times in PBS and diluted as required. To avoid mycobacterial clumping, the cell suspension was sonicated briefly then allowed to stand for 5 min to allow large clumps to settle, leaving behind a suspension of single cells.[13]

2.2. In vitro infection of THP-1 monocytes

Cells from the human myelomonocytic cell line THP-1 (ATCC TIB-202) were cultured, activated with phorbol myristate acetate (20 ng/ml; Sigma) and infected at ratios of 5 (2.5×10^6) and 10 (5×10^6) mycobacteria to one macrophage, as described previously.[17] Ten strains each of the CAS1 and Beijing genotypes were used (Table 1). Supernatants were collected at time intervals of 18 and 48 h post-infection and were than aliquoted and stored at −70 °C until used.[32] The supernatants were spun at 800 *g* for 2 min to pellet any cellular debris and measured for TNF-α activity. Endotoxin contamination was excluded using the E-Toxate kit (*Limulus* amoebocyte lysate test for detection and semi-quantification of endotoxin; Sigma, St Louis, MO, USA).

TNF-α was measured at 18 h as described previously.[33] No significant difference in TNF-α secretion induced by H37Rv was seen between multiplicity of infection (MOI) 5 and 10 bacilli/cell (MOI-5: 34 ± 4 pg/ml; MOI-10: 39 ± 7 pg/ml). Similarly, CAS1 (MOI-5: 25 ± 4 pg/ml; MOI-10: 30 ± 9 pg/ml) and Beijing strains (MOI-5: 28 ± 2 pg/ml; MOI-10: 41 ± 12 pg/ml) also showed no significant difference; therefore we chose MOI-5 for our experiments

2.3. Whole blood assay

BCG vaccinated, healthy volunteers ($n = 8$), five female and three male staff of Aga Khan University, were included in the study. The age range of individuals was 25–45 years, with a mean of 34.6 years. No subjects had any coexisting medical conditions and none were pregnant or breastfeeding. All had been BCG vaccinated at birth and had a BCG scar and a negative chest X-ray.

Heparinzed venous blood was taken from the eight endemic control volunteers. Each clinical strain was tested in two endemic controls. Cultures of venous whole blood from each endemic control were infected with two strains of the CAS1 genotype and two of the Beijing genotype from the 20 clinical strains used in the THP-1 assay. Two Beijing and two CAS1 isolates did not grow well enough in vitro to be used in the whole blood assay; therefore eight CAS1 and eight Beijing strains were used. The WBA method used was previously described by Hussain et al.[34] All experiments were performed in triplicate. The whole blood cells were infected with *M. tuberculosis* at 5×10^5 CFU/ml. The plates