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MODEL SYSTEMS

Ultra low dose aerosol challenge with *Mycobacterium tuberculosis* leads to divergent outcomes in rhesus and cynomolgus macaques

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SUMMARY

Well characterised animal models that can accurately predict efficacy are critical to the development of an improved TB vaccine. The use of high dose challenge for measurement of efficacy in Non-human primate models brings the risk that vaccines with the potential to be efficacious against natural challenge could appear ineffective and thus disregarded. Therefore, there is a need to develop a challenge regimen that is more relevant to natural human infection. This study has established that ultra-low dose infection of macaques via the aerosol route can be reproducibly achieved and provides the first description of the development of TB disease in both rhesus and cynomolgus macaques following exposure to estimated retained doses in the lung of less than 10 CFU of *Mycobacterium tuberculosis*. CT scanning *in vivo* and histopathology revealed differences in the progression and burden of disease between the two species. Rhesus macaques exhibited a more progressive disease and cynomolgus macaques showed a reduced disease burden. The ability to deliver reproducible ultra-low dose aerosols to macaques will enable the development of refined models of *M. tuberculosis* infection for evaluation of the efficacy of novel tuberculosis vaccines that offers increased clinical relevance and improved animal welfare.

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1. Introduction

Tuberculosis (TB) is a major global health problem, with 9 million new cases and nearly 1.5 million deaths annually [1]. With a third of the world's population estimated to be infected, and the effects of TB infection compounded by the emergence of multi-drug resistant strains and HIV co-infection, there is an urgent need for improved interventions, including a vaccine. The only currently available vaccine is *Mycobacterium bovis* Bacille Calmette Guerin (BCG) which protects children from developing severe TB disease [2], but it is unsuitable for use in people whose immune system is compromised, and has mostly failed to protect against pulmonary TB in adults [3].

The lack of a defined immunological correlate of protection for TB means that, in order to assess efficacy, candidate TB vaccines must enter large clinical trials involving thousands of at-risk individuals in endemic countries [4]. Therefore, preclinical animal models that can accurately predict the effectiveness of vaccines in humans through challenge studies are critical to achieving the goal of an improved TB vaccine. Because of their close similarity to humans, non-human primates (NHP) are excellent potential models of tuberculosis [5–7]. Reviews of published studies using the TB NHP model [5–7] reveal that the selection of model parameters, including the macaque species and route and size of the challenge dose, can affect the outcome of experimental TB exposure in NHPs. Both rhesus [8–15] and cynomolgus macaque species [11,16–19] are used to evaluate the efficacy of new TB vaccine candidates. Both species recapitulate aspects of human TB but which provides the most appropriate model for vaccine evaluation is a subject of debate.

The quantity of *M ycobacterium tuberculosis* administered to evaluate vaccine efficacy is critical to the outcome of an efficacy study. The dose needs to be sufficient to induce a consistent and

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measureable disease burden in all exposed animals but not so large as to overwhelm any protection provided by the host response. The readouts used routinely to measure the efficacy of new TB vaccines in NHP models such as changes in clinical parameters, bacterial burden and qualitative gross pathology and histopathological scoring systems have limited sensitivity [20]. This has necessitated the use of high doses of TB for challenge, which far exceed the estimated doses encountered during natural exposure, and bring the risk that potentially useful vaccines could be disregarded. Consequently, there is a growing view that a lower challenge dose (less than 10 infectious bacilli) that more closely resembles natural infection is required to refine the vaccine assessment process. The physical properties of *M. tuberculosis* (e.g. clumping) create difficulties in generating inocula containing very few bacilli from high titre stocks but the process of aerosolisation disrupts bacterial aggregates and generates aerosol particles which mostly contain single bacteria. Measurement of the outcome of very low dose challenge requires improved tools to provide more sensitive readouts of disease burden such as those offered by advanced *in vivo* imaging [21–23].

With the ultimate aim to establish an NHP model for vaccine assessment based upon ultra-low dose infection the present study was set up to provide proof of concept for the reproducible delivery of an ultra-low dose (ULD) of *M. tuberculosis* to macaques by the aerosol route; to characterise the outcome of ULD aerosol exposure; and compare the resulting disease in the two macaque species. Furthermore the potential benefit of computed tomography (CT) to provide sensitive measures of disease burden that could be used as readouts for evaluation of vaccine efficacy following low dose challenge was investigated.

2. Materials and methods

2.1. Experimental animals

Eight male rhesus macaques and eight male cynomolgus macaques, aged 3–4 years, were obtained from established UK breeding colonies. Genetic analysis of these colonies has previously confirmed the rhesus macaques to be of the Indian genotype and cynomolgus macaques of Indonesian genotype. Absence of previous exposure to mycobacterial antigens was confirmed by a tuberculin skin test and screening using an *ex-vivo* IFN-γ ELISPOT (MabTech, Nacka. Sweden) to measure responses to PPD (SSI, Copenhagen, Denmark), and pooled 15-mer peptides of ESAT6 and CFP10 (Peptide Protein Research LTD, Fareham, U.K.).

Animals were housed in compatible social groups, in accordance with the Home Office (UK) Code of Practice for the Housing and Care of Animals Used in Scientific Procedures (1989), (now updated to Code of Practice for the housing and Care of Animals Bred, Supplied or Used for Scientific Purposes, December 2014, and the National Committee for Refinement, Reduction and Replacement (NC3Rs), Guidelines on Primate Accommodation, Care and Use, August 2006 (NC3Rs, 2006)). Animals were sedated by intramuscular (IM) injection with ketamine hydrochloride (Ketaset, 100 mg/ml, Fort Dodge Animal Health Ltd, Southampton, UK; 10 mg/kg) for procedures requiring removal from their housing. None of the animals had been used previously for experimental procedures. All animal procedures were approved by the Public Health England Ethical Review Committee, Porton Down, UK, and authorised under an appropriate UK Home Office project license.

2.2. Clinical procedures

Animals were monitored daily for behavioural and clinical changes. Behaviour was evaluated for contra-indicators including depression, withdrawal from the group, aggression, changes in feeding patterns, breathing pattern, respiration rate and cough. Prior to blood sample collection, aerosol challenge and euthanasia, animals were weighed, examined for gross abnormalities and body temperature measured. Red blood cell (RBC) haemoglobin levels were measured using a HaemaCue haemoglobinometer (Haemacue Ltd, Dronfield, UK) to identify the presence of anaemia, and erythrocyte sedimentation rates (ESR) were measured using the Sediplast system (Guest Medical, Edenbridge, UK) to detect and monitor inflammation induced by infection with *M. tuberculosis*.

2.3. M. tuberculosis challenge strain

The Erdman K01 stock (HPA-Sept 2011) used for challenge was prepared from stocks of the *M. tuberculosis* Erdman strain K 01 (BEI Resources). A stock suspension was initially prepared from a 5 ml bacterial starter culture originally generated from colonies grown on Middlebrook 7H11 supplemented with oleic acid, albumin, dextrose and catalase (OADC) selective agar (BioMerieux, UK). A liquid batch culture was then grown to logarithmic growth phase in 7H9 medium (Sigma–Aldrich, UK) supplemented with 0.05% (v/v) Tween 80 (Sigma–Aldrich, UK). Aliquots were stored at −80 °C. Titre of the stock suspension was determined from thawed aliquots by enumeration of colony forming units cultured onto Middlebrook 7H11 OADC selective agar.

2.4. Aerosol exposure

2.4.1. Apparatus and procedure

The methodology and apparatus used to deliver *M. tuberculosis* via the aerosol route was as previously described [14,20,24]. In brief, the aerosols were generated from a suspension of *M. tuberculosis* at a pre-determined concentration (see below) using a 3-jet Collison nebuliser (BGI) and delivered, using a modified Henderson apparatus [25] controlled by an AeroMP (Biaera) control unit [26], to the nares of each sedated animal via a modified veterinary anaesthesia mask. A 'head-out', plethysmography chamber (Buxco, Wilmington, North Carolina, USA) enabled the aerosol to be delivered simultaneously with the measurement of respiration rate.

2.4.2. Quantification of ultra-low aerosol dose

The number of bacilli deposited and retained in the lungs of macaques cannot be measured directly and the quantification of the dose must be calculated from the concentration of viable organisms in the aerosol (C_{aero}) and the volume of aerosol inhaled by the animal. This 'presented dose' (PD) is the number of organisms which the animals inhale. C_{aero} is either measured directly using air sampling within the system or may be calculated using the concentration of organisms in the nebuliser (C_{neb}) and a 'spray factor' which is a constant derived from data generated for the specific organism with identical aerosol exposure parameters. The calculations to derive the PD and the retained dose (the number of organisms assumed to be retained in the lung) have been described previously for high/medium aerosol doses [20,24]. Direct measurement of C_{aero} would not be possible for an ultra-low dose challenge and therefore the dose calculations were based upon C_{neb} and the spray factor. Aerosol challenge data from previous experiments were used to develop and verify the calculation which would predict the retained dose, and a nebuliser concentration was selected to result in a retained dose of approximately 5 viable bacilli.

2.4.3. Macaque aerosol exposure

Four rhesus and four cynomolgus macaques (group A) were exposed to aerosols of *M. tuberculosis* five weeks before the second

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