Tuberculosis 105 (2017) 86–95

Contents lists available at ScienceDirect

Tuberculosis

journal homepage: http://intl.elsevierhealth.com/journals/tube

Quantitative and qualitative iNKT repertoire associations with disease susceptibility and outcome in macaque tuberculosis infection

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ARTICLE INFO

Article history:
Received 29 September 2016
Received in revised form
11 April 2017
Accepted 19 April 2017

Keywords:
Tuberculosis
Correlates of protection
Macaque
iNKT
CD1d

ABSTRACT

Correlates of immune protection that reliably predict vaccine efficacy against *Mycobacterium tuberculosis* (Mtb) infection are urgently needed. Invariant NKT cells (iNKTs) are CD1d-dependent innate T cells that augment host antimicrobial immunity through production of cytokines, including interferon (IFN)-γ and tumour necrosis factor (TNF)-α. We determined peripheral blood iNKT numbers, their proliferative responses and iNKT subset proportions after *in vitro* antigen expansion by α-galactosylceramide (αGC) in a large cohort of mycobacteria-naïve non-human primates, and macaques from Bacillus Calmette-Guerin (BCG) vaccine and Mtb challenge studies. Animals studied included four genetically distinct groups of macaques within cynomolgus and rhesus species that differ in their susceptibility to Mtb infection. We demonstrate significant differences in *ex vivo* iNKT frequency between groups, which trends towards an association with susceptibility to Mtb, but no significant difference in overall iNKT proliferative responses. Susceptible animals exhibited a skewed $CD4^+/CD8^+$ iNKT subset ratio in comparison to more Mtb-resistant groups. Correlation of iNKT subsets post BCG vaccination with clinical disease manifestations following Mtb challenge in the Chinese cynomolgus and Indian rhesus macaques identified a consistent trend linking increased $CD8^+$ iNKTs with favourable disease outcome. Finally, a similar iNKT profile was conferred by BCG vaccination in rhesus macaques. Our study provides the first detailed characterisation of iNKT cells in macaque tuberculosis infection, suggesting that iNKT repertoire differences may impact on disease outcome, which warrants further investigation.

1. Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (Mtb), is an ongoing pandemic due to imperfect diagnostic tools, rising drug resistance and lack of an effective vaccine [1,2]. Currently, Mtb causes the highest number of deaths related to infection globally. Tuberculosis is characterised by cavitating granulomas in the lung causing transmission via respiratory secretions [3]. Failure to resolve infection is due to a complex and prolonged interaction of the pathogen with the host, involving a multifaceted immune response which to date is only incompletely understood [4]. Historically, strong pro-inflammatory immune responses generating IFN-γ and TNF-α were assumed to provide protective immunity against Mtb infection, based on studies demonstrating that complete absence of these cytokines causes exacerbated disease [5,6]. However, there is increasing evidence that these cytokines play a more complex role in TB immunity, with an excessive Th1 response

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http://dx.doi.org/10.1016/j.tube.2017.04.011
1472-9792/

implicated in worsening pathology [7]. Importantly, there are no definitive correlates of protection against Mtb infection, due to the difficulties associated with longitudinal studies to dissect anti-mycobacterial immune responses and correlate them with disease outcome in humans [7,8]. Therefore, studies employing well-characterised animal models that accurately reflect human disease processes are required.

The widely used mouse model of TB infection has several limitations, as mice do not develop TB latency or caseating granuloma formation, which are hallmarks of human disease [9]. In contrast, non-human primates (NHP) are remarkably similar to humans in many aspects including their anatomy, immunology and clinical manifestations of TB [10,11]. Both rhesus and cynomolgus macaque species have been used to study Mtb infection and TB vaccine efficacy [10–14]. Importantly, animals that fall into rhesus and cynomolgus macaque species differ with regard to their genetic background and susceptibility to Mtb, a divergence also observed after challenge with other microorganisms, including human immunodeficiency virus and *Shigella* species [15–19]. Such differences may range from a strong ability to control infection in cynomolgus of Chinese or Indonesian genotype to reduced resistance in Mauritian cynomolgus [15,20,21]. Variations in susceptibility are likely due to immunological differences. Therefore, investigation into the adaptive and innate cellular immune processes that underpin these differences may provide novel disease correlates of protection that may ultimately help to inform the design of new vaccine candidates.

T cells are essential for a protective host immune response to Mtb [22–24], as they induce the effector antimicrobial functions of infected phagocytes. Identifying T cell-based correlates is a critical area of ongoing research within the TB field [7,25,26]. Invariant NKT cells (iNKTs) are innate T cells that recognise CD1d-presented lipid antigens via an invariant T cell receptor comprised of Vα24 and Vβ11 in humans, and the Vα24 chain is highly conserved in macaque [27,28]. iNKTs are critical for an effective immune response in a variety of diseases and comprise between <0.01% and 1% of T cells in the peripheral circulation in humans [29]. Despite their low numbers, iNKTs rapidly home to sites of infection and proliferate upon antigen exposure to control disease [30]. Furthermore, they exhibit anti-TB protective functions in mouse studies both *in vitro* [31] and *in vivo* [32]. Moreover, iNKT-mediated protection against Mtb infection is demonstrated by their early release of granulysin [33,34], IFN-γ [35] and GM-CSF [31]. Indeed, iNKTs are targets for vaccine adjuvants and incorporation of α-galactosylceramide (αGC), a potent iNKT agonist, with Bacillus Calmette-Guerin (BCG) markedly augmented the priming of $CD8^+$ T cells and increased vaccine efficacy in mice [36]. Furthermore, peripheral blood iNKT numbers are reduced in human TB patients with iNKTs exhibiting an activated phenotype [37,38], indicating a role for these innate T cells in the host immune defence against TB.

Non-human primate models allow in depth exploration of immunity to Mtb while detailing disease progression and outcome [9,11,39]. However, studies investigating iNKTs in these well-characterised NHP models are lacking. This study aimed to determine whether iNKT-based correlates of protection against TB can be identified in this model. We studied iNKT in four distinct groups of macaques that differ in genetic background and susceptibility to Mtb infection: Chinese cynomolgus (CC), Indonesian cynomolgus (IC), Mauritian cynomolgus (MC) and Indian rhesus (RM) macaques in BCG vaccine and Mtb challenge studies.

2. Materials and methods

2.1. Experimental animals

The animals used in this study were rhesus macaques of Indian origin (RM), Chinese cynomolgus macaques (CC), Mauritian cynomolgus macaques (MC) and Indonesian cynomolgus macaques (IC) obtained from established breeding colonies in the United Kingdom (MC, IC, RM) and China (CC). All animals were between 4 and 10 years old at the time of sample collection and were naïve to prior exposure to mycobacterial antigens (*M. tuberculosis* infection or environmental mycobacteria), demonstrated by a negative tuberculin test while in their original breeding colony and by the IFN-γ-based Primagam test kit (Biocor; CSL, Kansas, US) or screening using an ex-vivo IFN-γ ELISPOT (MabTech, Nacka, Sweden) to measure responses to mycobacterial antigens: purified protein derivative (PPD) batch RT50 (Statens Serum Institut (SSI), Copenhagen, Denmark), and 15-mer peptide pools of ESAT-6 and CFP-10 (Peptide Protein Research Ltd., Fareham, U.K.) just prior to the start of the study.

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), and the National Committee for Refinement, Reduction and Replacement (NC3Rs) Guidelines on Primate Accommodation, Care and Use, August 2006. Animals were sedated by intramuscular (IM) injection of 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 and each socially compatible group was randomly assigned to a particular study treatment. All animal procedures and study design were approved by the Public Health England, Porton Down Ethical Review Committee, and authorised under an appropriate UK Home Office project license.

2.2. BCG vaccination

Macaques were immunised intra-dermally in the upper left arm with 100 μl BCG vaccine, Danish strain 1331 (SSI, Copenhagen, Denmark). Vaccinations were administered within 1 h of vaccine reconstitution. The viability of the BCG vaccine was confirmed to be within the expected range for the batch on each occasion.

2.3. M. tuberculosis challenge strain

The Erdman K01 stock (HPA-Sept 2011) used for challenge was prepared from stocks of the Mtb Erdman strain K01 (BEI Resources, Manassas, VA, USA). 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, Marcy-l'Étoile, UK). A liquid batch culture was then grown to logarithmic growth phase in 7H9 medium (Sigma-Aldrich, Gillingham, UK) supplemented with 0.05% (v/v) Tween 80 (Sigma-Aldrich, UK). Aliquots were stored at −80 °C. The 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

The methodology and apparatus used to deliver Mtb via the aerosol route was as previously described [11]. In brief, monodispersed bacteria in particles were generated using a 3-jet Collison nebuliser (BGI, Butler, NJ, USA) and, in conjunction with a modified Henderson apparatus [17], delivered to the nares of each sedated primate via a modified veterinary anesthesia mask. Challenge was performed on sedated animals placed within a 'head-out', plethysmography chamber (Buxco, Wilmington, NC, USA) to