Tuberculosis xxx (2016) 1–12

Contents lists available at ScienceDirect

Tuberculosis

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

HOST GENETICS OF SUSCEPTIBILITY

Dissecting host factors that regulate the early stages of tuberculosis infection

Neha Agrawal [a, 1], Chandrika Bhattacharyya [b, 2], Ankur Mukherjee [b, 2], Ubaid Ullah [a, 3], Bhaswati Pandit [b], Kanury V.S. Rao [a, 4], Partha P. Majumder [b, *]

[a] Immunology Group, International Centre for Genetic Engineering and Biotechnology, 110067 New Delhi, India
[b] National Institute of Biomedical Genomics, Kalyani, 741251 West Bengal, India

ARTICLE INFO

Article history:
Received 1 November 2015
Received in revised form
13 June 2016
Accepted 10 July 2016

Keywords:
Tuberculosis
Human PBMC
In vitro granuloma model
Cytokines
Genotype

SUMMARY

Incomplete understanding of mechanisms involved in the host–pathogen interactions constrains our efforts to eliminate tuberculosis. In many individuals, resulting from immune response to mycobacterial infection organised structures called granulomas are formed. To identify host responses that may control at least the early stages of infection, we employed an *in vitro* granuloma model. Here, human PBMCs were infected with live *Mycobacterium tuberculosis* in culture, and the appearance of granuloma-like structures was monitored over the next several days. Production of cytokines and chemokines in culture supernatants was monitored at various times, and the resulting temporal profiles were examined for possible correlations with either granuloma formation, or bacterial growth. While a positive association of TNF-α and IFN-γ secretion levels with extent of granuloma formation could clearly be identified, we were, however, unable to detect any statistically significant relationship between any cytokine/chemokine and bacterial growth. Examination of specific host cellular biochemical pathways revealed that either modulation of neutral lipid homeostasis through inhibition of the G_i-protein coupled receptor GPR109A, or regulation of host metabolic pathways through addition of vitamin D, provided a more effective means of controlling infection. A subsequent genotypic analysis for a select subset of genes belonging to pathways known to be significant for TB pathology revealed associations of polymorphisms with cytokine secretions and bacterial growth independently. Collectively therefore, the present study supports that key metabolic pathways of the host cell, rather than levels of relevant cytokines/chemokines might be more critical for regulating the intracellular mycobacterial load, in the context of granuloma formation.

1. Introduction

Tuberculosis (TB) remains a major public health burden despite the achievement of a 2% decline in TB incidence in accordance with the Millennium Development Goals [1]. Successful elimination of TB will require a deeper understanding of host responses against the pathogen and their roles in either controlling or exacerbating the infection outcome. Despite extensive past and ongoing research, the objective of identifying host immune parameters that distinguish between resistance and susceptibility to infection has remained elusive [2,3]. The current need is to identify novel molecular signatures that may better achieve this objective [4,5].

Following primary exposure to *Mycobacterium tuberculosis*, some individuals actively proceed towards development of disease while others, termed latently infected, show 5–10% lifetime risk of disease progression [6]. Development of primary TB or reactivation of latent TB or remaining latently infected throughout life [7], therefore, constitutes the spectrum of TB pathology. Here, host genetics might play an important role in differential host–*M. tuberculosis* interactions [8]. Encounter of tubercle bacilli

* Corresponding author. Fax: +91 33 25892150.
E-mail addresses: nehaagr25@gmail.com (N. Agrawal), cb1@nibmg.ac.in (C. Bhattacharyya), am2@nibmg.ac.in (A. Mukherjee), uullah@btk.fi (U. Ullah), bp1@nibmg.ac.in (B. Pandit), kanury@icgeb.res.in (K.V.S. Rao), ppm1@nibmg.ac.in (P.P. Majumder).
[1] Present address: National Institute of Biomedical Genomics, Kalyani, 741251 West Bengal, India.
[2] Authors contributed equally to this work.
[3] Present address: Molecular Immunology Group, Turku Centre for Biotechnology, 20520 Turku, Finland.
[4] Present address: Drug Discovery Research Centre, THSTI, Faridabad 121001, India.

http://dx.doi.org/10.1016/j.tube.2016.07.009
1472-9792/

with a host initiates a series of chemo-attractant mechanisms which result in recruitment of various types of immune cells at the site of infection and development of granulomas [3]. These 'tubercles' or 'granulomas' are considered hallmark of active TB disease [9]. In human granulomas, macrophages may acquire appearance of epitheloid cells, or fuse to form multinucleated giant cells (MGCs) [10], or differentiate into foamy macrophages [11]. T-lymphocytes are found as either a discrete band of cells at the periphery of granulomas or distributed diffusely among the other cell types [12]. However, whether granulomas favour host defense or bacterial persistence is a matter of debate and further investigation [13].

Significant differences of human granulomas at the level of cellular organisation and microenvironment restrict recapitulation of human TB pathology by non-human study models [14]. To gain an understanding of the pathophysiology of tuberculosis, a model of granuloma is required to determine the nature of host–pathogen interactions that might take place during latency. In this connection the *in vitro* granuloma model used in the present study has been shown to successfully capture early events of *M. tuberculosis* interaction with cells of human immune system and therefore, is useful in investigating both spatial and temporal aspects of human granuloma development [15–18].

In the present study, we exploited the *in vitro* granuloma system to identify immune signatures that may correlate with the bacterial burden or granuloma development. We screened 87 healthy individuals for a set of phenotypic responses using this model to investigate: (i) time-dependent extracellular secretion of cytokines and chemokines, (ii) ability to sustain or clear infection, and (iii) extent of granuloma formation. Subsequently, we analysed the data to detect possible correlation between the temporal pattern of cytokine/chemokine secreted by these cultures with either the granulomatous response, or with the control of bacterial growth. Here, we produced experimental support for the heterogeneity of human immune responses when exposed to a low dose of *M. tuberculosis*. We identified TNF-α and IFN-γ as positive regulators of granuloma formation. However, we were unable to detect any correlation between cytokine/chemokine production and *M. tuberculosis* replication or killing in this model system. Interestingly though, in contrast to the lack of sensitivity of responses to either cytokines or chemokines, bacterial burden in *in vitro* granulomas could be substantially reduced by modulating metabolic pathways of the host macrophage. Thus, inhibition of GPR109A, a receptor that regulates neutral lipid homeostasis [19], or addition of calcitriol (1,25-dihydroxyvitamin D3; physiologically active form of vitamin D), that activates vitamin D-dependent pathways and modulates lipid metabolism [20], was found to lead to a marked reduction in the infection load. These findings therefore suggest that, rather than the external cytokine milieu, key intracellular regulatory pathways of the host macrophage may play a more important role in controlling the bacterial load at least in our *in vitro* model system. This interpretation could be further corroborated by a genotypic analysis of select host-specific genes. Here, distinct polymorphisms in genes involved in metabolic pathways were found to associate with phenotypic outcomes of bacterial growth in *in vitro* granulomas and cytokine/chemokine protein concentrations in supernatant.

2. Materials and methods

2.1. Ethical statement

The study was approved by Institutional Ethics Committees of International Centre for Genetic Engineering and Biotechnology, New Delhi, and National Institute of Biomedical Genomics, Kalyani. Blood was drawn with written informed consent from volunteers and all the methods were carried out in accordance with the approved guidelines.

2.2. Isolation of PBMCs

Donors were non-tuberculous, BCG vaccinated, young (25–30 years) individuals of upper economic strata having no close contact with TB patients. All donors were HIV negative and did not show any active TB up to 3 years later. Some donors ($n = 41$) tested positive for tuberculin skin test (TST) and some ($n = 33$) tested negative, while the status of few ($n = 13$) was not known. Peripheral blood mononuclear cells (PBMCs) were isolated from freshly collected heparinised blood by Ficoll density gradient centrifugation from donors. Equal densities of 4×10^5 cells per well were seeded in 48 well vacuum gas plasma treated culture plates (BD biosciences, other types of coated or uncoated plates were not supporting *in vitro* granuloma adherence over the time of experiment) for successive experiments. GlutaMAX medium (Invitrogen) supplemented with 10% heat-inactivated foetal calf serum (FCS) was used for cell cultures. Before infection, cells were pre-incubated for 12 h at 37 °C in a humidified, 5% CO_2 atmosphere. Bacterial single cell suspensions of H37Rv *M. tuberculosis*, which were prepared every time as described in the following method, were used to infect these cells.

2.3. Bacterial cultures

M. tuberculosis were grown in Middlebrook 7H9 medium supplemented with 10% Albumin–Dextrose–Catalase, 0.04% glycerol and 0.05% Tween 80 and harvested at 0.6 $O.D_{595}$ following storage at −80 °C. To check viability, bacteria were revived from randomly selected stocks for 2 h in 7H9 medium and colony forming units (CFUs) were enumerated by plating serial dilutions of single cell suspension on 7H11 medium supplemented with 10% Oleic acid–Albumin–Dextrose–Catalase and 0.5% glycerol. Approximately 7×10^6 CFUs/ml bacterial single cell suspensions were prepared following the described process from the same batch of aliquots at each time of infection in all successive experiments.

2.4. Formation of in vitro granulomas

Previous reports on *in vitro* granuloma cultures illustrate use of varied doses of viable *M. tuberculosis* inoculums ranging from 0.001 to 0.1 MOI (multiplicity of infection) for human PBMCs and reported appearances of aggregates after day 1 of infection which successively acquired granuloma like morphology in 4–5 days with continued monitoring for a time period of 10–14 days [15,17,18]. To determine optimal conditions of *in vitro* granuloma formation in our lab, PBMCs were isolated from 3 independent human blood samples and infected with *M. tuberculosis*, H37Rv at MOIs of 0.1, 0.01 and 0.005 to further monitor cell viability and granuloma formation. Cell viability was monitored by MTT assay (as described later in this section). No significant inter-individual cell viability differences were observed with 0.005 MOI and simultaneously induced granulomas were also more compact (Supplementary Figure S1a–d). Assuming the approximate count of macrophages in PBMCs to be 5%, the approximate effective MOI becomes 0.1. Infection of PBMCs with 0.005 MOI followed incubation for 4 h and amikacin (100 μg/ml) treatment for 2 h for removal of extracellular bacteria for all the successive experiments. *In vitro* granuloma cultures were maintained in GlutaMAX media supplemented with 10% heat-inactivated FCS for 9 days. Since human serum was not incorporating any considerable difference in number of granulomas compared to FCS, therefore FCS was used in all the experiments

Please cite this article in press as: Agrawal N, et al., Dissecting host factors that regulate the early stages of tuberculosis infection, Tuberculosis (2016), http://dx.doi.org/10.1016/j.tube.2016.07.009