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Development of immune-biomarkers of pulmonary tuberculosis in a rabbit model

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SUMMARY

Tuberculosis (TB) causes extensive morbidity and mortality worldwide with approximately 10 million new cases of active disease emerging mostly from a pool of two billion individuals latently infected with *Mycobacterium tuberculosis* (*M. tb*) every year. The underlying host immune responses that drive *M. tb* infection to active disease or latency are not well understood. We propose that identification and characterization of host immune biomarkers will be helpful to better understand the mechanisms that drive this process, and may, in addition, lead to the development of better diagnostic tools for TB. We have previously reported the profiles of plasma immune biomarkers in pulmonary TB patients in endemic countries, and in *M. tb*-infected nonhuman primates. However, biomarker profiling for a cost-effective and user-friendly animal model relevant to human disease, such as rabbit, has not been developed. One challenge in the analysis of circulating cytokines/chemokines for rabbit model of TB is the limited availability of validated immune-reagents. Here we report the use of a commercially available multiplex microbead human cytokine/chemokine panels as development platform for rabbit immune reagents. The results demonstrate their utility to determine circulating analytes and define their profiles related to TB in the rabbit model. In addition, we report the profiles of circulating anti-*M. tb* antibodies in the plasma of rabbits with active pulmonary TB. These studies show that the pattern of expression of circulating immune biomarkers correlate with TB pathology in rabbits, and are similar to those defined in pulmonary TB patients.

1. Introduction

Worldwide, two billion people are latently infected with *Mycobacterium tuberculosis* (*M. tb*), the etiological agent of tuberculosis (TB) and approximately 10% of those infected develop active TB in their lifetime [1]. Nearly 10 million new cases and 1.5 million deaths were attributed to TB in 2014 (http://www.who.int/mediacentre/factsheets/fs104/en/). In order to develop better strategies to detect and combat TB, understanding of the underlying mechanisms of TB pathology needs to be improved. Active pulmonary TB is an inflammatory disease and is increasingly viewed as an imbalance of immune responses to *M. tb* infection [2]. Because host immune response underlies TB pathogenesis, we hypothesized that studies on immune-biomarkers profile will provide insight into the molecular basis of TB.

Serious limitations exist in studies on TB patients due to a variety of reasons including lack of temporal knowledge post-infection, limited access to affected tissues, cost of clinical studies etc. These limitations necessitate studies to be performed in animal models. Several animal models such as mouse, rabbit, guinea pig and nonhuman primate have been used to study TB and have substantially enhanced our understanding of *M. tb* infection and pathogenesis. Although in general, mouse model of *M. tb* infection is considered economical and convenient, characteristics of infection and disease pathogenesis in the most common mouse models do not effectively reflect the human TB [3]. Nonhuman primate model represents human TB most closely but high cost, ethical

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issues and BSL3 handling difficulties limit its routine use [3]. Both rabbit and guinea pig models of TB generally offer a combination of a close representation of human disease, cost-effectiveness and relative ease of handling. Rabbits, similar to humans, are more resistant to *M. tb* infection, compared to guinea pigs that succumb rapidly once the disease is established [3]. In contrast, the rabbit model of pulmonary TB manifests various stages of disease pathology more consistently, and closely, to human TB [4,5]. However, a practical challenge in conducting advanced studies in the rabbit model is the limitation in the availability of immunological reagents. Recently, developments of rabbit specific immunological reagents are emerging, which should further improve the usefulness of this model in TB research. In this report, we used multiplex microbead panels containing 41 human cytokines/chemokines, as well as antibodies against 31*M. tb* antigens, to assess their utility in characterizing the host immune responses in a rabbit model of pulmonary TB. The multiplex immunoassays reported here may contribute significantly as a novel set of reagents to study TB pathogenesis in this animal model.

2. Materials and methods

2.1. Rabbit infection and sample collection

A total of 26 female New Zealand White rabbits were used in this study. All the animals were specific-pathogen-free and housed individually before and after pulmonary infection with *M. tb.* HN878 via aerosol challenge using "snout-only" apparatus (CH technologies, NJ) as described before [6]. Experimental procedures involving rabbits and post-procedural care were performed according to the approved IACUC guidelines of Rutgers University. TB infection was confirmed in experimentally infected rabbits by necropsy and by *M. tb* culture as described earlier [7]. Briefly, portions of *M. tb* infected rabbit lungs (40% of total weight) were homogenized in sterile saline and serial dilutions of the homogenate was plated on Middlebrook 7H11 agar plates. After 4–6 weeks of incubation at 37 °C/5% CO2, the number of *M. tb* colonies were counted and calculated for the total lungs. *M. tb* infected rabbits were sacrificed at T = 0 (3 h), 4 weeks and 12 weeks post infection (p.i.) (n = 7, 7, and 12 per each time point, respectively). Blood samples (EDTA treated) from rabbits were centrifuged at 1,800 g for 15 min at 15 °C to separate plasma, which were stored frozen at −80 °C in small aliquots. To minimize the effects of frequent freeze-and-thaw, fresh single aliquots were thawed on ice prior to each experiment.

2.2. Recombinant M. tb antigens

Based on our previous studies, a panel of thirty one *M. tb* antigens was used in multiplex microbead assay [8,9]. Briefly, *M. tb* genes corresponding to respective antigens were cloned and expressed in *Escherichia coli* as recombinant polyhistidine-tagged products and purified to near-homogeneity as previously described [10]. Plasmid vectors for expression of eight recombinant antigens were obtained from the TB Resource Center at Colorado State University (Fort Collins, CO): Rv3875 (ESAT6), Rv3874 (CFP10), Rv2031c (HspX), Rv3804c (antigen 85a [Ag85a]), Rv1886c (Ag85b), Rv0129c (Ag85c), Rv3841 (Bfrb1), and Rv3418c (GroES). The recombinant antigens were expressed and purified at the University of California, Davis (UCD; Davis, CA), and the University of Arid Agriculture (UAAR), Rawalpindi, Pakistan, as described elsewhere [8]. Other recombinant *M. tb* antigens (n = 14) were expressed and purified at the Infectious Disease Research Institute (IDRI; Seattle, WA) as previously described [11]: Rv2875 (MPT70), Rv1984c (CFP21), Rv1980c (MPT64), Rv0934 (P38 or PstS1), Rv1860 (MPT32), Rv0054, Rv3874-Rv3875 (CFP10-ESAT) fusion, Rv3873, Rv3619, Rv2220, Rv0831c, Rv1009, Rv1099, and Rv2032. Six of the antigens (Rv1926c, Rv2878c, Rv1677, Rv3881c, Rv1566c, and Rv3507) were expressed and purified at the University of California, Irvine (Irvine, CA), as previously described [12,13]. Membrane extracts (n = 3) from H37RV, HN878, CDC1551 were obtained from TB Resource Center at Colorado State University (Fort Collins, CO) [14].

2.3. Multiplex antibody assay and data collection

Antibodies against each of the 31 *M. tb* antigens were determined in the plasma of rabbits by using the xMAP technology platform (Luminex Corp, Austin, TX) as previously described [15,16]. Through carbodiimide linkages, each of the *M. tb* antigens was covalently coupled to carboxylated microbeads (Luminex Corp, Austin, TX), and multiplex assays were performed to obtain the data as median fluorescence intensity (MFI) [8]. Briefly, rabbit plasma samples were diluted at 1:400 in 2% Prionex (bio-WORLD, Dublin, OH), and 50 μl of this diluted plasma was mixed with the 31-plex bead mixture per well. For detection of rabbit IgG, biotinylated horse anti-rabbit IgG (Vector Laboratories, Burlingame, CA) at a 1:1000 dilution in 2% Prionex was used and incubated at room temperature for 30 min on an orbital shaker at 500 rpm. Following incubation with the secondary antibody, beads were washed two times. To detect biotinylated IgG, 100 μl of streptavidin-phycoerythrin (CalTag, Burlingame, CA) was added at a dilution of 1:500 in wash buffer. The assay was incubated at room temperature for 15 min. Beads were washed twice with wash buffer, resuspended in 100 μl of wash buffer per well, and analyzed in the Luminex-100 instrument.

2.4. Multiplex cytokine/chemokine assay

MILLIPLEX MAP Human Cytokine/Chemokine Magnetic Bead Panel was purchased from EMD Millipore (Billerica, MA) which the manufacturer empirically determined to be cross-reactive to rabbit cytokines/chemokines. Assays were performed according to the manufacturer's instructions. Briefly, 25 μl undiluted plasma was mixed with 25 μl antibody coated microspheres. The assay was incubated overnight at 4 °C, washed twice with wash buffer. 25μl detection antibody was added and incubated for 1 h at room temperature, followed by the addition of 25 μl streptavidin-phycoerythrin solution and incubation for 30 min at room temperature, washed twice and then 150 μl sheath fluid was added and analyzed in the Luminex 100 instrument. There were 41 immune molecules/analytes (cytokines/chemokines) in the assay kit that included: sCD40L, VEGF, TNF-β, TNF-α, TGF-α, RANTES, PDGF-AB/BB, PDGF-AA, MIP-1β, MIP-1α, MDC (CCL22), MCP-3, MCP-1, IP-10, IL-17A, IL-15, IL-13, IL-12 (p70), IL-12 (p40), IL-10, IL-9, IL-8, IL-7, IL-6, IL-5, IL-4, IL-3, IL-2, IL-1Ra, IL-1β, IL-1α, IFN-γ, IFN-α2, GRO-α, GM-CSF, G-CSF, Fractalkine, Flt-3 ligand, FGF-2, Eotaxin, EGF. The concentration (pg/ml) of each cytokine/chemokine in the multiplex panels was measured based on a 6-point standard curve using BioPlex 6.1 software (Bio-Rad, Hercules, CA). All the measurable analyte concentrations were within the dynamic range of the respective standard curves of cytokines/chemokines.

2.5. Data analysis

For measurements of cytokines/chemokines, scatter grams were generated, and p-values were determined by Mann–Whitney test using GraphPad Prism version 6.05 (GraphPad software Inc., La Jolla California, http://www.graphpad.com/scientific-software/prism). For the analysis of antibody data, cutoff values were calculated using