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IMMUNOLOGICAL ASPECTS

Differences in immune cell function between tuberculosis positive and negative Asian elephants

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SUMMARY

Tuberculosis is an important health concern for Asian elephant (*Elephas maximus*) populations worldwide, however, mechanisms underlying susceptibility to *Mycobacterium tuberculosis* are unknown. Proliferative responses assessed via brominated uridine incorporation and cytokine expression measured by real-time RT-PCR were evaluated in peripheral blood mononuclear cell (PBMC) cultures from 8 tuberculosis negative and 8 positive Asian elephants. Cultures were stimulated with *Mycobacterium bovis* purified protein derivative (PPD-B), *M. tuberculosis* culture filtrate protein (CFP)-10, and *Mycobacterium avium* PPD (PPD-A). Following stimulation with PPD-B, proliferation was higher ($\alpha = 0.005$) in positive samples; no significant differences were detected following CFP-10 or PPD-A stimulation. Tumor necrosis factor (TNF)-α, interleukin (IL)-12, and interferon (IFN)-γ expression was greater in samples from positive elephants following stimulation with PPD-B ($\alpha = 0.025$) and CFP-10 ($\alpha = 0.025$ TNF-α and IL-12; $\alpha = 0.005$ IFN-γ). Stimulation with PPD-A also produced enhanced IL-12 expression in positive samples ($\alpha = 0.025$). Findings suggested that differences in immune cell function exist between tuberculosis positive and negative elephants. Proliferative responses and expression of TNF-α, IL-12, and IFN-γ in response to stimulation with PPD-B and CFP-10 differ between tuberculosis positive and negative elephants, suggesting these parameters may be important to tuberculosis immunopathogenesis in this species.

1. Introduction

Tuberculosis is an important health concern for elephant populations worldwide. The disease most commonly affects Asian elephants (*Elephas maximus*), and the majority of cases are due to *Mycobacterium tuberculosis*, the cause of human tuberculosis [1–4]. Since 1994, more than 50 culture confirmed cases of tuberculosis have been documented in U.S. captive elephants [2]. Recent studies have also reported confirmed cases and increased seroprevalance in populations of domesticated Asian elephants in range countries [5,6]. Elephant infections are typically chronic and subclinical; consequently diagnosis of infected individuals is challenging [1–3,7]. The current gold standard test, trunk wash mycobacterial culture, is insensitive [1,2,8,9]. Newer serologic tests have been developed and are recommended for screening. These tests have improved sensitivity and good specificity, however, validation is on-going [1,8–11]. In addition to individual animal and herd health concerns, elephant mycobacterial infection has significant public health implications. Throughout the world, captive elephants interact closely with human handlers for work and exhibition providing opportunity for exposure and potential zoonotic and/or anthropozoonotic transmission of disease [1,3,12,13]. The threat of disease transmission also extends to wild populations of elephants.

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Though infection has not been documented in wild elephants to date, captive working elephants in range countries frequently mingle with free-ranging elephants providing opportunity for disease transmission [1]. Public health vigilance and effective conservation of this endangered species require a better understanding of tuberculosis pathogenesis.

Unfortunately, the mechanisms underlying tuberculosis susceptibility in elephants are unknown, and information regarding elephant immune function is scarce [14–17]. In humans and other studied species, disturbances in the normal balance between cell-mediated (T_H1) and humoral (T_H2) immune responses are central to tuberculosis pathogenesis [18–28]. Evaluation of systemic immune responses in humans via measurement of cytokines has shown that inadequate T_H1 responses are a feature of active disease [19,27,29–34]. Considering altered immune responses to tuberculosis are instrumental in determining disease susceptibility and influencing pathogenesis in humans, it is plausible that immune function alterations may similarly contribute to Asian elephant tuberculosis susceptibility.

In a previous study, mRNA levels of several T_H1 and T_H2 cytokines significant in the pathogenesis of human tuberculosis were measured in peripheral whole blood samples from 106 (15% tuberculosis seropositive) Asian elephants using elephant-specific, real time RT-PCR assays [15]. Cytokine levels were measured in the absence of any mitogenic or antigenic stimulation. Though significant differences in levels of examined cytokines were not detected, the data illustrated some trends in cytokine expression between the two groups that warranted further investigation. Consequently, the current study was undertaken to measure proliferative responses and cytokine mRNA expression in peripheral blood mononuclear cell (PBMC) cultures from 8 tuberculosis negative and 8 tuberculosis positive North America-based Asian elephants following stimulation with mycobacterial antigens.

2. Materials and methods

2.1. Animals/sample collection

Samples were collected from 16 captive, North America-based, Asian elephants. For the purposes of this study, positive cases were designated as elephants with *M. tuberculosis* positive trunk wash cultures and/or seroreactivity to both the Elephant TB STAT PAK® screening test (Chembio, Medford, NY) and multiple antigen print immunoassay (MAPIA). Negative cases were those with no history of positive trunk wash culture or serology and no history of exposure to a known positive elephant. All study animals were zoo or retired performance elephants accustomed to human handlers and trained to cooperate voluntarily with routine veterinary examinations and procedures. Using minimal manual restraint, a total of 20 ml of peripheral whole blood was collected from the ear vein of each animal into collection tubes containing heparin sulfate (BD Biosciences, San Jose, CA). All samples were received for processing at the laboratory within 24 h of collection. In addition to blood samples, information regarding age, sex, current medical treatments and pertinent chronic inflammatory conditions was obtained for each of the study animals. Prior to experiments utilizing samples from the 16 study animals, preliminary experiments were conducted using samples from 2, tuberculosis negative Asian elephants to determine optimal parameters for culture of elephant PBMCs. Variables evaluated included: cell culture medium composition, concentration of viable PBMCs/ml, mitogen and antigen concentration for stimulation and incubation times following mitogen and antigen exposure [35]. Results of these preliminary experiments were used to determine the optimal parameters for culture and stimulation of elephant PBMCs utilized in the current study.

2.2. Isolation of PBMCs

Upon receipt, whole blood smears were examined via light microscopy to determine the relative proportions of monocytes and lymphocytes in each sample. Peripheral blood mononuclear cells were isolated from whole blood samples using density gradient centrifugation. From each sample, 15 ml of room temperature heparinized whole blood were diluted with 33 ml of room temperature Hank's Balanced Salt Solution (HBSS; Fisher Scientific, Pittsburgh, PA). The diluted sample was divided into six equal 8 ml aliquots, and each aliquot was carefully layered over 4 ml of room temperature Ficoll-Paque™ Plus, density 1.077 ± 0.001 g/ml (GE Healthcare, Uppsala, Sweden) in 15 ml sterile, plastic conical vials. Separation was achieved through centrifugation at 400 RCF for 30 min. Following centrifugation, the superficial buffer and plasma layers were removed from each vial via aspiration without disturbing the mononuclear cell layer interface. Next, the mononuclear cell layer was removed via aspiration and transferred to a new 15 ml vial. Mononuclear cells were washed with HBSS; individual aliquots from each sample were recombined and suspended in 37 °C cell culture medium. Cell culture medium consisted of RPMI 1640 medium with HEPES and L-glutamine (HyClone; Thermo Scientific, Waltham, MA) supplemented with 10% heat inactivated fetal calf serum (MP Biomedicals, Solon, OH), 1% 100 mM sodium pyruvate (Invitrogen, Carlsbad, CA), 1%, 10 mM minimal essential medium (Sigma–Aldrich, St. Louis, MO), 1% 5000 U penicillin/5000 Fg/ml streptomycin (Invitrogen), 0.1% 10 mg/ml gentamycin sulfate (Invitrogen) and 3.66×10^{-4}% beta mercaptoethanol (2×10^{-5} M; Fisher Scientific). Mononuclear cell viability was assessed directly in a 100 μl aliquot of each sample through evaluation of trypan blue dye (Sigma–Aldrich) retention (viable cells are able to pump dye out excluding the stain; nonviable cells retain stain). Each sample was then diluted with cell culture medium to a final concentration of 1×10^6 viable cells/ml.

2.3. Proliferation assays

For each sample, 100 μl aliquots (1×10^5 viable cells), in triplicate, were plated onto 96 well, clear plastic, flat bottom culture plates (Falcon® 3872 Primaria®; Becton Dickinson, Lincoln Park, NJ). Samples were incubated for 5 days at 37 °C, 5% CO_2 in the presence of 20 μg/ml *Mycobacterium bovis* purified protein derivative (PPD-B; Prionics, Zurich, Switzerland), 20 μg/ml *M. tuberculosis* culture filtrate protein (CFP)-10 (BEI Resources, Manassas, VA), and 20 μg/ml *Mycobacterium avium* PPD (PPD-A; Prionics). Unstimulated sample aliquots and cell-free medium controls were included to serve as negative controls. Aliquots cultured in the presence of 6 μg/ml concanavalin A (ConA; Sigma–Aldrich) were included as positive controls. Following the 5-day incubation, proliferating cells were distinguished and quantified via brominated uridine (BrdU) incorporation using a commercially available cell proliferation 5-bromo-2′deoxyuridine ELISA kit (Roche Diagnostics Corporation; Indianapolis, IN) according to manufacturer's instructions. Colorimetric reactions were assessed via spectrophotometry at 20 min post addition of kit substrate (SPECTRAmax PLUS; Molecular Devices, Sunnyvale, CA) and analyzed using SOFTmaxPRO software (Version 3.1.1; Molecular Devices). Absorbance values obtained via spectrophotometry for each sample triplicate were averaged to produce a single representative value. Any sample in which triplicate values varied more than one standard deviation from the mean were rejected and reanalyzed. Then, absorbance values for stimulated sample aliquots were divided by absorbance

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