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Humoral and lung immune responses to *Mycobacterium tuberculosis* infection in a primate model of protection ☆,☆☆

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ABSTRACT

Recently we reported (Mehra et al., 2013), that lung granulomas from *Mycobacterium bovis* Bacille Calmette–Guérin (BCG)-vaccinated cynomolgus macaques exhibit upon challenge with *M. tuberculosis* a more balanced expression of α- and β-chemokines, relative to comparable samples from sham-vaccinated animals by comparative transcriptomics. Here, we studied the recruitment of immune cells to blood and lungs in *M. tuberculosis*-infected macaques as a function of prior BCG-vaccination. Vaccination initially enhanced the levels of both macrophages and lymphocytes in blood. In contrast, significantly more $CD4^+$ lymphocytes were later recruited to the lungs of sham-vaccinated animals compared with earlier times/BCG vaccinated animals. BCG-vaccination had a short-lived impact on the anti-*M. tuberculosis* response. *M. tuberculosis* continued to replicate in the lung even in the wake of increased $CD4^+$ T cell recruitment to primate lungs, indicating that immune subversive mechanisms are key to its survival *in vivo*.

Introduction

Tuberculosis is responsible for the death of over 1.5 million people every year [1]. This situation has worsened due to the emergence of drug-resistance [2], HIV co-infection and the insufficient protection of the Bacillus Calmette–Guérin (BCG) vaccine, which is unable to protect adults against pulmonary TB [3,4]. The efficacy of BCG in protecting against adult pulmonary TB is unsatisfactory [5]. New and efficacious vaccines against TB are therefore urgently needed [6,7]. This requires both a better understanding of the correlates of protection in appropriate experimental models [8,9], and also a clearer understanding of the shortcomings of BCG such that they can be avoided with future vaccine design.

Nonhuman Primates (NHPs), such as rhesus [10–13] or cynomolgus [11,14] macaques are excellent models of *Mycobacterium tuberculosis* (*Mtb*) infection [15]. Upon experimental infection via either the intrabronchial or the aerosol route, these animals recapitulate the entire breadth of the human TB infection syndrome, including acute TB [10] characterized by massive caseous immunopathology [13] and asymptomatic, latent infection (LTBI). Furthermore, in macaques, LTBI can be reactivated by SIV co-infection [12], TNFα inhibition [12,16] or CD4-depletion [17]. More importantly, macaques demonstrate a spectrum of lung pathological outcomes upon experimental infection with *Mtb*, similar to naturally infected human beings [10–13,15,18–20]. Akin to humans, vaccination with BCG can protect NHPs against *Mtb* challenge, but the protection is incomplete [11,21,22]. These observations reinforce our contention that macaques best represent the human TB infection syndrome, allowing a study of pathology as well as protective responses not possible in other model systems [23].

It is known that prior BCG vaccination leads to a higher expression of β-chemokines in lung lesions following *Mtb* challenge [11].

Abbreviations: NHP, Nonhuman Primate; TB, tuberculosis; *Mtb*, *Mycobacterium tuberculosis*; BCG, Bacille Calmette–Guerin; BAL, broncho-alveolar lavage.

☆ The Tulane National Primate Research Center facilities are accredited by the American Association for Accreditation of Laboratory Animal Care and licensed by the US Department of Agriculture. All animals were routinely cared for according to the guidelines prescribed by the NIH Guide to Laboratory Animal Care. NHP studies were conducted following the recommendations of the institutional animal care and use committee. Humane endpoints were pre-defined in this protocol.

☆☆ The procedures reported in this manuscript were approved by the relevant oversight committees (IACUC and IBC).

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Vaccinated animals expressed a better balance of α- and β-chemokines in lung granulomas at week 5, relative to unvaccinated NHPs, which only expressed high levels of α- but not β-chemokines. Unsurprisingly, more macrophages were present in the lesions of vaccinated relative to sham-vaccinated macaques at earlier time points, as many β-chemokines promote specific monocyte/macrophage chemotaxis.

We reasoned that the presence of significantly more macrophages in the lesions of BCG-vaccinated primates causes a more robust antigen-presentation against the pathogen resulting in the generation of a more effective adaptive immune response. Here we compared both systemic and local cellular responses to *Mtb* infection in the two groups of BCG and sham vaccinated-NHPs by phenotyping for effector cells in blood, broncho-alveolar lavage (BAL) and in granulomatous as well as non-granulomatous regions of the lung at necropsy.

Materials and methods

Animals, vaccination, challenge, sample collection and phenotypic analyses of immune cells

Blood and BAL samples were obtained from BCG- and sham-vaccinated cynomolgus macaques (2/group), which were challenged with highly virulent *Mtb* Erdman 17 weeks post-vaccination [11]. Lung (granuloma as well as adjoining normal) samples were obtained from necropsy of two animals each in both groups at week 5 and 10 post-infection, a cellular fraction obtained by suspending the tissue samples in complete Roswell Park Memorial Institute (RPMI) medium containing collagenase and proteinase-K (1 h, 37 °C) and immunophenotyped as described [18]. The experimental design and the schedule/intervals at which the various blood, BAL and lung samples were collected for immunophenotyping are shown in Fig. 1. For flow cytometry, cells were stained with appropriately diluted concentrations of specific monoclonal antibodies are shown in Table 1.

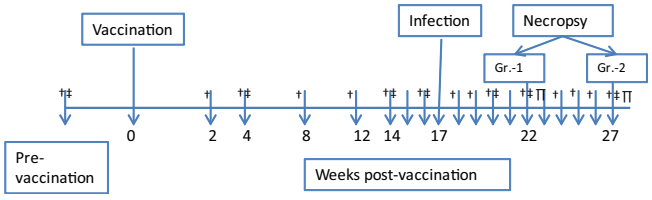

Fig. 1. Vaccination, challenge and sampling schedule. Cynomolgus macaques were divided into two groups (BCG- and sham-vaccinated control) of four per group as follows: Animals Group 1: IF16 & IF21 (BCG, TB + 5 weeks); IF23 & IF18 (saline, TB + 5 weeks). Animals Group 2: IF20 & IF17 (BCG, TB + 10 weeks); IF19 & IF22 (saline, TB + 10 weeks). Groups 1 and 2 were sacrificed after 5 and 10 weeks post-challenge with *Mycobacterium tuberculosis* Erdman (250–500 CFU, bronchoscopically), respectively. Whole blood (†), BAL (‡) and lung (∏) samples were collected at indicated time points.

Table 1
List of antibodies used for FACS analysis.

Antibody	Fluorochrome	Cat#	Company	Volume used per reaction (μl)
CD8	PE-TR	MHCD0817	Caltag	3
CXCR4	PE-Cy5	555975	BD Biosciences	20
CD4	PerCP-Cy5.5	552838	BD Biosciences	10
CD14	AL700	557923	BD Biosciences	10
CD69	APC Cy7	557756	BD Biosciences	5
CD3	PB	558124	BD Biosciences	7
CASP3	AL647	558124	BD Biosciences	20
CD68	FITC	F7135	Dako	20
CD163	PE	556018	BD Biosciences	20

Data evaluation and statistical analysis

Data was analyzed using FlowJo version 9.5.2 and statistical comparisons utilized ONE-way ANOVA with Bonferroni multiple hypothesis correction within GraphPad Prism, a P value of <0.05 was considered significant.

Results

Recruitment of macrophages in peripheral blood and lungs in response to Mtb infection in BCG-vaccinated and sham-vaccinated NHPs

Significantly more macrophages ($CD14^+$ $CD68^+$) were recruited to peripheral blood of BCG vaccinated compared to sham-vaccinated NHPs at an early stage (week 3) post-*Mtb* infection (Fig. 2A). This is consistent with our earlier report [11] that more macrophages are present within early granulomatous lesions derived from BCG-vaccinated rather than sham-vaccinated NHPs. The increase in blood macrophages is possibly, in part due to increased trafficking of these cells to the lungs of vaccinated animals. That observation, using confocal microscopy, is itself consistent with our transcriptomics data, where TB lesions derived from BCG-vaccinated, *Mtb*-challenged animals expressed greater amounts of CCL2 and CCL3 (molecules primarily responsible for macrophage recruitment) at week 5 [11] but not at the later time-points. The timing of the immunophenotyping observations was also consistent with the chemokine expression data. Similarly, our immunophenotyping data also indicates that while early on (at week 3), more macrophages were recruited to the lungs of animals that received vaccination, by the later time point (week 5), the higher recruitment of macrophages to the lung of vaccinated animals dissipated and in fact more macrophages were recruited to the lungs of sham vaccinated animals. It is important to note that *Mtb* replication levels, while undetectable for the first few weeks, were significantly higher in the sham-vaccinated animals relative to the BCG-vaccinated animals at the latter time-point (week 5). Thus, the higher macrophage recruitment levels in the sham-vaccinated animals at week 5 could be attributed to differences in antigen load. Monocytes generated in bone marrow enter the peripheral blood and in response to the appropriate chemotactic signal home to lungs where they differentiate into activated macrophages that can recognize and begin innate phagocytosis of *Mtb*. Thus, one of the mechanisms by which BCG vaccination may confer partial protection against *Mtb* infection may be via increased accumulation of innately activated macrophages.

Recruitment of lymphocytes in peripheral blood and lungs in response to Mtb infection in BCG-vaccinated and sham-vaccinated NHPs

Granuloma formation requires the interplay between both innate and adaptive immunity. Hence, we analyzed the recruitment of T cells following *Mtb*-infection as a function of vaccination. NHPs in the BCG-vaccinated group exhibited a significant increase in the peripheral blood levels of total $CD3^+$, $CD3^+CD4^+$ and $CD3^+CD8^+$ cells at 3 weeks post-infection (Fig. 2B–D). By week 5, the total number of $CD3^+$, $CD4^+$ and $CD8^+$ T cells present in the peripheral blood of sham-vaccinated NHPs were higher than in the vaccinated NHPs and the differences were no longer statistically significant (Fig. 2B–D), although there remained more T cells in the vaccinated group. The absolute lymphocyte counts further increased at later time-points in the unvaccinated (week 7 and 9) (data not shown). We also analyzed if $CD4^+$ cells from the vaccinated group also exhibited a higher level of activation, as determined by the co-expression of CD69 (week 8) as well as CD28 and CD95 (week 3 and 8) (data not shown). Distinct populations of T cells were typed