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

Noton K. Dutta[#a,2], **James McLachlan**[#b], **Smriti Mehra**[a,b], and **Deepak Kaushal**[a,b,*]

[a] Tulane National Primate Research Center, Covington, LA, USA

[b] Department of Microbiology & Immunology, Tulane University Health Sciences Center, New Orleans, LA, USA

[#] These authors contributed equally to this work.

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*.

Keywords

M. tuberculosis; BCG; Animal model; Granuloma; Macrophage; Lymphocyte

⋆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).

* Corresponding author at: Department of Microbiology & Immunology, Tulane National Primate Research Center, 18703 Three Rivers Road, Covington, LA 70433, USA. Tel.: +1 (985) 871 6254; fax: +1 (985) 871 6260. dkaushal@tulane.edu (D. Kaushal)..
[2]Present address: Center for Tuberculosis Research, Johns Hopkins School of Medicine, Baltimore, MD, USA.

Author contributions

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Conflict of interest

The authors declare no conflict of interest in connection with this manuscript.

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]. 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.

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 as belonging to naïve ($CD28^+CD95^-$), central memory ($CD28^{+-}$ $CD95^+$) or effector memory (CD28 $CD95^+$) phenotypes. However, we were unable to detect any significant differences in these sub-populations between the vaccinated and the sham-vaccinated group. Our results showing a higher absolute recruitment of $CD4^+$ T cells in the absence of any increase in their activation levels indicates that BCG vaccination allows for an earlier recruitment of potentially antigen-specific T cells upon *Mtb* infection. This may potentially represent another possible mechanism by which BCG partially protects against TB.

While significantly more lymphocytes were present in whole blood immediately following infection, a different result was observed in lungs. In BAL, significantly higher numbers of $CD3^+$, $CD4^+$ and $CD8^+$ T cells were obtained from sham-vaccinated rather than BCG-vaccinated NHPs at all times observed (weeks 3, 5 and 8) post-*Mtb* infection (Fig. 3). Hence, the recruitment of lymphocytes to the lung occurred largely in an antigen-dependent manner, as a higher *Mtb* load was prevalent in the lungs of sham- rather than BCG-vaccinated macaques.

Next, we analyzed the total number of lymphocytes and their activation status in the lung lesions derived from the two groups of animals, relative to adjoining non-lesion areas. At week 5, a statistically significant higher number of $CD4^+$ T cells were present in the lung lesions derived from sham-vaccinated, relative to vaccinated NHPs (Fig. 4A). Importantly, this difference, at least at week 5, was restricted to the granulomatous lesions, because the $CD4^+$ T cell counts were not different in normal tissue isolated from lung regions adjoining granulomatous lesions (Fig. 4A). We conclude that at this time, the T cells being recruited in an antigen-specific manner were specifically routed to the granuloma. These differences increased in both magnitude and significance in lesions, but not in normal tissue at week 10 (Fig. 4B). We also analyzed the total $CD8^+$ T cell levels in these samples and observed no differences between the BCG vaccinated and sham-vaccinated animals and between granuloma and adjoining tissue at either 5 or 10 weeks (Fig. 4C, D). Thus, the excess recruitment of T cells, in response to the higher replication of *Mtb* in the sham-vaccinated group, only manifested itself in the case of CD4 T cells.

Discussion

Long-term control of TB requires the development of efficacious vaccines [24]. This in turn requires an understanding of the immune responses that have the potential to protect against increasingly virulent *Mtb* infections. The outcome of infection with *Mtb* – acute TB, latent infection or reactivation, is decided at the level of the granuloma [25,26].

Primate granulomas exhibit highly organized architecture, commonly with central necrosis surrounded by a peripheral rim of fibrosis [11,18]. This is critical for containing *Mtb* replication, and conversely, permitting the latent survival of the pathogen [27,28]. Recently, it has been proposed that *Mtb* may actively promote granulomatous inflammation as part of its life cycle, since granulomas allow it to persist and disseminate [29–31]. However, *Mtb* must manage this Th1 response through novel mechanisms [32,33]. Hence, we characterized the cellular response in NHP granuloma's, as an addendum to characterization at the molecular level [11,13,18] and compared it to that observed in blood and BAL.

Vaccinated animals initially recruited significantly higher numbers of macrophages, as well as $CD3^+$ and $CD3^+CD4^+$ lymphocytes in peripheral blood upon *Mtb* challenge, relative to sham-vaccinated animals. However, these differences rapidly normalized and by 5 weeks post-*Mtb* infection, higher lymphocyte recruitment occurred in sham-vaccinated animals, in an antigen load-dependent manner. Moreover, immunophenotyping of both lung tissue and BAL showed higher $CD4^+$ T cell counts in sham-vaccinated relative to BCG-vaccinated macaques during early time-points (Fig. 2). The differences manifested themselves primarily in the granulomatous lesions obtained from sham-vaccinated relative to BCG-vaccinated macaques at week 5 and further increased at week 10. The differences were not observed for the surrounding non-granulomatous normal tissue and were specific to lesion tissue only. Significant differences were also not observed for $CD8^+$ T cells at either 5 or 10 weeks. Thus, recruitment and accumulation of likely antigen-specific, Th1-type, activated $CD4^+$ T cells in granulomatous lesions occurred as a function of antigen load. However, the accumulation of these $CD4^+$ T cells in the granulomas of sham-vaccinated NHPs failed to check both the replication of *Mtb* and the onset of acute TB at this site [11].

Since $CD4^+$ T cells are absolutely required for the protective immune response to *Mtb* infection [34], our results suggest that their recruitment to the lung by itself is insufficient in controlling the infection. This underscores the ability of *Mtb* in modulating host immunity and persisting in the wake of protective immune responses.

One mechanism by which *Mtb* may survive in lungs is by inducing IDO, which encodes indoleamine-2,3,-dioxygenase. Its expression is highly induced in lungs following *Mtb* infection in an antigen-specific manner and occurs exclusively within macrophages in the inner part of the granuloma closer to the central necrotic region [11]. Since IDO exerts powerful T cell suppressive effects, this expression pattern could prevent activated T cells from accessing the central regions of the granuloma, potentially preventing the exacerbation of immunopathology. A similar mechanism has been uncovered in lung granulomas formed by *Listeria monocytogenes* infection [35].

While preventing immunopathology, IDO may also inadvertently provide *Mtb* with a way to survive in the face of activated CD4 cells, by forming a physical barrier. This likely allows distance to be maintained between macrophages infected with *Mtb*, and $CD4^+$ cells capable of eliminating the pathogen. Antigens secreted by the necrosis of macrophages infected with *Mtb* potentially causes increased lymphocyte influx to the lesion, but the expression of IDO around the ring-wall prevents them from directly engaging *Mtb*. Hence, an increased antigen-specific $CD4^+$ T cell response in the sham-vaccinated NHPs is unable to check *Mtb* replication in NHP lung granulomas. Removal of IDO by inhibitors or siRNA, or the removal of T cells from this microenvironment, may in fact overcome this inhibitory effect. Experiments are underway to address this question. In this regard, it is important to point to the work by Rubin and coworkers [36], who recently found that *Mtb* can in fact synthesize tryptophan, thus countering the immune-function of IDO in the granuloma.

Acknowledgments

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Abbreviations

NHP	Nonhuman Primate
TB	tuberculosis
Mtb	Mycobacterium tuberculosis
BCG	Bacille Calmette–Guerin
BAL	broncho-alveolar lavage

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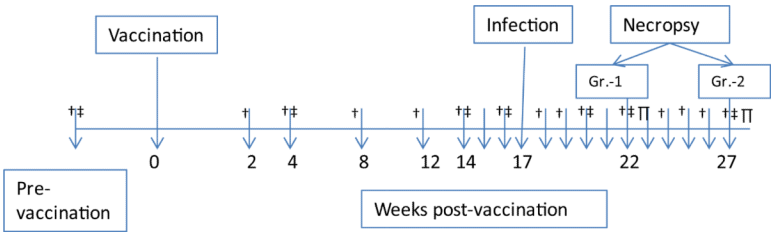

Fig. 1.

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 (g=P) samples were collected at indicated time points.

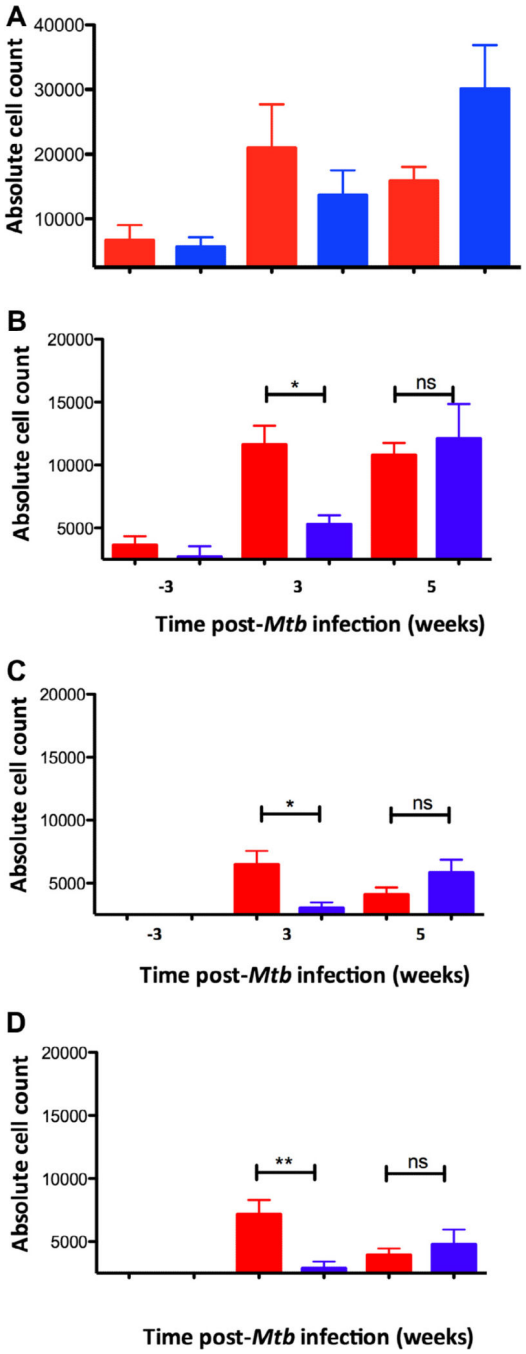

Fig. 2.

Accumulation of macrophages and lymphocytes in whole blood following *Mtb* infection as a function of prior BCG vaccination. Absolute cell counts in whole blood are shown (on Y-axis) for BCG-vaccinated (red) and sham-vaccinated (blue) NHPs. The numbers on the X-axis indicate weeks prior to or post-*Mtb* infection. (A) $CD68^+$ macrophages in whole blood. (B) $CD3^+$ cells, (C) $CD3^+CD4^+$ cells, (D) $CD3^+CD8^+$ cells in whole blood. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

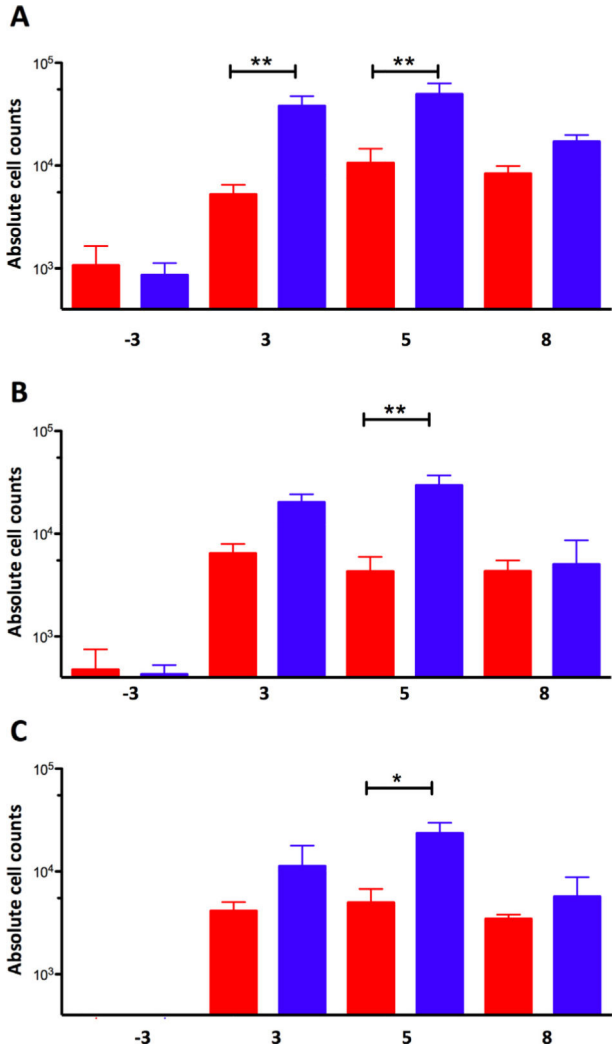

Fig. 3.

Accumulation of macrophages and lymphocytes in lung samples, following *Mtb* infection as a function of prior BCG vaccination. Absolute cell counts in BAL samples are shown (on the Y-axis) for BCG-vaccinated (red) and sham-vaccinated (blue) NHPs. X-axis in all cases represents time post-*Mtb* challenge. Thus, a time-point denoted as 3 corresponds to a post-vaccination time-point 3-weeks prior to *Mtb* challenge. (A) $CD3^+$ cells. (B) $CD3^+CD4^+$ cells. (C) $CD3^+CD8^+$ cells. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

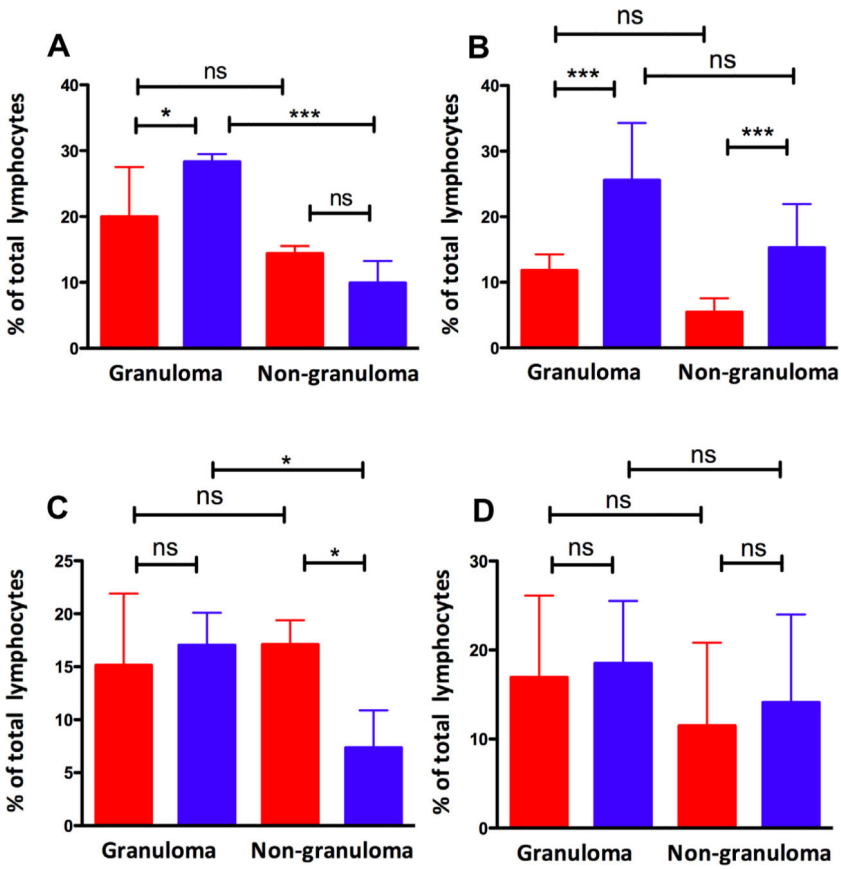

Fig. 4.

$CD4^+$ T cells expressed as a % of total lymphocytes are shown for lung (granuloma as well as non-granuloma) samples on the Y-axis for BCG-vaccinated (red) and sham-vaccinated (blue) NHPs at both 5 (A) and 10-week (B) post-*Mtb* infection. $CD8^+$ T cells expressed as a % of total lymphocytes are shown for lung (granuloma as well as nongranuloma) on the Y-axis for BCG-vaccinated (red) and sham-vaccinated (blue) NHPs at both 5 (C) and 10-week (D) post-*Mtb* infection. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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