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Induction and maintenance of a phenotypically heterogeneous lung tissue-resident CD4$^+$ T cell population following BCG immunisation

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ABSTRACT

Tuberculosis (TB) is the biggest cause of human mortality from an infectious disease. The only vaccine currently available, bacille Calmette-Guérin (BCG), demonstrates some protection against disseminated disease in childhood but very variable efficacy against pulmonary disease in adults. A greater understanding of protective host immune responses is required in order to aid the development of improved vaccines. Tissue-resident memory T cells (T_{RM}) are a recently-identified subset of T cells which may represent an important component of protective immunity to TB. Here, we demonstrate that intradermal BCG vaccination induces a population of antigen-specific CD4$^+$ T cells within the lung parenchyma which persist for >12 months post-vaccination. Comprehensive flow cytometric analysis reveals this population is phenotypically and functionally heterogeneous, and shares characteristics with lung vascular and splenic CD4$^+$ T cells. This underlines the importance of utilising the intravascular staining technique for definitive identification of tissue-resident T cells, and also suggests that these anatomically distinct cellular subsets are not necessarily permanently resident within a particular tissue compartment but can migrate between compartments. This lung parenchymal population merits further investigation as a critical component of a protective immune response against *Mycobacterium tuberculosis* (*M. tb*).

1. Introduction

Tuberculosis (TB), caused by infection with *Mycobacterium tuberculosis* (*M. tb*), presents a major challenge to global health, claiming 1.7 million lives in 2016 [1]. The only licensed vaccine against TB, bacille Calmette-Guérin (BCG), was developed almost a century ago [2]. When BCG is administered intradermally in early life, it is protective against disseminated forms of TB in childhood [3]. However, efficacy against pulmonary disease in adulthood, the most common form of TB disease, is very variable [4].

In murine models of TB, BCG provides significant protection against infection [5–8]. Despite strong evidence supporting a critical role for CD4$^+$ T cells producing interferon-gamma (IFN-γ) in this protection [8,9], frequencies of *M. tb*-specific CD4$^+$ T cells in the blood and lymphoid organs of humans and mice do not correlate with protection [10,11]. Similarly, magnitude and frequency of vaccine-induced IFN-γ responses fail to predict protective immunity [10–12]. A better understanding of the underlying mechanisms of vaccine-mediated protection, and generation of T cell memory in response to vaccination, is critical to rational development of more efficacious vaccines.

Tissue-resident memory T cells (T_{RM}), a recently-identified subset of memory T cells, may play an important role in protective immunity to TB. T_{RM} persist in non-lymphoid tissues without re-circulating through the body and are present locally at sites of infection in multiple different tissues, including the lungs [13–16]. They are able to mount a rapid *in situ* response to pathogenic challenge and can coordinate recruitment of immune cells to tissue sites [16–18]. Development of an intravascular staining technique has enabled the study of T_{RM}, allowing definitive discrimination between cells resident within the parenchyma of an organ and those present within the vasculature [16,19–21].

Several studies have investigated CD4$^+$ T_{RM} in the lungs within the context of *M. tb* infection [17,22–24], but their induction following BCG vaccination has not been well-characterised. Connor et al. [25] suggest that BCG-induced protection depends on lymphocyte migration to the lungs and retention of lung memory

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$CD4^+$ T cells. Perdomo et al. [26] describe a transient influx of $CD4^+$ and $CD8^+$ T cells into the parenchyma of the lung following intratracheal BCG vaccination. However, neither of these studies utilised the intravascular staining technique for definitive identification of tissue-resident cells. This is critical, as previous studies utilising intravascular staining reveal that >95% of $CD4^+$ T cells and >99% of total lymphocytes isolated from naïve murine lung via standard methods were in fact present in the vasculature of the lung rather than the parenchyma [17,21].

Other studies have employed the use of intravascular staining to investigate responses to novel TB vaccines [27–31]. Woodworth et al. [27] found that mice immunised with a subunit TB vaccine generated polyfunctional $CD4^+$ T cells which preferentially localised to the parenchyma of *M. tb*-infected lungs upon adoptive transfer. Carpenter et al. [28] demonstrated that vaccination with mycobacterial peptides resulted in a secondary $CD4^+$ T cell response against *M. tb* challenge, comprised of antigen-specific cells preferentially localising to the lung parenchyma. Both of these vaccines conferred protection against *M. tb* infection, highlighting the exciting potential role of this subset in protective immunity.

The phenotype of $CD4^+$ T cells induced by BCG vaccination has been described by several studies [5,32,33], but it is unclear how these phenotypes are distributed in the parenchymal and vascular compartments of the lung, as no studies have separately identified these populations with respect to BCG vaccine-induced responses. It is now important to establish whether BCG-induced lung parenchymal cells exhibit a unique phenotype, identifying them as tissue-resident. A number of studies have used expression of CD69 to define tissue-residence in the lung [16,19,20,34,35]. However, it is unclear how reliably this identifies lung T_{RM} in the context of TB vaccination. It is also important to determine whether the phenotype of these cells provides further knowledge regarding their functional potential. Whilst T_{RM} have been shown to express high levels of CD44 and low levels of CD62L, in common with effector memory T cells (T_{EM}) [36]; they also exhibit a unique transcriptional profile, different from that of other memory T cell subsets [37], which confirms their classification as a separate population.

Here, we utilise the intravascular staining technique to comprehensively characterise the development of an antigen-specific tissue-resident $CD4^+$ T cell population over the course of 12 months following intradermal BCG vaccination. We determine that BCG induces a population of these cells which are still present in the lung parenchyma 12 months post-immunisation. They display phenotypic and functional heterogeneity, reinforcing the importance of the intravascular staining technique for their definitive identification in the absence of unique phenotypic markers of lung location.

2. Results

2.1. Frequency of $CD4^+$ T cells is greater in the lung vasculature than parenchyma post-BCG or placebo immunisation

Following intradermal vaccination with BCG or placebo, intravascular anti-CD45 staining allowed discrimination between $CD4^+$ T cells present in the lung parenchymal tissue and those present in the lung vasculature (Fig. 1a). At all time points investigated, up to 12 months post-vaccination, frequencies and absolute numbers of $CD4^+$ T cells in the lung vasculature were significantly higher than in the parenchyma, for both BCG and placebo-immunised mice ($P < 0.0001$) (Fig. 1b and c). For the first 12 weeks post-immunisation, frequencies of $CD4^+$ T cells in the lung vasculature were approximately 9-fold greater than in the parenchyma. At 26 and 52 weeks post-immunisation, frequencies of $CD4^+$ T cells in the lung parenchyma were significantly greater than for all previous time points ($P < 0.05$) and frequencies of $CD4^+$ T cells in the lung vasculature were significantly lower than for all previous time points ($P < 0.05$). The actual number of $CD4^+$ T cells in the lung parenchymal compartment did not alter significantly between any of the time points measured post-immunisation. There were significantly fewer $CD4^+$ T cells in the lung vascular compartment at week 26 (4.5×10^5) compared to weeks 1 (6.5×10^5, $P = 0.0022$) and 6 (6.8×10^5, $P = 0.0004$) in both BCG and placebo-immunised mice.

2.2. BCG vaccination induces antigen-specific $CD4^+$ T cells in the lungs, spleen and blood

In order to investigate the development of antigen-specific $CD4^+$ T cells following BCG vaccination, lymphocytes isolated from the lungs, spleen and peripheral blood were stimulated with a pool of TB10.4 peptides before intracellular cytokine staining (ICS) to identify $CD4^+$ T cells producing interferon-gamma (IFN-γ), interleukin-2 (IL-2) and tumour necrosis factor-alpha (TNF-α) (Supplementary Fig. 1). Boolean gating allowed analysis of all $CD4^+$ T cells producing any of these cytokines independently or in combination (cytokine$^+$). BCG vaccination induced significant populations of antigen-specific $CD4^+$ T cells in the lung, spleen and peripheral blood, compared to placebo immunisation ($P < 0.05$) (Fig. 2a). Antigen-specific $CD4^+$ T cells were identified at all time points from week 3 post-BCG vaccination in the lung vasculature and from week 5 post-BCG vaccination in the lung parenchyma, spleen and peripheral blood.

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2.3. BCG induces the highest frequencies of antigen-specific $CD4^+$ T cells in the lung, with no difference in frequency between parenchyma and vasculature

There was no significant difference in frequency of antigen-specific $CD4^+$ T cells present in the lung vascular and parenchymal compartments at any time point post-BCG vaccination (Fig. 2b). At week 5 post-vaccination, both the lung parenchyma and vasculature contained significantly higher frequencies of antigen-specific $CD4^+$ T cells (2.36% and 2.49% respectively) when compared to peripheral blood (0.51%, $P = 0.0146$ & $P = 0.0074$ respectively). The lung vascular compartment contained significantly higher frequencies of antigen-specific $CD4^+$ T cells when compared to spleen (0.82%, $P = 0.0338$). At week 12 post-vaccination, only the lung parenchymal compartment contained significantly higher frequencies of antigen-specific $CD4^+$ T cells (3.48%) compared to spleen (1.34%, $P = 0.0055$) and peripheral blood (0.85%, $P = 0.0002$). At week 52 post-vaccination, only the lung vascular compartment contained significantly higher frequencies of antigen-specific $CD4^+$ T cells (3.68%) compared to spleen (1.17%, $P = 0.0004$) and peripheral blood (1.03%, $P = 0.0001$).

2.4. BCG-induced antigen-specific $CD4^+$ T cells display a dominance of multifunctional cells in the lungs, spleen and blood

Boolean gating analysis was used to identify populations of antigen-specific $CD4^+$ T cells producing IFN-γ, IL-2, TNF-α or any combination of the three. Triple-positive ($IFN\gamma^+IL\text{-}2^+TNF\text{-}\alpha^+$) and double-positive ($IFN\gamma^+IL\text{-}2^-TNF\text{-}\alpha^+$) $CD4^+$ T cells were detectable in all compartments at all time points post-BCG vaccination except week 1, when there were no significant populations of antigen-specific $CD4^+$ T cells in any compartment. Representative data for week 5 is shown (Fig. 3), with data for all other time points avail-

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