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Protective efficacy of recombinant BCG over-expressing protective, stage-specific antigens of *Mycobacterium tuberculosis*

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ABSTRACT

Tuberculosis (TB) remains a major cause of mortality and morbidity worldwide, yet current control strategies, including the existing BCG vaccine, have had little impact on disease control. CysVac2, a fusion protein comprising stage-specific *Mycobacterium tuberculosis* antigens, provided superior protective efficacy against chronic *M. tuberculosis* infection in mice, compared to BCG. To determine if the delivery of CysVac2 in the context of BCG could improve BCG-induced immunity and protection, we generated a recombinant strain of BCG overexpressing CysVac2 (rBCG:CysVac2). Expression of CysVac2 in BCG was facilitated by the *M. tuberculosis hspX* promoter, which is highly induced inside phagocytic cells and induces strong cellular immune responses to antigens expressed under its regulation. Intradermal vaccination with rBCG:CysVac2 resulted in increased monocyte/macrophage recruitment and enhanced antigen-specific $CD4^+$ T cell priming compared to parental BCG, indicating CysVac2 overexpression had a marked effect on rBCG induced-immunity. Further, rBCG:CysVac2 was a more potent inducer of antigen-specific multifunctional $CD4^+$ T cells ($CD4^+IFN\text{-}\gamma^+TNF^+IL\text{-}2^+$) than BCG after vaccination of mice. This improved immunogenicity however did not influence protective efficacy, with both BCG and rBCG:CysVac2 affording comparable level of protection aerosol infection with *M. tuberculosis.* Boosting either BCG or rBCG:CysVac2 with the CysVac2 fusion protein resulted in a similar improvement in protective efficacy. These results demonstrate that the expression of protective antigens in BCG can augment antigen-specific immunity after vaccination but does not alter protection against infection, further highlighting the challenge of developing effective vaccines to control TB.

1. Introduction

Tuberculosis (TB) remains a leading cause of mortality worldwide, and the only preventive immunoprophylaxis available today is vaccination with *Mycobacterium bovis* Bacille de Calmette et Guérin (BCG). BCG is an attenuated strain of *M. bovis* that was the first live attenuated vaccine used in humans and is still the most widely used vaccine in the world. However, despite its efficacy in preventing severe forms of TB in children, it is only partly effective against pulmonary TB in adults [1]. Consequently a better vaccine is needed to control the spread of TB.

Data from field trials indicate that, for reasons that are still poorly understood, the protective response induced by BCG vaccination wanes after about 10–15 years [2]. Nevertheless BCG is an excellent vehicle for delivering recombinant antigens since it can be readily manipulated to express a range of recombinant proteins [3]. Furthermore, since it is a live vaccine, the expression and delivery of recombinant antigens can be sustained over time compared to subunit vaccines, and, due to its complex cell wall components, it possesses potent immunostimulatory properties. Many components of the mycobacterial cell wall have the ability to bind pattern recognition receptors (PRRs) on the surface of immune cells. These include, but are not limited to, lipoarabinomannan (LAM) and phosphatidylinositol mannoside (PDIM) that bind to TLR2; and trehalose 6,6′-dimycolate (TDM) that is recognised by the Mincle receptor (reviewed in [4]). Indeed TDM is believed to account for most of the immunostimulatory effect of Complete Freund's Adjuvant, which contains heat-killed *M. tuberculosis* [5], and its adjuvant properties have been used for TB vaccine delivery [6]. Due to its immunostimulatory properties, BCG

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is the most effective immunotherapy used for treatment of bladder cancer [7] and has been proposed as a therapeutic to prevent relapse of multiple sclerosis [8] and as treatment of diabetes mellitus type I [9]. For these reasons, BCG is considered an ideal vector for the delivery of foreign antigens, and recombinant BCG strains (rBCG) have been trialled to protect against a wide variety of bacterial and viral disease [10,11].

In order to develop a more effective vaccine against TB, research has focused on modifying the currently used BCG to improve its capacity to induce protective immune responses. This has been pursued by using a number of different strategies, including: (i) improving the antigen repertoire of BCG by expressing mycobacterial antigens absent from the vaccine [12]; (ii) overexpressing shared immunodominant antigens [13,14]; (iii) expressing immunomodulatory cytokines and chemokines to improve the quality and longevity of T cell memory responses [15]; and (iv) conferring BCG the capacity to escape the phagosome to improve antigen presentation [16]. The latter strategy has now entered Phase II vaccine trials in humans [16].

In the current study, BCG was genetically modified by conferring on the vaccine the ability to express CysVac2, a fusion protein consisting of the early secreted antigen Ag85B combined with CysD, a component of *M. tuberculosis* sulphate assimilation pathway expressed during chronic infection in mice [17]. Vaccination with the CysVac2 fusion protein was previously shown to induce a higher level of protection against chronic *M. tuberculosis* infection than BCG [17]. Therefore it was hypothesised that the overexpression of CysVac2 by BCG may further improve its protective efficacy. The expression of the CysVac2-encoding sequence was facilitated by the *M. tuberculosis hspX* promoter, which is highly induced inside phagocytic cells and induces strong immune responses to antigens expressed under its regulation [14]. In order to improve the knowledge of the early innate immune response to BCG in the skin, the site where the vaccine is delivered in humans, we also examined the recruitment of innate immune cells to the vaccination site and their association with rBCG:CysVac2.

2. Materials methods

2.1. Bacterial strains and plasmids

M. tuberculosis H37Rv and BCG Pasteur were grown at 37 °C in Middlebrook 7H9 medium (Becton Dickinson, BD) supplemented with 0.5% glycerol, 0.02% Tyloxapol, and 10% albumin-dextrose-catalase (ADC) or on solid Middlebrook 7H11 medium (BD) supplemented with oleic acid–ADC. Kanamycin and hygromycin (Sigma Aldrich) were used when needed at a final concentration of 100 μg/mL and 50 μg/mL respectively.

The construction of BCG expressing the CysVac2 fusion protein was performed by using the pJEX93 expression vector, an *E. coli*/mycobacterial shuttle vector in which gene expression is under the control of the inducible *M. tuberculosis hspX* promoter [14]. *M. tuberculosis* H37Rv genes *fbpB* (encoding Ag85B) and *cysD* (encoding CysD) were amplified by PCR, cloned into pJEX93 and transformed into BCG resulting in the generation of the rBCG:CysVac2 strain. Correct sequence and alignment were determined and the expression of the fusion protein confirmed by Western Blot with polyclonal antisera recognising Ag85B (Fig. S1A). A band of 71 kDa was observed in rBCG:CysVac2 lysates but not in lysates from BCG containing control vector, thus confirming expression of the fusion protein in the rBCG:CysVac2 strain. BCG strains expressing the mCherry reporter protein, $BCG^{mCherry}$ and rBCG:$CysVac2^{mCherry}$, were constructed by transformation of the pNIP-40-mCherry plasmid [18] into BCG or rBCG:CysVac2, respectively. Fluorescence of the strains was confirmed by flow cytometry (Fig. S1B).

2.2. Animals

Female C57BL/6 (6–8 weeks of age) were purchased from the Animal Resources Centre (Perth, Australia). P25 transgenic TCR (p25-TgTCR) mice (specific for residues 240–254 of *M. tuberculosis* Antigen 85B (Ag85B) were bred at the Centenary Institute [14,19]. All mice were maintained in specific pathogen-free condition and experiments were performed with the approval of the University of Sydney Animal Care and Ethics Committee (approval number K75/9-2012/3/5846). Animals were randomly assigned to experimental groups.

2.3. Vaccination and aerosol M. tuberculosis infection of mice

For intradermal (i.d.) vaccination, 5 μL of bacterial suspension containing approximately 5×10^5 CFUs or PBS was injected intradermally (i.d.) into each ear under a surgical Leica M651 microscope (Leica, Wetzlar, Germany) using an ultrafine syringe (29 G, BD Biosciences), as previously described by [20]. For subcutaneous (s.c.) vaccination approximately 5×10^5 CFU of bacteria in 200 μL of PBS were injected at the base of the tail with an insulin syringe. The CysVac2/MPL-DDA vaccine was prepared as described previously [17].

Eight weeks after the final vaccination, mice were challenged with aerosolized *M. tuberculosis* H37Rv, using an inhalation exposure apparatus (Glas-Col) with an infective dose of approximately 100 viable bacilli per lung. Bacterial load was determined at different timepoints after challenge by plating serially-diluted homogenates of lung and spleen onto 7H11 media. Colonies were enumerated after approximately 18 days growth.

2.4. Cell preparation and immunogenicity studies

For adoptive transfer studies, splenocytes from p25-TgTCR (p25) mice were prepared and labeled with CFSE (Molecular Probes-Invitrogen, USA) as described [35]. C57BL/6 mice (CD45.2) received i.v 5×10^5 CFSE-labeled p25 splenocytes (CD45.1) and the next day were immunized with 5×10^5 CFU of BCG or rBCG:CysVac2. After 4 days, the auricular LN was harvested, single-cell suspensions prepared and stained with the specified monoclonal antibodies (Table S1) and examined by flow cytometry to quantify the extent of proliferation of adoptively transferred cells (CFSE dilution) and their ability to produce cytokine in response to re-stimulation with antigen (see below).

For cell preparations from the ear, dorsal and ventral pinnae of each ear were separated using tweezers, and cells from the ears were dissociated with 1 mg/mL collagenase type I (Worthington) and DNase (10 U/ml; Worthington) and then passaged through a cell strainer. Single cell suspensions were prepared from the lung, LN and spleen as previously described [17]. PBMCs were isolated from whole blood as previously described [17]. Antigen-specific IFN-γ producing cells were detected by ELISPOT assay as described previously [21]. All antigens were used at a concentration of 10 μg/ml.

2.5. Intracellular cytokine staining and flow cytometry

Single-cell suspensions from the ears were stained with relevant fluorophore-conjugated mAbs specific for myeloid populations (Supplementary Table S1). To assess antigen-specific cytokine induction by T cells, PBMCs or single-cells suspensions from the LN, lung and spleen were stimulated for 12–16 h with p25 peptide FQDAYNAAGGHNAVF (5 μg/mL) in the presence of

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