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Short communication

Evaluation of the immunogenic capability of the BCG strains BCGΔBCG1419c and BCGΔBCG1416c in a three-dimensional human lung tissue model

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ABSTRACT

Tuberculosis (TB) still remains as an unmet global threat. The current vaccine is not fully effective and novel alternatives are needed. Here, two vaccine candidate strains derived from BCG carrying deletions in the *BCG1416c* or *BCG1419c* genes were analysed for their capacity to modulate the cytokine/chemokine profile and granuloma formation in a human lung tissue model (LTM). We show that the clustering of monocytes, reminiscent of early granuloma formation, in LTMs infected with BCG strains was similar for all of them. However, BCGΔBCG1419c, like *M. tuberculosis*, was capable of inducing the production of IL-6 in contrast to the other BCG strains. This work suggests that LTM could be a useful *ex vivo* assay to evaluate the potential immunogenicity of novel TB vaccine candidates.

1. Background

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* is a chronic disease, which in 2016 caused almost 1.7 million deaths and an estimated 10.4 million new cases [1]. The only available TB vaccine, *Mycobacterium bovis* (*M. bovis*) Bacillus Calmette Guérin (BCG) exhibits variable efficacy and no effect in preventing latent pulmonary TB. Therefore, among other strategies for an improved TB vaccine, genetic modulation of the existing BCG strains is exploited [2]. Granulomas are dynamic clusters of immune cells that are typically formed around sites of infection with *M. tuberculosis* [3], typically harbouring infected macrophages in the core. *M. tuberculosis* replicates inside macrophages, however extracellular infection characterised by biofilm-like formation has been reported in susceptible mice and guinea pigs [4,5]. The genes *BCG1416c* and *BCG1419c* of *M. bovis* BCG, are homologous to *Rv1354c* and *Rv1357c*, which encode for c-di-GMP diguanylate cyclase and phosphodiesterase, respectively. These genes regulate the levels of mycobacterial second messenger c-di-GMP [6], a molecule linked to biofilm formation in mycobacteria [7]. Recently, the BCGΔBCG1419c strain was shown to provide protection after *M. tuberculosis* challenge *in vivo*, including increased proliferation of T $CD4^+$ $IFN\gamma^+$ and T $CD8^+$ $IFN\gamma^+$ lymphocytes [7]. Moreover, BCGΔBCG1419c displayed significant changes related to surface pellicle production, such as persistence within lungs of infected mice and tolerance to nitrosative stress [8]. The use of three-dimensional (3D) tissue models that better resemble the air/liquid environment of lungs *in vitro* is a promising route for the evaluation of new vaccines. Here, we compared *M. tuberculosis*, BCG and BCG-derived mutants in c-di-GMP diguanylate cyclase- and phosphodiesterase-encoding genes with respect to granuloma formation and release of inflammatory mediators in a human lung tissue model.

2. Methods

2.1. Ethical approval and biological samples

Peripheral blood, collected at the blood bank at Linköping University Hospital, was obtained from healthy donors who had

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given written consent for research use of the donated blood in accordance with the Declaration of Helsinki.

2.2. BCG strains and cell culture

All experiments with *M. tuberculosis* (H37Rv) and the BCG strains BCG-WT, BCGΔBCG1416c, BCGΔBCG1419c and BCGΔBCG1419c::Rv1357 were carried out in the BSL3 facility at the Faculty of Medicine and Health Sciences, Linköping University following the methodology previously described [8,9]. The mutant strains were generated as previously described. Briefly, the 5′ and 3′-flanking regions (arms) of both genes were individually amplified by PCR and cloned into pYUB854 [10], to then transform the resulting plasmids, into BCG Pasteur by electroporation and selection on 7H10 OADC plates with 50 μg/mL hygromycin. We cultured the resulting colonies, isolated genomic DNA, and characterized by PCR the candidates for double cross-over homologous recombination events, where replacement of the BCG genes for the hygromycin one occurred [8].

2.3. Lung tissue model and bacterial load

The infection of macrophages and the development of the air-exposed models were carried out following the methodology previously described [11]. Briefly, the *Mycobacterium*-infected macrophages were mixed with freshly prepared uninfected monocytes (1:5 ratio) and added in the tissue model prior to addition of epithelial cells. Introduction of infected macrophages is to ensure productive infection of lung tissue model depicting intracellular bacteria. For experiments with non-transformed BCG strains, samples were processed following a standard immunofluorescence staining using an anti-*Mycobacterium tuberculosis* rabbit polyclonal primary antibody (Ab905 from Abcam, Cambridge, UK) and a goat-anti-rabbit secondary antibody coupled to Alexa Fluor 688 (Invitrogen, Carlsbad, CA). Monocytes were labelled with the red fluorescent dye PKH26 (purchased from Sigma-Aldrich). Bacterial numbers were quantified measuring the total GFP fluorescence intensity [9]. Samples were analysed using a LSM700 Confocal microscope (Zeiss, Gottingen, Germany) after 7 days of culture.

2.4. Image analysis of cell clusters

The volume of cellular clusters was determined from the models by confocal imaging. Twelve different areas of each model were analysed using Imaris Image Processing Software (Version 8.0, Bitplane AG, Switzerland). The analysis of GFP-based cellular clusters was carried out using Huygens Professional Software. Two regions of interest were considered from each individual image, one region to detect GFP-bacteria (positive areas) and one region for non-green fluorescence signal (negative areas). The volume ratio of cell clusters was calculated by dividing the red fluorescence signal obtained from the positive over the negative regions. This ratio represents the fold change in the volume at the sites of bacterial infection, compared to the sites without bacteria.

2.5. Cytokine and chemokine analysis

The expression of cytokines and chemokines was quantified from supernatants after 5 days of infection using a Cytometric Bead Array Kit (BD, San Diego, CA) following the manufacturer's instructions in a Gallios flow cytometer (Beckman Coulter).

2.6. Statistics

The statistical analysis was carried out using GraphPad (Prism, version 5) software. Data is presented as mean or median of individual groups from 2 to 4 experiments. Statistical comparisons between groups were carried out using a Kruskal Wallis test followed by Dunn's post-test. A value of $p < 0.05$ was considered significant.

3. Results

First, we investigated whether formation of monocyte clusters reminiscent of early granuloma [11,12] can be found in the tissue model in response to infection with the BCG strains carrying deletions in *BCG1416c* or *BCG1419c* or in the complemented strain BCGΔBCG1419c::Rv1357c. To this end, developing lung tissue models were infected with the tested strains and incubated for 7 days. Confocal imaging of the tissue models showed that models infected with the virulent *M. tuberculosis* strain H37Rv displayed clusters of monocytes at the sites of infection whereas the clusters observed in the models infected with the different BCG strains were less prominent (Fig. 1A). Quantitative image analyses of Z-stacks obtained using confocal microscopy were done using two approaches; the cumulative volumes of the cell clusters were determined for all conditions (Fig. 1B) and for those strains, in which transformation with a GFP-reporter plasmid was possible (all strains except the BCGΔBCG1419c::Rv1357c, because it already harbours two antibiotic resistance genes and could not be transformed with the GFP-plasmid), the ratio of red fluorescence of monocytes in areas with bacteria vs. red fluorescence in areas void of bacteria was determined (Fig. 1C). The tested BCG strains did not display altered capacity to cause aggregation of monocytes as compared to BCG-WT, however, there was a tendency for all strains to cause more monocyte clustering compared to uninfected controls. Only the virulent *M. tuberculosis* strain significantly altered the distribution of monocytes in the model towards clustering around the site of infection. Next, we analysed the bacterial load within the tissue model after infection with the different BCG strains transformed with GFP. To this end, image analysis of the cumulative intensity of the GFP-labelled bacteria was done in tissue models infected with the GFP-expressing strains (Fig. 1D). We found no difference in bacterial load among the BCG strains, and only H37Rv displayed a significantly higher bacterial load (Fig. 1 D), reflecting replication during the 7 days of incubation (method described in [9]). Finally, we investigated the secretion pattern of a panel of cytokines and chemokines from the infected tissue models, including IL-6, IL-8, RANTES, IP-10 and MCP-1 (Fig. 2) and IL-1β, IL-12, TNF-α (not shown). Of all tested mediators, only IL-6, IP-10 and MCP-1 were significantly induced in the models infected with *M. tuberculosis*. The only BCG strain that, like H37Rv, induced IL-6 was the BCGΔBCG1419c mutant. This mutant also displayed enhanced secretion of MCP-1 (2.06-fold vs uninfected controls and 1.86-fold vs BCG-WT), however the result did not reach statistical significance (Fig. 2E).

4. Discussion

Granuloma formation is a pathological hallmark of *M. tuberculosis*-infected tissue [3]. Previously thought of as static frameworks that contain mycobacterial infection, granuloma are now described as dynamic structures that display heterogeneity in arrangement and fate [3]. During early granuloma formation, pathogenic mycobacteria are actively recruiting their cellular niche, the monocytes/macrophages, thereby promoting replication and dissemination in the tissue [12,13]. Granuloma formation is clearly dependent on the secretion of the virulence factor ESAT-6 via the Type VII secretion machinery [12,13] that is absent in BCG [14]. Here we report that infection of the human lung tissue model with BCG strains deficient in *BCG1416c* and *BCG1419c* induced monocyte

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