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Short communication

MspA-*Mycobacterium tuberculosis*-transformant with reduced virulence: The "unbirthday paradigm"

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ABSTRACT

Expressing *msp*A porin gene from *Mycobacterium smegmatis* in *Mycobacterium tuberculosis* attenuated this pathogen. Intracellular growth of the transformants into free-living amoeba and murine and human macrophages decreased. Furthermore, transformants decreased the microbicidal program of human monocyte-derived macrophages. BALB/c mice inoculated with transformants exhibited higher weights, lower histological lesions and lower *M. tuberculosis* inoculum in the liver, spleen and lungs than control mice challenged with wild-type *M. tuberculosis*. Preliminary evaluation indicated that mice inoculated with this transformant showed higher weights and lower numbers of lung nodules and tissular mycobacteria than control mice when challenged with wild-type *M. tuberculosis*. Similar to the paradoxical "unbirthday" gift coined by Lewis Carroll in *Alice's Adventures in Wonderland*, adding *msp*A gene reduced the virulence of *M. tuberculosis* and yielded a protective effect. Lost of non-virulence genes is a mechanism for virulence in mycobacteria. Engineering non-virulence genes in *M. tuberculosis* may yield strains with decreased virulence and increased immunogenicity.

1. Introduction

Since the seminal work of Louis Pasteur on *Pasteurella multocida* "attenuation" through axenic culture, most living vaccines, such as Bacille Calmettes Guérin (BCG), have been obtained through genome reduction via *in vitro* culture. This gave birth to a major paradigm in bacteriology suggesting that pathogenic bacteria acquire specific virulence genes clustered in so-called pathogenicity islands through lateral gene transfer to become virulent [1–4]. The virulence factors described in the facultative intracellular pathogens *Shigella* [5,6], *Salmonella* [3], *Listeria monocytogenes* [7] and *Brucella abortus* [8] affect various steps of bacteria-host cell interactions, including adhesion, penetration, survival and intracellular multiplication to mediate bacterial escape from host immune responses [1–9].

As a consequence of virulence gene acquisitions, pathogens should exhibit a trend towards increased genome size. However, neutral post-genomic observations have shown that pathogens have smaller genomes than their non-pathogenic counterparts [10,11]. This phenomenon is particularly obvious for *Rickettsia* and *Mycobacterium* species [12,13]. Despite such evidence, the current concept for virulence of the tuberculosis agent *M. tuberculosis* is that it acquired specific virulence genes by horizontal gene transfer [14].

Recently, it was discovered that harmless organisms harbored non-virulence genes, i.e. genes whose expression decreases or suppresses the virulence of a pathogen and, as a consequence, must be turned-off or deleted in pathogens. For example, the pathogenic *Shigella sonnei* [15] and *Yersinia pestis* [16] lack the lysine decarboxylase gene present in harmless, closely related *Escherichia coli*. In mycobacteria, it was shown that MspA, the major outer membrane porin (OmpA) of rapidly-growing *Mycobacterium smegmatis*, was absent in slow-growing pathogenic mycobacteria, including *M. tuberculosis* [17,18]. In *M. smegmatis*, MspA constitutes a central pore for hydrophilic solutes [17,19] which renders *M. smegmatis* susceptible to nitric oxide [20]. Accordingly, its deletion increases the survival of *M. smegmatis* in macrophages [20].

Here, we hypothesized that MspA could be a non-virulence gene in *M. tuberculosis* which acquisition would decrease pathogenicity in *M. tuberculosis*, referring to this concept as the "unbirthday

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hypothesis," modeled after the increased benefits of paradoxically receiving more presents for non-birthdays instead of a birthday, as explained to Lewis Carroll's character Alice in Alice's Adventures in Wonderland [21]. We expressed the *M. smegmatis mspA* gene in *Mycobacterium bovis* BCG [22,23] and *M. tuberculosis* [24] to expand the genomic content of both bacteria and evaluate the behavior and virulence of these bacteria using several in-vitro and in-vivo approaches. The results show that expanding the genomic content of *M. tuberculosis* attenuates the virulence of this pathogen.

2. Results

2.1. Transformation of mycobacteria with the putative non-virulence gene

A 636-base pair (bp) DNA fragment, containing mspA, was PCR-amplified from the *M. smegmatis* mc2 155 strain and cloned into the 5792-bp plasmid pVV16 under the control of the hsp60 promoter. Sequencing the insert revealed no amino acid replacement in mspA. After the electroporation of the transformed and control plasmids into the *M. bovis* BCG Tokyo (BCG) and *M. tuberculosis* H37Rv strains, the presence of mspA-positive transformants was verified through PCR and RT-PCR using specific primers (Supplementary Table 1) (Supplementary Fig. 1, Supplementary Figs. 2a–b). MspA is a heat-stable porin23; therefore, after selective protein extraction at a high temperature, sodium dodecyl sulfate (SDS)PAGE was performed and showed a clear signal characteristic of the tetrameric form of MspA [25,26] in extracts from *M. smegmatis* BCG and *M. tuberculosis* transformants (Supplementary Fig. 2c). However, these signals were not observed in BCG and *M. tuberculosis* controls. The monomeric form of MspA (~20 kDa) was further observed in extracts from *M. smegmatis* and transformants after heating the tetrameric protein in 80% dimethyl sulfoxide (DMSO) at 100 °C (Supplementary Fig. 2d). After gel extraction, the proteins spots were identified as *M. smegmatis* MspA with 21% sequence coverage and 29% sequence similarity. We therefore verified the expression of this putative non-virulence gene in BCG and *M. tuberculosis* transformants.

2.2. The putative non-virulence gene decreases intracellular growth

After four days co-culture with the amoeba *Acanthamoeba polyphaga*, the number of viable BCG transformants was significantly smaller than that of the controls ($p \leq 0.05$) (Supplementary Fig. 3b). On day 12, the number of colony-forming units (cfu) for the BCG transformants was 10 ± 3 compared with 95 ± 9 for the controls ($p \leq 0.05$) (Supplementary Fig. 3b). Similarly, after eight days co-culture, the number of *M. tuberculosis* transformants was significantly smaller than that of the controls ($p \leq 0.05$). On day 12, the number of cfu for the transformants was $2 \times 10^5 \pm 6 \times 10^4$ versus $8 \times 10^3 \pm 5 \times 10^3$ for the controls (Fig. 1a).

In the bone marrow-derived macrophages (BMDMs) co-culture, the survival of the transformants was significantly lower than that of the controls ($p \leq 0.05$) (Supplementary Figs. 3c and 1b). On day 14, the number of cfu was $1 \times 10^7 \pm 6 \times 10^6$ for the BCG transformants and $3 \times 10^8 \pm 7 \times 10^7$ for the controls, and $4 \times 10^5 \pm 1 \times 10^5$ cfu were observed for the *M. tuberculosis* transformants versus $1 \times 10^6 \pm 7 \times 10^5$ cfu for the controls.

In co-culture with human macrophages derived from blood monocytes (hMdMs), the survival of BCG and *M. tuberculosis* transformants was significantly lower than that of the controls ($p \leq 0.05$). On day 14, we recovered $1 \times 10^6 \pm 1 \times 10^5$ cfu for the BCG transformants versus $5 \times 10^6 \pm 3 \times 10^5$ cfu for the controls (Supplementary Fig. 3d). As for *M. tuberculosis*, on day 14, we obtained $5 \times 10^3 \pm 7 \times 10^2$ cfu for the transformants and $2 \times 10^4 \pm 2 \times 10^3$ cfu for the controls (Fig. 1c).

2.3. The putative non-virulence gene disarms microbicidal program in macrophages

We used high-throughput approach based on whole genome microarray to investigate the activation pattern in response to wild-type and transformant *M. tuberculosis*. PCA analysis revealed that both strains of *M. tuberculosis* induced a transcriptional pattern markedly distinct from unstimulated monocyte-derived macrophages (MDMs); the differences between wild-type and transformants, were lower than those between both strains of *M. tuberculosis* and control cells (Fig. 2A). The hierarchical clustering confirmed that *M. tuberculosis*-stimulated cells were present in a cluster different from unstimulated cells. Among stimulated cells, the patterns of wild-type- and transformants-stimulated cells were on two different branches of the dendogram (Fig. 2B). Using Venn diagrams, we identified a core response to *M. tuberculosis* that consisted of 572 genes (473 up-modulated and 99 down-modulated genes). We identified a specific signature for *M. tuberculosis* in which responses to wild-type and transformants were differently enriched. We found a greater enrichment with down-modulated genes in responses to the transformant; in contrast wild-type response was poorly enriched (Fig. 2C). Hence, while core response markedly consisted of up-modulated genes, the specific response to transformants was made of down-modulated genes.

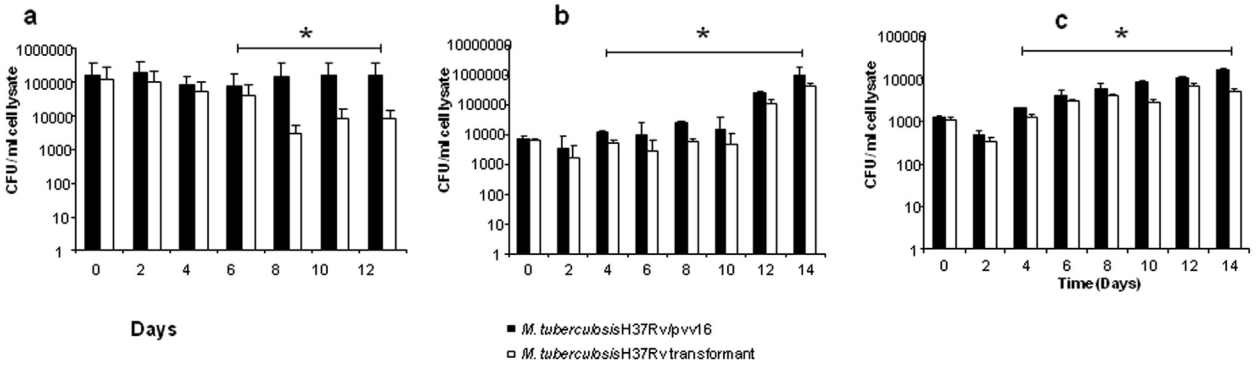

Fig. 1. Intracellular fate of *M. tuberculosis* H37Rv in different eukaryotic hosts. Colony-forming unit estimates for *M. tuberculosis* H37Rv/pVV16 and *M. tuberculosis* H37Rv/pVVMspA in *A. polyphaga* (a), BMDM (b) and hMdM (c). The data points represent the means of triplicate wells, and the standard errors are represented using error bars. Statistically significant differences are indicated by asterisk * ($p < 0.05$).