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SHORT COMMUNICATION

Vaccination of mice with recombinant bacille Calmette-Guérin harboring *Rv1357c* protects similarly to native BCG

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SUMMARY

Despite the availability of a *Mycobacterium bovis* bacille Calmette Guérin (BCG) vaccine, tuberculosis (TB) remains a global public health problem. In this study, we introduced the c-di-GMP phosphodiesterase gene *Rv1357c*, implicated in regulating mycobacterial replication within macrophages, into BCG Pasteur, and tested the resulting strain for its capacity to serve as a vaccine against TB in a murine model. Modified BCG was more phagocytosed than its parental strain, but halted bacterial replication, and protected against *M. tuberculosis* challenge similarly to unmodified BCG.

KEY WORDS: tuberculosis; vaccine; c-di-GMP

THE WORLDWIDE TUBERCULOSIS (TB) burden remains at close to 9 million new cases per year, with an annual estimated death toll close to 2 million.[1] It is known that the efficacy of the current vaccine, *Mycobacterium bovis* bacille Calmette-Guérin (BCG), which confers better protection against meningitis and miliary TB than against other forms of TB, is variable (0% to 80%).[2] As a means of improving the current BCG vaccine, BCG has been genetically modified and tested in new vaccine candidates. For example, Δ*ureC*:*Hly* (VPM1002), which expresses listeriolysin (Hly) derived from *Listeria monocytogenes*, enables BCG to escape from the endosome.[3] A Phase I clinical trial has been completed, and a dose-escalation randomized controlled trial comparing VPM1002 and BCG in healthy adults in South Africa is ongoing.[4] Other approaches for vaccine development have recently been reviewed[4] and will not be further discussed here.

In 2005, a BCG transposon mutant in *Rv1357c* was reported to have attenuated replication within macrophages[5] (the actual gene was *BCG1419c*, as the mutant was created in BCG). It was not known whether *BCG1419c* transcription was eliminated or enhanced as a consequence of tranposon insertion (Graham Stewart, personal communication). *Rv1357*c encodes for a cyclic diguanosine monophosphate (c-di-GMP) phosphodiesterase.[6] We hypothesized that by expressing *Rv1357c* in multiple copies, we might confer on BCG the capacity to augment its antigen-presenting capabilities in vivo by extending its intra-macrophage fitness, thus improving its vaccine efficacy.

The Committee of Ethics from Instituto Salvador Zubirán approved all experiments performed with mice.

We aimed to construct a recombinant modified BCG strain by cloning the polymerase chain reaction (PCR) amplified *Rv1357c* gene under the control of its native promoter. For this, we used *M. tuberculosis* H37Rv genomic DNA and primers Rv1357c-5F (5′-ACGTAAGCTTTAGCGCGTAACT-3′, HindIII site underlined) and Rv1357c-3R (5′-CGGGGTAATCGA ATGGATCA-3′), to amplify the gene using Pfu Ultra High Fidelity (Agilent Technologies, Wilmington, DE, USA), digesting with HindIII and SspI and ligating into the HindIII and PvuII sites of pMV261. The resulting plasmid was named pMF261Rv1357c; the identity and fidelity of the amplified gene were confirmed by DNA sequencing, after which transformation was performed in *M. bovis* BCG Pasteur 1173P2 by electroporation, resulting in a strain referred to throughout this work as Pasteur+Rv1357c. We grew triplicate cultures of BCG Pasteur and Pasteur+ Rv1357c as surface pellicles in 7H9 oleic acid albumin dextrose complex media for 2 weeks at 37ºC and 5%CO_2, isolated RNA, synthesized cDNA using 60 ng total RNA as template, and calculated expression values of *BCG1419c* (primers BCG1419c-F, 5′-CCGCGACCAGGTCAACA-3′, BCG1419c-R,

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5′-GCCACGCGCATCGAA-3′, probe FAM-TCGCCA TTATCTGTCC) relative to *sigA* (primers SA-F, 5′-TGGCGTTTCTCGACCTGATC-3′, SA-R, 5′-GGA GAACTTGTACCCCTTGGTGTA-3′, probe 5′-VIC-CGGTGGAGAAGTTC-3′) by real-time qPCR using Taqman Universal PCR master mix (Applied Biosystems, Foster City, CA, USA) with the following parameters: 1 cycle at 50°C for 2 min, 1 cycle at 95°C for 10 min, and 28 cycles at 95°C for 15 s and 60°C for 1 min. Reactions were run in a StepOne thermocycler (Applied Biosystems) using 6 ng cDNA, and expression values were determined as 2-ΔΔCt, as described.[7] We found the expression of *Rv1357c* to be 3.6 ± 1.33 higher in Pasteur+Rv1357c than in the Pasteur parent strain.

We compared the capacity of both strains to grow in J774A1 murine macrophages by infecting them as already described,[8] eliminating bacteria not internalized after 2 h by replacing the culture media. We used 5.2×10^6 colony-forming units (cfu; Pasteur) or 5.4×10^6 cfu (Pasteur+Rv1357c) with a multiplicity of infection 10:1, and found that although the inocula were not different, Pasteur+Rv1357c was significantly more phagocytosed at time zero (Figure 1, $P = 0.008$, Student's *t*-test, double-tailed), a difference that persisted after 24 h incubation (Figure 1, $P = 0.001$, Student's *t*-test, double-tailed). Experiments were performed in triplicate, on three different occasions, with similar results. Pasteur+Rv1357c was significantly more phagocytosed than Pasteur, possibly due to the expression of surface adhesion molecules or secreted proteins that enhanced bacterial spread.

We compared the capacity of Pasteur+Rv1357c, its parent BCG, as well as other BCG strains that had shown good (Phipps) and moderate (SSI1331) protection against *M. tuberculosis* challenge according to previous results,[9] to protect against virulent *M. tuberculosis*.

We used 8000 cfu intradermally applied vaccine to promote survival among BALB/c mice (groups of 10 animals) after intratracheal challenge with 2.5×10^5 cfu of the virulent Beijing-family *M. tuberculosis* strain 27,[10] and found no difference between BCG Pasteur and its isogenic derivative harboring *Rv1357c* into pMV261, 2 months post-infection by the log-rank (Mantel-Cox) test, although both strains protected better than BCG SSI1331, with 80% survival for Pasteur- and Phipps-vaccinated mice compared to 20% survival observed with SSI1331 (Figure 2A). Likewise, both Pasteur and Pasteur+Rv1357c protected better than BCG SSI against bacterial replication in the lungs of vaccinated mice after 2 and 4 months post-infection ($P = 0.018$ and 0.015, respectively for Pasteur+Rv1357c vs. SSI); our results for BCG Pasteur are in agreement with previous findings,[9] in which SSI-vaccinated mice showed high tissue damage and delayed-type hypersensitivity response, suggesting that SSI1331 induces a high pro-inflammatory response post-challenge. The presence or absence of *Rv1357c* in BCG Pasteur made no difference (Figure 2B, $P = 0.4$ and 0.3, respectively).

In conclusion, despite increased mycobacterial phagocytosis by macrophages created by the multiple copies of *Rv1357c*, its vaccine properties were not altered, either due to the fact that the expression of protective antigens in the recombinant strain were not modified, or due to a possible growth defect in vivo of this strain, which might have hindered antigen presentation in a more efficient manner. Plasmid is stable in vivo for at least 4 weeks after BALB/c intravenous infection, as the strain grows on plates containing kanamycin at 25 μg/ml after this post-

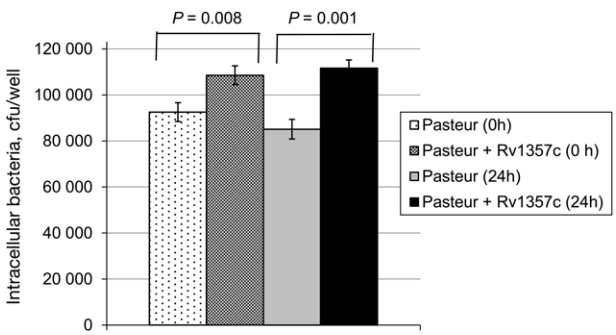

Figure 1 Mycobacterial phagocytosis by J774A.1 murine macrophages is enhanced by the presence of multicopies of the *Rv1357c* gene. Experiments were performed in triplicate, and were repeated on three different dates with similar results. Error bars indicate standard deviation. cfu = colony-forming units.

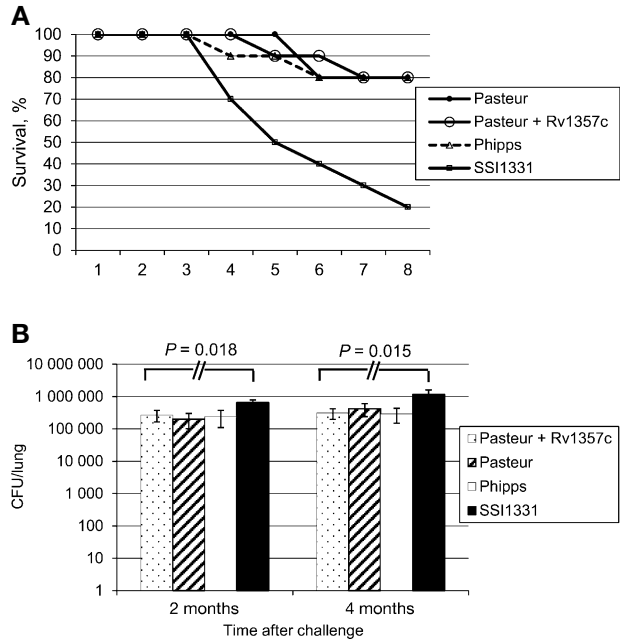

Figure 2 The presence of multicopies of the *Rv1357c* gene results in similar protection to that produced by the parent BCG. **A.** Mouse survival at the indicated time points (weeks). **B.** Replication in lungs of *M. tuberculosis* Beijing, strain 27, 2 and 4 months post-infection, in different BCG-vaccinated groups.

infection interval (Flores-Valdez et al., in preparation). Immune pathways were not analyzed in this study, given that protection markers were not significantly affected by the change induced in the parent strain. It would be worthwhile comparing our modified strain in other available TB models (aerosol, intravenous, guinea pigs, C57BL/6, hybrid mice) that differ from the intratracheal exposure in BALB/c mice used in the present work, as well as determining the strain's in vivo replication and antigen repertoire.

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RÉSUMÉ

En dépit de la disponibilité du vaccin du bacille Calmette Guérin (BCG) *Mycobacterium bovis*, la tuberculose (TB) reste un problème mondial de santé publique. Dans cette étude nous avons introduit le gène c-di-GMP phosphodiestérase *Rvl357c* (impliqué dans la régulation de la réplication mycobactérienne au sein des macrophages) dans le BCG Pasteur et nous avons testé la souche qui en résulte pour sa capacité d'être utilisée comme vaccin contre la TB dans un modèle murin. Le BCG modifié a été phagocyté davantage que sa souche parentale, mais a arrêté la réplication bactérienne et a donné une protection similaire à celle du BCG non modifié dans un test de provocation par *M. tuberculosis*.

RESUMEN

La tuberculosis (TB) sigue siendo un problema de salud pública mundial, a pesar de disponerse de una vacuna, *Mycobacterium bovis* bacille Calmette Guérin (BCG). En este trabajo, introdujimos al gen *Rv1357c*, que codifica para una fosfodiesterasa de c-di-GMP, implicada en regular la replicación micobacteriana en macrófagos, hacia BCG Pasteur, y comparamos la capacidad de esta nueva vacuna contra la TB en un modelo murino. La cepa BCG modificada fue fagocitada en mayor proporción que la cepa parental, a pesar de lo cual no hubo diferencias respecto a BCG sin modificar, tanto en la capacidad de detener la replicación micobacteriana como en la supervivencia de ratones vacunados, después del reto con *M. tuberculosis*.