Disruption of the SapM locus in *Mycobacterium bovis* BCG improves its protective efficacy as a vaccine against *M. tuberculosis*

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Mycobacterium bovis bacille Calmette-Guerin (BCG) provides only limited protection against pulmonary tuberculosis. We tested the hypothesis that BCG might have retained immunomodulatory properties from its pathogenic parent that limit its protective immunogenicity. Mutation of the molecules involved in immunomodulation might then improve its vaccine potential. We studied the vaccine potential of BCG mutants deficient in the secreted acid phosphatase, SapM, or in the capping of the immunomodulatory ManLAM cell wall component with α-1,2-oligomannoside. Both systemic and intratracheal challenge of mice with *Mycobacterium tuberculosis* following vaccination showed that the SapM mutant, compared to the parental BCG vaccine, provided better protection: it led to longer-term survival. Persistence of the SapM-mutated BCG *in vivo* resembled that of the parental BCG indicating that this mutation will likely not compromise the safety of the BCG vaccine. The SapM mutant BCG vaccine was more effective than the parental vaccine in inducing recruitment and activation of $CD11c^+MHC$-$II^{int}CD40^{int}$ dendritic cells (DCs) to the draining lymph nodes. Thus, SapM acts by inhibiting recruitment of DCs and their activation at the site of vaccination.

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INTRODUCTION

One-third of the world's population is infected with *Mycobacterium tuberculosis* (*M. tb*), the cause of tuberculosis (TB). The World Health Organization estimates that about 8–10 million new TB cases occur annually worldwide. TB is one of the three leading causes of death from a single infectious agent, the others being malaria and HIV-AIDS, and approximately 2 million deaths are attributable to TB annually (WHO Report, 2009).

The only currently licensed vaccine for TB is *Mycobacterium bovis* bacille Calmette-Guerin (*M. bovis* BCG), an attenuated strain of *M. bovis*, which has been administered to over 4 billion people since 1921, when it was first used. When administered at birth, *M. bovis* BCG confers consistent and reliable protection against disseminated disease in the first decade of life (Rodrigues et al, 1993). However, the protection conferred against pulmonary TB in adolescents and adults is much more

variable (Colditz et al, 1994). The most clinically advanced current approach for developing more effective prophylactic TB vaccines is to prime with *M. bovis* BCG and boost some time later with selected immunodominant antigens in the form of proteins or viral vectors. However, improvements in the priming vaccine will expectedly be necessary to accomplish the full potential of the booster vaccines as well (Barker et al, 2009; STOP-TB-Partnership, 2009).

Most work in this field is directed by the hypothesis that BCG is 'missing something' that is present in *M. tb* and that either this 'component' has to be incorporated in BCG to improve vaccine-induced protection or, conversely, that *M. tb* should be attenuated to the low virulence of BCG while preserving its immunodominant antigens. Examples of the former approach are recombinant BCG strains overexpressing immunodominant *M. tb* antigens (Horwitz & Harth, 2003; Pym et al, 2003). Examples of the latter are virulence factor knock-outs (Copenhaver et al, 2004), *M. tb* auxotrophic mutants (Samban-damurthy et al, 2005), and signal transduction mutants (Martin et al, 2006). Moreover, improving the induction of phagosome maturation and apoptosis in phagocytes (Grode et al, 2005; Hinchey et al, 2007; Velmurugan et al, 2007) is being pursued to increase cross-presentation and thus vaccine efficacy.

Compared to the BCG vaccine, a few engineered live vaccines are sufficiently promising to be tested in Phase I clinical trials or advanced preclinical work (Parida & Kaufmann, 2010; STOP-TB-Partnership, 2009). However, none of these manipulated vaccine strains is expected to provide full protection, and only a few of them have demonstrated improvement in long-term survival of experimental animals challenged with *M. tb* (Hinchey et al, 2007; Horwitz & Harth, 2003; Martin et al, 2006; Sun et al, 2009). Combinations of the above-mentioned manipulated strains are being investigated, but new manipulations that enhance the protective immune response by different, synergistic mechanisms are also needed.

All transgene-overexpressing and gene-inactivation mutants of BCG and of *M. tb* are likely to be considered as GMOs by regulatory authorities, complicating their implementation to some extent. In the safety assessment of these organisms, the safety of the parent organism is important. Therefore, it is not surprising that attenuated *M. tb* strains have raised more issues and required a more precautionary approach than derivatives of *M. bovis* BCG (Kamath et al, 2005; Walker et al, 2010). Amongst the BCG derivatives, those overexpressing a virulence gene like *Listeria monocytogenes* listeriolysin have been allowed to go into human clinical trials upon adequate preclinical safety evaluation. Nevertheless, if a *M. bovis* BCG strain that is more protective than the licensed strain could be developed by targeted inactivation of endogenous genes rather than by expression of heterologous virulence genes, safety concerns could likely be further assuaged and also the stability of the genetic manipulation could be assured in a more straightforward way. In this regard it is important to note that, as in attenuated *M. tb* (Hinchey et al, 2007), the secA2 mutation in BCG in combination with anti-oxidant gene mutations (such as SodA) enhances the BCG vaccine's efficacy in mice (Sadagopal et al, 2009), through a mechanism that involves increased induction of apoptosis in phagocytes. Experiments on long-term survival of *M. tb*-challenged animals have yet to be reported for this improved BCG derivative.

We hypothesized that a better vaccine could be developed by generating a *Mycobacterium* strain that does not inhibit phagosome maturation (Briken et al, 2004) or by removing the immunomodulatory molecules of BCG. We adopted the latter approach by removing two candidate immunomodulatory components: the secreted acid phosphatase SapM and capping of the cell wall lipoarabinomannan with α-1,2-oligomannosides (which is then called ManLAM).

We selected the secreted SapM phosphatase as a candidate because it had been indirectly implied in the continuous removal of phosphatidylinositol-3-phosphate (PI3P) from the membranes of phagosomes containing live Mycobacteria (Vergne et al, 2005), which reduces their fusion with late endosomes and thus contributes to blocking phagosome maturation. When macrophages phagocytose latex beads coated with ManLAM, it is the α-1,2-oligomannosyl capping that is essential for inducing inhibition of phagosome maturation (Fratti et al, 2003). However, it was later shown that phagocytosed mycobacteria do not need these caps to inhibit phagosome maturation (Appelmelk et al, 2008). Nevertheless, the cap structures are critical for the reported immunomodulatory properties of ManLAM: *in vitro* they skew cytokine secretion by human monocyte-derived dendritic cells (DCs) in response to LPS towards a Th2 profile, which might interfere with the induction of a protective anti-TB immune response.

Given the above indications that SapM and the ManLAM caps might affect the interactions between *Mycobacterium* and antigen-presenting cells (APC), we were motivated to study *M. bovis* BCG with mutations in these molecules as vaccines against TB.

In recent work (Batni et al, submitted for publication), we describe the generation and polymerase chain reaction (PCR)-based rapid screening of an ordered *M. bovis* BCG transposon insertion mutant library. As part of this work, we identified mutants in SapM and in the α-1,2-mannosyltransferases Mb2203 and Mb1661c. Whereas the Mb2203 mutant specifically lacks the α-1,2-oligomannosyl capping of ManLAM, the Mb1661 mutant has much lower levels of LAM rather than a specific deficiency in the capping of this molecule (Kaur et al, 2006, 2008). Therefore, the Mb2203 mutant is more suitable for studying the specific function of α-1,2-oligomannosyl capping, whereas the Mb1661c mutant (Appelmelk et al, 2008) can be used to study the effect of reduced abundance of ManLAM in the cell wall.

We evaluated these transposon insertion mutants of *M. bovis* BCG as TB vaccines in mice. We show that the SapM mutant (but not the ManLAM mutants) is a better vaccine than the parental *M. bovis* BCG strain, even when assessed in the most stringent tests (long-term survival of TB-challenged animals). While analysing this improved vaccine efficiency, we found that the SapM mutation does not act by counteracting the inhibition of phagosomal maturation in APCs (and neither does ManLAM capping mutation). Surprisingly, it acts by counteracting inhibition of DC migration to and activation in the lymph

nodes draining the subcutaneous site of BCG vaccination. Boding well for potential synergism of combined-mutation vaccine design, other validated mechanisms of action of improved live-*Mycobacterium* vaccines were not at play: when using the SapM mutant BCG, reactive oxygen species (ROS) production in APCs was not increased (Hinchey et al, 2007; Sadagopal et al, 2009) and neither was induction of autophagy (Jagannath et al, 2009).

RESULTS

The SapM mutant is more protective than *M. bovis* BCG wild-type (WT) as well as the Mb2203 and Mb1661c mutants in BALB/c mice

BALB/c mice were vaccinated subcutaneously with 10^5 CFU of the parental or mutant *M. bovis* BCG strains. Three months post-vaccination, animals were intravenously infected with 5×10^4 CFU of luminescent *M. tb* H37Rv. The mice were divided into two groups. One group was sacrificed for analysis of bacterial counts and the other was monitored for mortality and weight loss.

Mice in the first group were sacrificed 2, 4 or 8 weeks post-infection, and the number of bacteria in spleen and lungs was determined by luminometry, a validated method which correlates very well with CFU counting (Bonay et al, 1999). Parental BCG and all three mutant strains provided significant protection against growth of *M. tb*: the number of bacteria in spleen and lung were 10-fold to 100-fold lower than in the unvaccinated mice infected with *M. tb* (Fig 1A and B and Suppl. Fig 1A and B). At 2 and 4 weeks post-infection, no significant differences in lung or spleen bacterial load was found between the mutant BCG vaccinated groups and the parental BCG vaccinees. However, 8 weeks post-infection, the number of bacteria in lungs of mice vaccinated with the SapM::T mutant was about four times lower than in mice vaccinated with parental BCG ($p < 0.05$; Fig 1A), but this was not the case for the ManLAM mutants (Suppl. Fig 1A and B). This difference was mainly due to a 10-fold higher bacterial amplification between weeks 4 and 8 in parental BCG vaccinated mice compared to a stable load in the SapM vaccinees.

Mice in the second group were monitored weekly for survival and weight loss for 11 months. Vaccination with all BCG strains delayed cachexia as compared to non-vaccinated mice (Suppl. Fig 1D). Whereas unvaccinated mice started to lose weight about 15 weeks after infection, BCG-vaccinated animals kept their initial body weight for about 10 weeks longer. Afterwards, cachexia developed in BCG vaccinated mice as well, but mice vaccinated with Mb1661c::T or SapM::T mutant maintained their weight for the longest time. Vaccination with the ManLAM capping mutants or the SapM::T mutant resulted in significantly longer survival as compared to non-vaccinated mice (SapM::T: $p < 0.005$; Mb2203::T: $p < 0.01$; Mb1661c::T: $p < 0.05$), whereas vaccination with the parental BCG did not (Fig 1C and D and Suppl. Fig 1C). Moreover, mice vaccinated with the SapM mutant survived significantly longer than mice vaccinated with the parental BCG (median survival time (MST) was 32 weeks for

mice vaccinated with the SapM mutant and 26.5 weeks for mice vaccinated with parental BCG; $p = 0.015$; Fig 1C and D).

We repeated the experiment using intratracheal *M. tb* challenge 3.5 months post-vaccination. The number of bacteria in spleen and lungs was determined 4 and 8 weeks post-infection. Again, parental and all three mutant BCG strains conferred significant protection against *M. tb* as shown by 10-fold to 100-fold reduction in bacteria in spleen and lung as compared to unvaccinated TB-infected mice. Again, 8 weeks post-infection, mice vaccinated with the SapM::T mutant had three to four times less bacteria in spleens and lungs than mice vaccinated with parental BCG (Fig 1E and F), whereas this was not the case for the ManLAM mutant BCG (Suppl. Fig 1E and F). A second set of groups of mice was monitored weekly for 17 months for mortality. Again, mice vaccinated with the SapM mutant survived significantly longer than mice vaccinated with the parental BCG (MST was 56 weeks for SapM mutant vaccinees and 48 weeks for mice vaccinated with parental BCG, $p = 0.05$; Fig 1G and H). Remarkably, and as also observed in the i.v. infection experiment, the survival curves of parental BCG and SapM mutant vaccinees show a similar shape until about 40–50% of the infected mice have died. After that, the survival curve of the SapM mutant vaccinees bends off while the parental BCG vaccinees die rapidly. As a consequence, significantly more SapM mutant vaccinees than parental BCG vaccinees survive at the time-points beyond 50 weeks post-challenge ($p < 0.05$, see Fig 1H).

These results focused our attention on the SapM mutation as a basis for improving the BCG vaccine, so we report the data obtained with this mutant in the figures of the main text. To maintain clarity of these figures, we placed results for all experiments on the ManLAM mutant BCG strains in Supplementary Materials. These strains are important for drawing conclusions on the function of ManLAM capping in *Mycobacterium*–APC interactions, but they also serve as controls. In that context, they demonstrate that the SapM mutant phenotypes are specific to the mutation of SapM and not merely due to the transposon used for mutagenesis.

The SapM mutant BCG is indistinguishable from parental BCG in persistence and lung granulomatous response

To evaluate the safety of the mutant BCG strains, we tested them for parameters related to chronic mycobacterial infection. To analyse bacterial persistence *in vivo*, BALB/c mice were infected intravenously with *M. bovis* BCG or *M. bovis* BCG mutants (Mb2203, Mb1661c or SapM). The bacterial load in the lungs and spleen was determined 2, 4, and 12 weeks post-infection. Two weeks after infection, the load of the parental strain in the lung was slightly higher than the loads of the mutants. Four weeks post-infection, the mutants reached similar numbers of CFU in the lungs and spleen as the WT *M. bovis* BCG (Fig 2A and B, Suppl. Fig 2A and B). Overall, bacterial numbers in lung and spleen decreased over time, indicating partial clearance.

The uptake of mycobacteria of the *M. tb* complex by alveolar macrophages typically results in a local inflammatory response (van Crevel et al, 2002) mediated by the production of IL-12, interferon (IFN)-γ, and tumour-necrosis factor (TNF) and leads
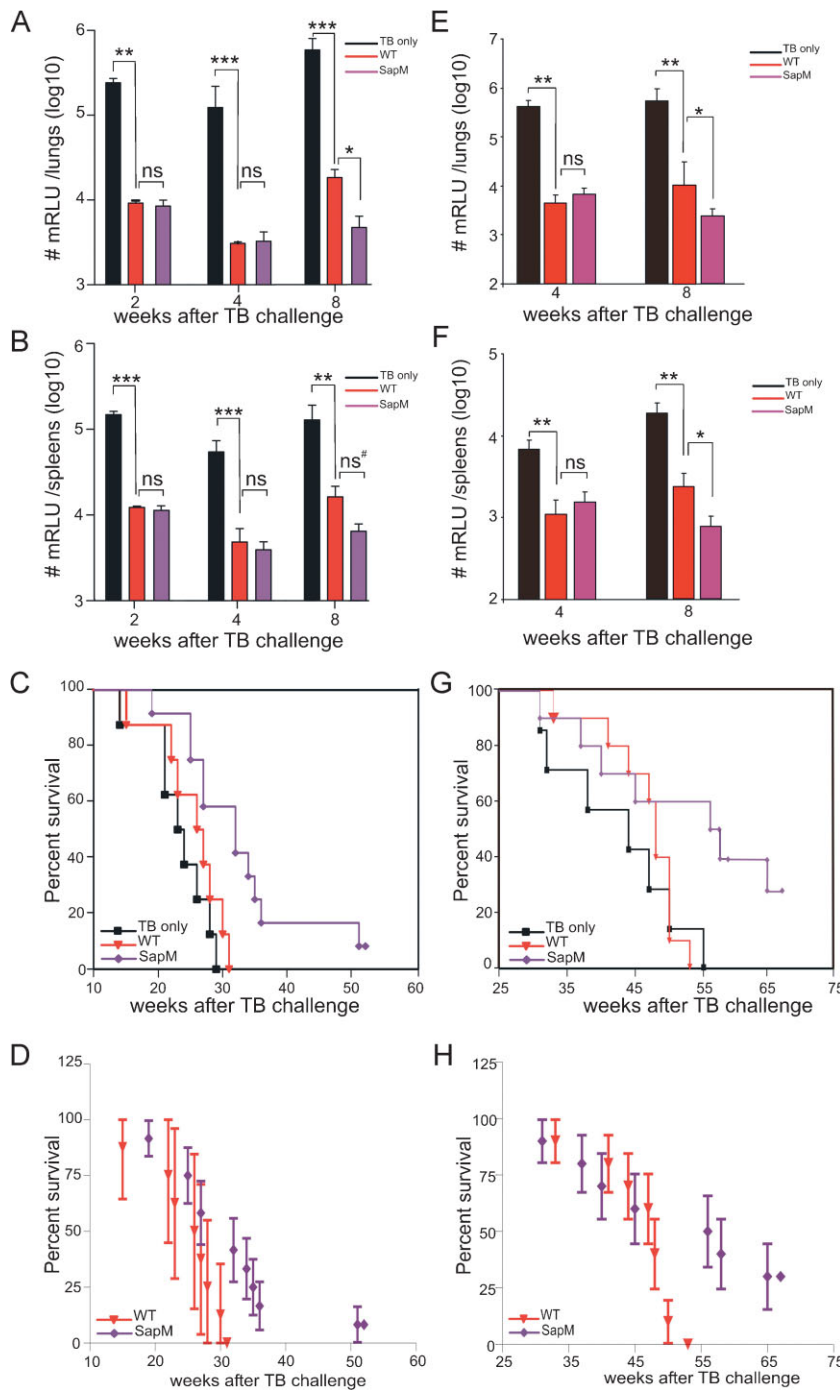
Figure 1. Protective efficacy of *M. bovis* BCG WT *versus* mutant SapM::T (Mb3338) in BALB/c mice.

A,B. BALB/c mice were vaccinated subcutaneously with the parental or mutant *M. bovis* BCG strain. Three months post-vaccination, animals were intravenously infected with luminescent *M. tuberculosis* H37Rv. Mice were sacrificed 2, 4, and 8 weeks post-infection, and the number of bacteria in lungs (A) and spleens (B) was determined by luminometry. Individual mice were evaluated for RLU, and averages and error bars of +/− 1× standard deviation (s.d.) are shown. (One-way ANOVA, Bonferroni's multiple comparison test; ***$p < 0.0001$; **$p < 0.0012$; *$p < 0.05$; ns, not significant).

C,D. Mortality of mice was monitored weekly for 11 months. The percentage of survival is shown. The mean survival time (MST) for unvaccinated mice was 23.5 weeks, and for mice vaccinated with BCG WT and SapM::T 26.5 weeks and 32 weeks, respectively ($p = 0.0145$; Kaplan–Meier, Bonferroni). We have included the 95% confidence intervals (CI) for the survival proportions in panel D.

E,F. BALB/c mice were vaccinated subcutaneously with the parental or SapM mutant *M. bovis* BCG. After 3.5 months, animals were intratracheally infected with luminescent *M. tuberculosis* H37Rv. Mice were sacrificed 4 or 8 weeks post-infection and the number of bacteria in lung (E) and spleen (F) was determined by luminometry. Individual mice were evaluated for RLU, and averages and error bars of +/− 1× s.d. are shown. (One-way ANOVA, Bonferroni's multiple comparison test; ***$p < 0.0001$; **$p < 0.0012$; *$p < 0.05$; ns, not significant; ns#, not significant with 95% CI of difference from −0.14 to 0.946).

G,H. Mortality was also monitored weekly for 17 months. The percentage of survival is shown. The MST for unvaccinated mice was 44 weeks, and for mice vaccinated with BCG WT and SapM::T, respectively, it was 48 and 56 weeks ($p = 0.05$; Kaplan–Meier, Bonferroni; Panel G). Panel H shows survival with indication of the 95% CI for the survival proportions. At the time-points beyond 50 weeks post-challenge, 95% CI on the survival proportions of SapM vaccinees do not overlap with survival proportions of the WT vaccinees, demonstrating a significant difference ($p < 0.05$).

to granuloma formation (Cooper et al, 1993; Kindler et al, 1989). Progressive granuloma formation is a hallmark of chronic mycobacterial infection, which is associated with a partial shift to Th2-type immunity (Harris et al, 2008; Jiao et al, 2003). To assess whether the lung inflammatory responses to the SapM mutant BCG and the parental BCG are different, the two types of bacteria were instilled in the trachea of mice and cytokines in the bronchioalveolar lavage (BAL) were measured 4 weeks post-infection. We detected IFN-γ and TNF but very little or no IL-2,

IL-4, IL-5, IL-6, IL-10, or IL-12p70. There was no significant difference between infection with the parental and the mutant bacteria (Fig 2C, Suppl. Fig 2C, Suppl. Table 1). To study granuloma formation, mice were vaccinated intravenously or intratracheally with parental or mutant *M. bovis* BCG. Livers and lungs were removed 3 weeks or 4 weeks post-vaccination, and granulomas were counted in paraffin sections stained with haematoxylin–eosin (H&E). Livers of mice vaccinated with Mb1661c::T or Mb2203::T had slightly fewer granulomas than
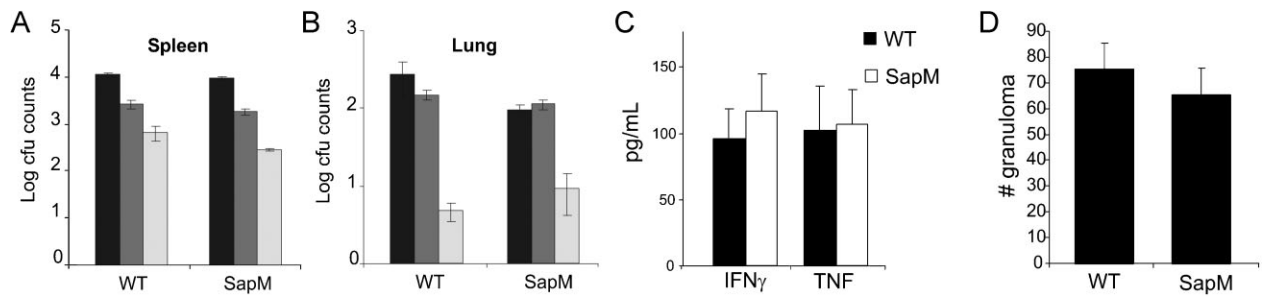
Figure 2. Bacterial replication of *M. bovis* BCG WT *versus* SapM mutant in spleen and lungs of mice and immune response in the lungs following infection.

A,B. BALB/c mice were intravenously infected with *M. bovis* BCG WT or with SapM mutant and sacrificed after 2 weeks (black bars), 4 weeks (dark grey bars) or 12 weeks (light grey bars; 5 mice/group). Lung and spleen homogenates were plated in duplicates on 7H10 agar for counting CFUs. The mean and standard errors (SEM) of each group are shown. No difference was detected between the SapM mutant and the WT ($p > 0.1$, Mann–Whitney *U*-test).

C. C57BL/6 mice were infected intratracheally with *M. bovis* BCG WT or with SapM mutant. After 4 weeks, five mice/group were killed and BAL was performed. The mean levels of TNF and IFN-γ in the BALF and SEM of each group are representative of two independent experiments. ($p > 0.05$, Mann–Whitney *U*-test).

D. C57BL/6 mice were infected intravenously with *M. bovis* BCG WT or with mutants. After 3 weeks, five mice/group were killed and livers were removed aseptically. Quantitative studies on H&E-stained paraffin sections to identify and count granuloma were performed by direct microscopic examination (3.5× magnification) and analysed with ImageJ software. The SEM of each group are shown.

mice vaccinated with *M. bovis* BCG WT or with SapM::T (Fig 2D, Suppl. Fig 2D–F).

These data show that the SapM mutant BCG strain does not persist longer than parental WT BCG in immunocompetent mice, a prerequisite for its safe use as a vaccine.

Neither inactivation of SapM nor ManLAM capping deficiency affects BCG uptake by macrophages and dendritic cells

To study how disrupting the SapM locus and creating a deficiency in ManLAM capping affects the efficiency of binding and phagocytosis of the mycobacteria, *M. bovis* BCG WT and mutants were labelled with fluorescein isothiocyanate (FITC) and their uptake by murine Mf4/4 macrophages was analysed by flow cytometry. Comparison of the percentage of FITC-positive macrophages showed that WT *M. bovis* BCG and the SapM and capping mutants were bound or taken up by murine macrophages with similar efficiency (Suppl. Fig 3A). Confocal and electron microscopy analysis demonstrated that the macrophages counted as FITC$^+$ had all taken up FITC-BCG and that the bacteria were not just attached to the cell surface (Suppl. Fig 3B–D). Measurement of the mean FITC intensities of the macrophages showed that similar amounts of bacteria were taken up under all the conditions tested (Suppl. Fig 3A). We conclude that neither SapM deficiency nor deficiency in ManLAM capping strongly affects the binding and uptake of the bacteria by macrophages.

Similarly, we analysed the uptake of the parental *M. bovis* BCG and the SapM mutant by bone marrow-derived dendritic cells (BM-DCs). Again, WT *M. bovis* BCG and the SapM mutant were taken up by murine DCs with the same efficiency (Fig 3A).

Neither inactivation of SapM nor ManLAM deficiency affects fusion of lysosomes with BCG-containing phagosomes

It has been proposed that both SapM and ManLAM affect inhibition of the maturation of phagosomes containing Mycobacteria (Vergne et al, 2003, 2005). To find out if the *M.*

bovis BCG mutations affect phagosome maturation in macrophages, we performed colocalization experiments. Mf4/4 murine macrophages were infected with FITC-labelled *M. bovis* BCG and subsequently labelled with Lysotracker Red. We examined colocalization of BCG-containing phagosomes with
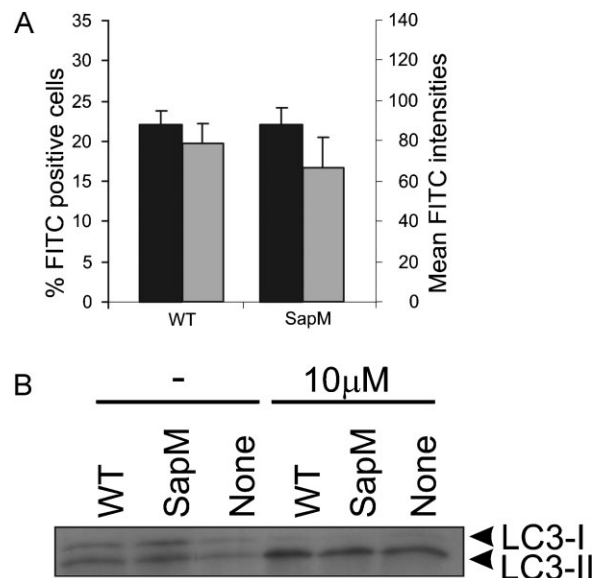

Figure 3. Uptake of *M. bovis* BCG WT and SapM mutants by BM-DCs and autophagy induction upon infection.

A. BM-DCs were infected for 24 h with FITC-labelled mycobacteria (*M. bovis* BCG WT or SapM::T mutant, as indicated) at a MOI of 2. The number of FITC-positive DCs and mean FITC intensities are shown as averages and SEM of five independent experiments.

B. BM-DCs (either pretreated with 10 µM rapamycin or not treated) were infected with *M. bovis* BCG WT or with SapM mutant at MOI of 2 and incubated at 37°C. After 24 h, cells were lysed and the expression of LC3 was revealed by western blot.

lysosomes by confocal microscopy (Suppl. Fig 4A). No significant differences in colocalization of lysosomes with FITC-containing phagosomes were seen between infection with the parental strain and any of the mutants. These findings were confirmed by analysing the survival of the SapM, Mb2203, and Mb1661c mutants in macrophages: survival and replication of *M. bovis* BCG mutants in Mf4/4 macrophages was not significantly different from that of the WT during 48 h after infection (Suppl. Fig 4B).

Infection with parental and mutant BCG strains induces similar ROS levels in macrophages

The ability of mycobacteria to survive in the hostile environment of macrophages also depends on their ability to scavenge ROS. Importantly, this scavenging of ROS species appears to block the induction of apoptosis of phagocytes, which reduces mycobacterial antigen cross-presentation. It was recently shown that immunogenicity of *M. bovis* BCG can be enhanced by reducing the activity and secretion of microbial antioxidants (Sadagopal et al, 2009). Therefore, we compared intracellular ROS production following infection of macrophages with *M. bovis* BCG WT or with mutants. We detected ROS production by means of dihydro-dichloro-fluorescein diacetate (CM-H_2DCFDA), a cell-permeant indicator of ROS that becomes fluorescent when the acetate groups are removed by intracellular esterases and ROS-mediated oxidation of the molecule within the cell. We document an increase in ROS production over time post-infection (Suppl. Fig 5A), but we observed no significant differences between *M. bovis* BCG WT and the mutants.

Parental and mutant BCG strains induce similar levels of autophagy in APCs following infection

Autophagy, which is a bulk protein and organelle degradation process essential for cell maintenance and viability, can enhance the efficacy of BCG as a vaccine by increasing antigen presentation in mouse DCs (Jagannath et al, 2009). Conversion of microtubule-associated protein 1 light chain 3 (LC3)-I to LC3-II was used to monitor autophagic activity following infection of macrophages and BM-DCs with *M. bovis* BCG WT or with mutants. We could not observe any difference in conversion to LC3-II upon infection with WT *versus* the BCG mutants (Fig 3B and Suppl. Fig 5B and C), which indicates that the improvement in vaccine efficacy cannot be attributed to a stronger induction of autophagy in APCs.

Effect of the SapM::T mutation on the activation of iNKT cells

Immunization with a BCG strain in which α-galactosylceramide (αGalCer), a potent NKT cell activator, is incorporated, stimulates maturation of DCs, augments the priming of Ag-specific CD8$^+$ T cell responses, and strengthens protection against challenge with *M. tb* (Venkataswamy et al, 2009). We analysed iNKT hybridoma cytokine responses and cell surface marker expression following coculture with BM-derived DCs (WT or CD1d$^{-/-}$) infected with *M. bovis* BCG (parental or SapM mutant). Before coculture, DCs were loaded with αGalCer or left unloaded. Coculture of iNKT hybridoma cells with DCs infected

with *M. bovis* BCG SapM::T yielded levels of IL-2 and IL-12p40 in the supernatant similar to those in coculture with DCs infected with *M. bovis* BCG WT. IL-2 production was strictly CD1d dependent, since IL-2 levels were below the limit of detection when DCs from CD1d$^{-/-}$ mice were used. This was not the case for the production of IL-12p40 (Suppl. Fig 6A). Expression analysis of activation surface markers on DCs (MHCII, CD80, CD86, PD-L1, and CD40) did not reveal differences between WT- and SapM::T-infected DCs. Expression of activation surface markers was also not strictly dependent on CD1d (Suppl. Fig 6B). Similarly, the expression of T-cell antigen receptor (TCR-β) and the activation marker CD69 in the iNKT hybridoma did not change significantly (Suppl. Fig 6C). These results are also in accordance with the findings that lipid and mycolic acid composition are identical in the parental *M. bovis* BCG and mutant strains (Batni et al, submitted for publication).

Ag85A and PPD-specific immune responses following vaccination with parental or with mutant BCG strains

We assessed T-cell immunity by comparing production of antigen-specific IFN-γ by splenocytes in response to restimulation with recAg85A (encoded by Rv3804c), the I-E^d restricted synthetic 20-mer peptide spanning amino acids 101–120 of Ag85A (p11) (Denis et al, 1998), and purified protein derivative (PPD). This was done 4 weeks after vaccination of BALB/c mice with the parental or SapM mutant BCG strain (Suppl. Fig 7). Cultured spleen cells from BALB/c mice vaccinated with the WT BCG or with the SapM locus mutant secreted substantial amounts of IFN-γ, demonstrating a Th1 response. However, no significant differences in the IFN-γ levels were observed between parental and SapM mutant vaccinees.

The SapM mutant BCG induces higher levels of pro-inflammatory, Th1-polarizing cytokines than parental BCG following infection of BM-DCs

To begin to assess whether the SapM mutation alters DC-mediated immune responses, BM-DCs were infected with parental *M. bovis* BCG or with its SapM mutant derivative for 6 h, after which the bacteria were washed away. After 24 and 48 h, the levels of IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12p40, IFN-γ, and TNF-α were measured in the BM-DC culture medium. The levels of IL-2, IL-4, IL-10, and IFN-γ, if any, were below the detection limit of our assay. However, production of TNF-α increased more than 10-fold at 24 h and was accompanied by robust increases in IL-1β and IL-6 (threefold and fourfold higher than with parental BCG) and a modest increase in IL-12p40 (Fig 4). These results demonstrate robust BM-DC activation and an augmented pro-inflammatory, Th1-skewing cytokine response.

Vaccination with the SapM locus mutant increases recruitment and activation of DCs at the draining lymph nodes

Following subcutaneous injection of BCG, local DCs are the primary host cells and they initiate adaptive immune responses. Differences in DC activation and/or trafficking to draining lymph nodes could have a major impact on the initiated immune
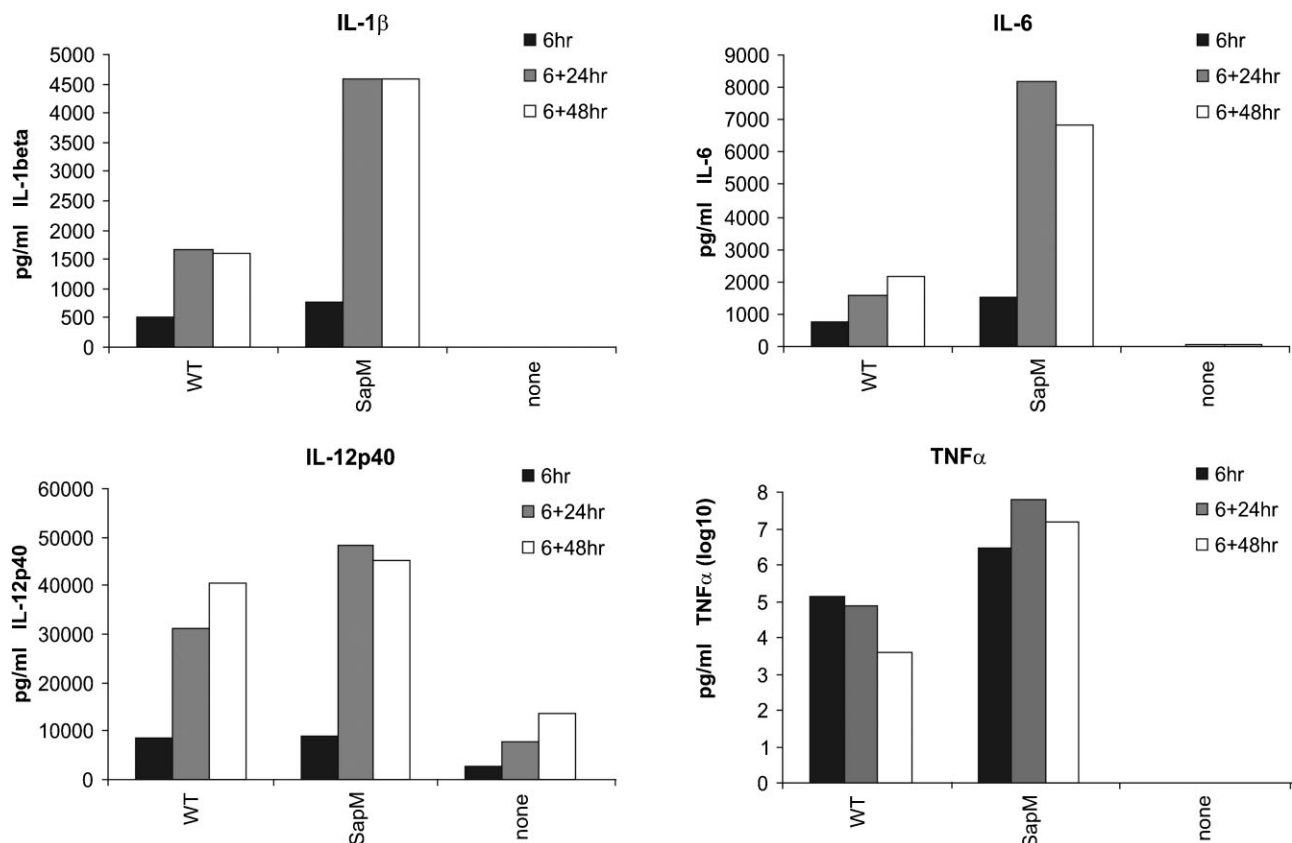
Figure 4. Cytokine production following infection of BM-DCs with *M. bovis* BCG WT or with SapM::T. BM-DCs were infected for 6 h with *M. bovis* BCG WT or with mutants at a MOI of 2. Six hours later, bacteria were washed away, fresh medium was added, and incubation was continued at 37°C for 24 or 48 h. At the indicated time points, supernatant was removed and analysed for the presence of cytokines by flow cytometry. This experiment is representative of three independent experiments.

response and on subsequent protection against *M. tb*. In this context, and following up on the *in vitro* lead that the SapM mutation induces much stronger pro-inflammatory cytokine responses in DCs, we studied the CD11c$^+$ DC cell population in the lymph nodes draining the site of s.c. immunization with the parental *M. bovis* BCG or with the SapM mutant. BALB/c mice were infected subcutaneously at the base of the tail with 10^6 CFU of the parental or the *M. bovis* BCG SapM mutant strain and sacrificed on days 1 and 7 post-infection. The infiltration of DCs and their activation status in the inguinal and brachial/axillary lymph nodes was examined by flow cytometry. On day 7 post-infection, the number of CD11c$^+$ cells (DCs) was clearly increased in the inguinal lymph nodes (ILNs; Fig 5A) and axillary/brachial lymph nodes (A/B LNs; Suppl. Fig 8A) of mice infected with the *M. bovis* BCG SapM mutant compared to the control group, but not in the mice infected with *M. bovis* BCG WT. A closer examination of mice infected with BCG WT and those infected with the BCG SapM mutant revealed a shift in the DC population towards a MHC-II^{int}CD40^{int} phenotype, mostly CD8a$^-$ cells, indicating swift migration of DCs to the draining lymph nodes (Fig 5B-6, Suppl. Fig 8B-9; Manolova et al, 2008). However, this population was much more abundant (from day 1 post-infection) in the ILNs in mice infected with *M. bovis* BCG

SapM compared to those infected with *M. bovis* BCG WT. In the A/B LNs, the increase in MHC-II^{int}CD40^{int} DCs was significantly different only on 7 days post-infection and was less prominent, possibly because A/B LNs lie further away from the site of vaccination. Besides increased recruitment of CD8a$^-$MHC-II^{int}CD40^{int} DCs to the draining lymph nodes upon vaccination with *M. bovis* BCG SapM, this population also displayed increased expression of CD80 and CD86 than obtained with parental BCG, demonstrating improved activation (Fig 6, Suppl. Fig 9). We conclude that vaccination with the *M. bovis* SapM mutant augments recruitment of DCs to the draining lymph nodes and their activation, which is a well-established prerequisite for effective BCG antigen presentation (Marino et al, 2004) and initiation of adaptive immunity.

Vaccination with the SapM locus mutant increases recruitment of inflammatory DCs at the draining lymph nodes

We further explored the CD11c$^+$ DC subtypes in the lymph nodes draining the site of s.c. immunization with the parental *M. bovis* BCG or with the SapM mutant. BALB/c mice were again infected subcutaneously at the base of the tail with 10^6 CFU of the parental or the *M. bovis* BCG SapM mutant strain and
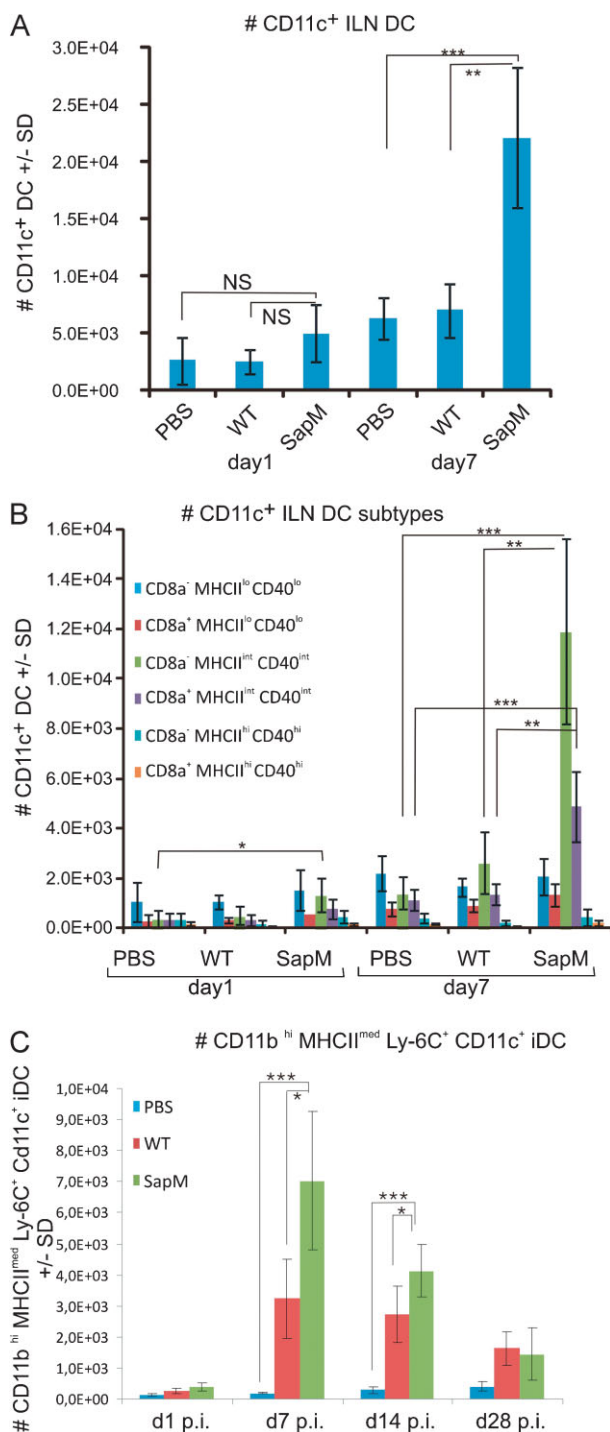
A. The number of CD11c$^+$ cells in the total ILN population.

B. Number of CD11c$^+$ ILN DC subtypes.

C. BALB/cJ mice were immunized subcutaneously at the base of the tail with *M. bovis* BCG WT or *M. bovis* BCG SapM mutant. One, 7, 14, and 28 days later, six mice/group were sacrificed, and ILNs were removed. Cells were prepared, labelled with different antibodies staining iDCs and analysed by flow cytometry. (Kruskal–Wallis, Bonferroni's multiple comparison test; $^{***}p < 0.005$; $^{**}p < 0.01$; $^*p < 0.05$; ns, not significant).

Figure 5. Trafficking and activation of DCs. BALB/cJ mice were infected subcutaneously with *M. bovis* BCG WT or *M. bovis* BCG SapM mutant. One and 7 days later, five mice/group were sacrificed, and ILN were removed. Cells were prepared, labelled with the different antibodies and analysed by flow cytometry.

sacrificed on days 1, 7, 14 and 28 post-infection. The infiltration of DCs in the ILNs was examined by flow cytometry. On days 7 and 14 post-infection, the number of CD11b^{hi}MHCII^{med}Ly-6C$^+$CD11c$^+$ inflammatory DCs (iDCs) was clearly increased in the ILNs (Fig 5C) of mice infected with the *M. bovis* BCG SapM mutant compared to the control group, but not in the mice infected with *M. bovis* BCG WT, demonstrating that vaccination with the *M. bovis* SapM mutant augments recruitment of iDCs to the draining lymph nodes.
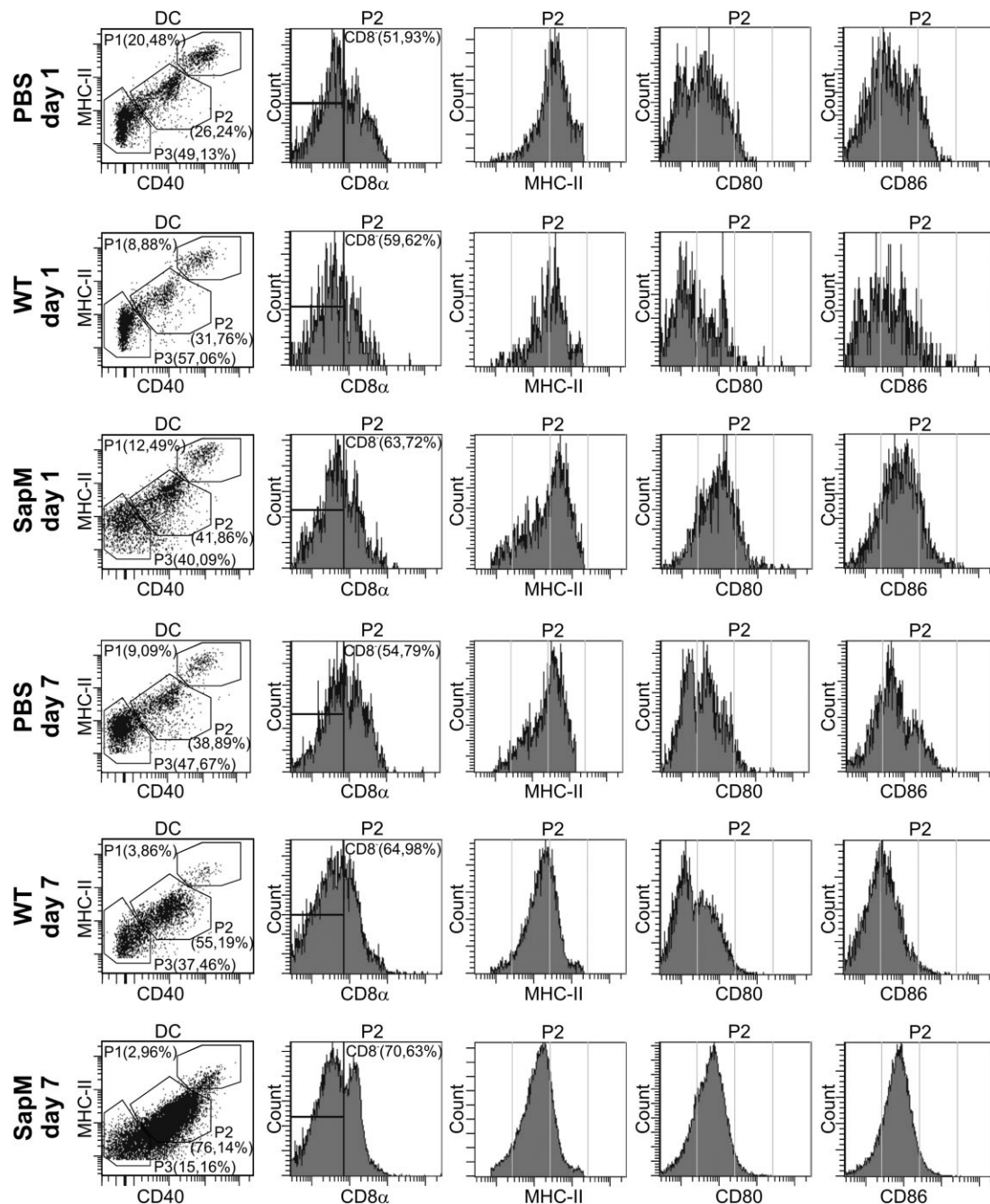
DISCUSSION

We demonstrate how a single null mutation in *M. bovis* BCG can result in a TB vaccine that provokes an immune response, which provides improved long-term survival upon both systemic and intratracheal challenge with *M. tb*. It is important to note that previously reported live TB vaccines that are currently in phase I clinical trials resulted in similar but not longer survival in mouse models (Hinchey et al, 2007; Sun et al, 2009). Moreover, these other vaccine improvements mainly delay mortality of the vaccinated mice but have very limited effect on the time at which all mice died. In contrast, onset of mortality in mice vaccinated with the SapM mutant BCG was very similar to that obtained with the WT BCG, but the survival curve is remarkably biphasic and the rate of mortality was substantially reduced later in the experiment. Consequently, some mice survived for a very long time after challenge. Although the parental BCG strain provided efficient protection in terms of reduced TB load early after infection, it did not significantly extend the survival of vaccinated mice, which is different from several other such studies. This is very likely due to differences in the *M. bovis* BCG substrains used and indeed vaccine efficacy variability amongst BCG substrains has been amply described in the literature (Castillo-Rodal et al, 2006).

Our observations bide very well for a next-generation vaccine in which the SapM mutation is incorporated in the improved BCGs (or attenuated *M. tb* strains). Such a vaccine would result in better initial control of the pathogen by a qualitatively enhanced immune response and the protection would be prolonged by the improved long-term protective mechanisms made possible by the SapM mutation. We are currently working on such combined-engineering BCG vaccines.

In terms of the safety of the SapM-mutated BCG strain, our *in vitro* studies showed that the three mutant BCG strains survived and replicated indistinguishably from parental BCG in macrophages. Analysis of bacterial replication in immunocompetent mice did not show any difference between parental and SapM mutant BCG. The number of granulomas formed in livers and lungs post-infection was also unaffected. From the assembly of these data we conclude that the safety of these mutated BCG strains in immunocompetent mice is equivalent to that of parental BCG. Further studies in immunodeficient mice will certainly be required before any clinical testing can be performed.

Figure 6. Phenotype and activation of CD11c^{+} DCs in ILN. The samples described in Fig 5A and B were further characterized. Representation of MHCII^{lo} CD40^{−} (P3), MHCII^{int} CD40^{int} (P2), MHCII^{hi} CD40^{hi} (P3) populations (**left column**). Representation of expression levels of CD8α, MHCII, CD80, and CD86 on cells in P2.

Remarkably, our results with *in vitro* infection of macrophages and BM-DCs strongly suggest that neither SapM nor ManLAM capping are necessary for arrest of phagosome maturation, even though this had been inferred from previous studies. However, the previous studies did not make use of mutant bacterial strains deficient in the molecules concerned. Our results are in agreement with the study by Appelmelk et al

(Appelmelk et al, 2008), who did not detect a defect in phagosome maturation arrest in their ManLAM capping-deficient strains.

In our mechanistic analysis of the improved protection induced by the SapM mutant BCG vaccine, we systematically analysed the different correlates of protection and the mechanisms that had been implicated in improved protection against

TB. The SapM mutation did not enhance ROS production in phagocytes, did not enhance autophagy in macrophages or BM-DCs, did not result in increased IFN-γ secretion by restimulated splenocytes and did not enhance iNKT cell responses to CD1d-mediated glycolipid antigen presentation. However, we found that the SapM mutated BCG elicited strongly enhanced secretion of TNF-α, IL-6, and IL1-β from BM-DCs. Accordingly, this improved vaccine mediated a robust increase in recruitment of DCs and their activation in the lymph nodes draining the site of vaccination, which resulted in a four to sixfold increased frequency of CD11c$^+$MHC-II^{int}CD40^{int} DCs, 7 days after vaccination. The CD11c$^+$MHC-II^{int}CD40^{int} CD8$^-$ DCs were also fully activated (CD80$^+$ and CD86$^+$), and we further phenotyped them as mainly iDCs being CD11b^{hi}MH-CII^{med}Ly-6C$^+$CD11c$^+$. We conclude that vaccination with the SapM mutant BCG probably enhances antigen presentation in a pro-inflammatory, Th1 skewing environment. Also in the context of DC-based cancer vaccines, it is generally accepted that reinjected DCs loaded with tumour antigens must be able to migrate to lymph nodes, encounter naive T cells and activate them towards a Th1 phenotype to induce efficient anti-tumoural cytotoxic responses (Gilboa, 2007; Schuler et al, 2003; Wang, 2001).

The SapM mutant BCG appears to achieve its enhanced protective efficacy by the novel mechanism of enhancing pro-inflammatory signalling in the DCs that phagocytose it. Thus, SapM secretion might have evolved as an important immuno-modulatory capability of Mycobacteria. It is intriguing that SapM is a phosphatase that might affect intracellular signal transduction cascades, although it is entirely unclear how the SapM protein could gain access to the DC cytoplasm, as BCG has been reported not to escape from the phagosome, in contrast to *M. tuberculosis*.

Our observations are also of interest in view of BCG genome evolution (Brosch et al, 2007; Kernodle, 2010). A region of the BCG genome called DU2 is prone to duplication and subsequent deletion events. The exact delineation of the duplicated region differs amongst the substrains of BCG, but it is striking that the SapM gene (Mb3338) belongs to a narrow genomic region, which is duplicated in all BCG substrains except the Pasteur lineage, in which the duplication of the SapM genomic region has been lost again. It will be most interesting to study the relevance of this SapM duplication for BCG vaccine efficacy.

In conclusion, we propose the inclusion of the SapM loss-of-function mutation in the current community endeavour to generate an improved priming vaccine based on live *Mycobacterium* for two main reasons: it has a strong potential for enhancing the protective immunity against TB, and it acts through a mechanism that differs from those involved in vaccines currently under investigation.

MATERIALS AND METHODS

Mycobacterial strains
M. bovis BCG 1721 (Master et al, 2008), a derivative of *M. bovis* BCG Pasteur, carrying a non-restrictive rpsL alteration was used as parental strain throughout these studies. *M. bovis* BCG WT and the mutants were grown in Middlebrook 7H9 broth (Difco) supplemented with 0.05% Tween-80, Middlebrook Oleic acid–albumin–dextrose–catalase (OADC supplement, Becton Dickinson) and antibiotics (100 μg/ml streptomycin for WT and additionally 50 μg/ml kanamycin for the mutants) when grown in liquid culture. The mutants are described in detail in Batni et al (submitted for publication).

Generation of bone marrow-derived dendritic cells
BM cells were cultured for 8 days in DC culture medium (RPMI 1640 containing GlutaMAX-I, supplemented with 5% v/v fetal calf serum (FCS), 50 μM β-mercaptoethanol and 20 ng/ml GM-CSF (produced in house). This procedure is adapted from (Lutz et al, 1999).

Laboratory animals
Female C57BL/6 mice and BALB/c mice were purchased from Janvier (France) and housed under specific pathogen-free conditions in microisolator units. At the beginning of the experiment, the mice were 7–8 weeks old. All experiments were approved by and performed according to the guidelines of the animal ethical committee of Ghent University and Scientific Institute of Public Health (SIPH), Belgium.

Vaccination with *M. bovis* BCG and challenge with *M. tb*
Female BALB/c mice were vaccinated subcutaneously with 10^5 of the parental or mutant *M. bovis* BCG strains grown on 7H9-OADC medium (30 mice/group). They were challenged intravenously 3 months after BCG vaccination with 5×10^4 CFU luminescent *M. tb* H37Rv grown for 2 weeks on liquid 7H9-OADC medium. For the intratracheal challenge, mice were rested for 3.5 months post vaccination, anaesthetized and instilled intratracheally with 2×10^5 CFU of luminescent *M. tb* H37Rv in 70 μl of phosphate buffered saline (PBS), as reported (Korf et al, 2009). Bacterial replication in spleen and lungs was monitored by luminometry. Mice were sacrificed on weeks 2, 4 and 8 post-infection (4–5 mice/group), as described in results. Luminescence was measured in a Turner Design Luminometer as flash emission (15 s integration time) using 1% *n*-decanal (Sigma) in ethanol as substrate. In this assay, only live bacteria are counted because emission of light is dependent on the presence of reduced flavin mononucleotide (FMNH$_2$), a co-factor that is found only in living cells. At least 10 additional mice/group were monitored for weight loss and mortality, as reported before (Romano et al, 2006).

Replication of *M. bovis* BCG in mice
At the age of 8 weeks, female BALB/cJ mice (Janvier) were infected intravenously with 1×10^6 CFU *M. bovis* BCG WT or mutants in 200 μl PBS. After 2, 4 and 12 weeks, five mice/group were sacrificed; lungs and spleens were removed aseptically and homogenized in PBS. Neat, 1/10 and 1/500 dilutions were made, 100 μl was plated in duplicate on 7H10-OADC agar and the colonies were counted after 21 days. The CFU counts were calculated from the highest dilution showing distinct colonies.

Granuloma formation and cytokine measurements following infection of mice with *M. bovis* BCG
C57BL/6 mice were anaesthetized and infected intravenously with 1×10^6 CFU or intratracheally with 0.5×10^6 CFU of *M. bovis* BCG WT or with mutants. After 3 and 4 weeks, five mice/group were sacrificed

The paper explained

PROBLEM:

One-third of the world's population is infected with *M. tb*, the etiologic agent of tuberculosis (TB). The World Health Organization estimates that about 8 to 10 million new TB cases occur annually worldwide. Together with malaria and HIV-AIDS, TB is one of the three leading causes of death from a single infectious agent, and approximately 2 million deaths are attributable to TB annually. Currently, only one vaccine is licensed: *M. bovis* BCG, an attenuated strain of *M. bovis*. Over the decades, more than 3 billion people have received this vaccine, typically in the first hours of life. Whereas the BCG vaccine protects against disseminated disease in childhood, it provides only partial protection at best against pulmonary TB in adolescents and adults.

In recent years, several other live candidate vaccines have been developed and some of them are being tested in preclinical and clinical trials. Only a few of them resulted in a modest improvement in long-term survival of experimental animals challenged with TB and further improvements are clearly desirable. We reasoned that inactivating immunomodulatory strategies of the live *M. bovis* BCG vaccine would be a promising avenue to generate an improved vaccine. Such strategy would make use of gene-knockouts, and such manipulations can be made in a genetically very stable way.

RESULTS:

To test the approach, we evaluated transposon insertion mutants of *M. bovis* BCG as TB vaccines in the mouse model and show that a mutant *M. bovis* BCG deficient in the secreted acid phosphatase SapM is a better vaccine than the parental *M. bovis* BCG strain. Vaccination of mice with the SapM mutant resulted in better protection than obtained with the parental BCG, both upon systemic and intratracheal challenge with *M. tb*, resulting in improved long-term survival. Remarkably, we observed that the SapM mutated BCG provides improved resistance to TB lethality especially at late time points after challenge, which is a very rare but also very desirable feature in this field.

Mechanistically, the SapM mutant BCG vaccine induces an increased recruitment and activation of iDCs to the draining lymph nodes as compared to parental BCG. This shows that the secreted SapM protein acts as an inhibitor of DC recruitment to and activation at the site of vaccination. The SapM mutant BCG thus appears to achieve its enhanced protective efficacy through a novel mechanism, *i.e.* by enhancing pro-inflammatory signalling in the DCs that phagocytose the vaccine.

IMPACT:

Since our vaccine acts through a different mechanism than already exploited in current designs, it has strong potential of further enhancing the obtained protective immunity against TB. Incorporation of the SapM mutation in strains that achieve better control of a TB challenge through other described mechanisms might lead to a truly protective TB vaccine.

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and livers and lungs were removed aseptically, fixed in 3.7% paraformaldehyde, and embedded in paraffin for thin sectioning. General morphology and inflammation were examined by H&E staining (Merck, VWR International). Quantitative studies were performed by direct microscopic examination and analysis with ImageJ software. Bronchoalveolar lavage (BAL) was performed 4 weeks post-infection, and cytokine levels in BAL fluid were determined using Cytometric Bead Array and Flex sets according to the manufacturer's instructions (BD Biosciences).

Flowcytometric analysis of binding/uptake of mycobacteria by macrophages and BM-DCs

Log-phase *M. bovis* BCG WT and mutants (OD = 0.8–1.2) were FITC-labelled by resuspending the bacterial pellet (10 mg wet weight) in 200 µl of FITC solution (0.1 mg/ml FITC isomer 1 (Sigma–Aldrich) diluted in 0.1 M NaHCO₃, pH 9), and incubating the suspension at 25°C for 1 h with gently shaking. Bacteria were then washed in PBS until the supernatant was clear of residual FITC. The bacteria were used for infection of BM-DCs at a multiplicity of infection (MOI) of 2 and incubated for the indicated durations at 37°C. Infected BM-DCs were centrifuged for 10 min at $150 \times g$ and washed in PBS. The number of FITC-positive BM-DCs was determined in FL1 on a FACScalibur flow fluorocytometer (Becton Dickinson) equipped with a 488-nm argon ion laser.

Induction of autophagy

To analyse autophagy following *M. bovis* BCG infection of BM-DCs, these cells were infected with *M. bovis* BCG WT or with mutants at MOI of 2 for 24 h. Autophagy was also induced with rapamycin as previously described (Gutierrez et al, 2004). Cells were lysed in NP-40 lysis buffer (1% NP-40, 200 mM NaCl, 5 mM EDTA, 10% glycerol, 10 mM Tris–HCl pH 7.5, protease inhibitors). We analysed 40 µg total protein by western blot (17.5% SDS–PAGE) by using monoclonal anti-LC3 antibody clone 5f10 (Nanotools, Enzo Life Sciences) and anti-mouse-HRP (Amersham). Anti-LC3-I recognizes a band of 18 kD and anti-LC3-II a band of 16 kD.

Cytokine production following infection of BM-DCs with *M. bovis* BCG WT or SapM::T

BM-DCs were infected for 6 h with *M. bovis* BCG WT or with mutants. Following infection, the BM-DCs were collected by centrifugation at $150 \times g$ for 10 min at 4°C. Cells were washed in PBS and returned to culture for 24 and 48 h. At the indicated time-points, supernatant was removed and analysed by flow cytometry for the presence of cytokines (BioPlex Pro Cytokine Express Assay, Biorad).

Lymph node DC trafficking and activation

At the age of 8 weeks, female BALB/cJ mice (Janvier) were infected subcutaneously at the base of the tail with 1×10^6 CFU *M. bovis* BCG

WT or with *M. bovis* BCG SapM mutant in 100 μl PBS (five mice/group). On days 1 and 7 post-infection, five mice/group were sacrificed; inguinal and brachial/axillary lymph nodes were removed aseptically, and homogenized in RMPI with 1% collagenase (shaking 45 min at RT). For DC subtyping, six mice/group were infected and sacrificed on days 1, 7, 14 and 28 post-infection. There, only ILNs were removed. Cells were prepared by crushing the lymph nodes on a 70 μm cell strainer and labelled with either CD11c-APC, MHCII-FITC, CD80-V450, CD40-PE, CD8a-PerCP and CD86-PE-Cy7 or Ly-6C-V450, MHCII-FITC, CD103-PE, B220-PerCP-Cy5.5, CD11c-APC, CD8a-PE-Cy7 and CD11b-APC-Cy7 and analysed on a LSRII flowcytometer (BD™) equipped with three lasers (488, 635 and 405 nm). Analysis was performed using FACSdiva™ software.

Statistical analysis

Results obtained in millirelative light units (mRLU) were converted to mean $\log_{10}$ mRLU/organ. Survival data were analysed using the method of Kaplan–Meier and Logrank test in Prism version 4.0 (GraphPad). The other data were analysed with one-way ANOVA or Kruskal–Wallis followed by Bonferroni's multiple comparison test or Mann–Whitney U-test, as indicated. p-values < 0.05 were considered statistically significant (More details on Materials and Methods Section are given in the Supportive Information).

Author Contributions

NF designed and performed experiments, analysed and interpreted data, co-wrote the manuscript. PB performed and analysed experiments (flowcytometry). AB generated and characterized the transposon mutant *M. bovis* BCGs. EH performed experiments (granuloma mice model). EP performed experiments. DV and BL performed *in vivo* experiments. BA and EP performed and analysed experiments (confocal microscopy). RDR performed and analysed experiments (electron microscopy). MW, PJ, DE and BNL contributed important reagents, analyses and interpretation of data. PB contributed important knowledge (granuloma mice model). KH designed, performed and analysed experiments. NC initiated and designed the study, coordinated the project, interpreted data and co-wrote the manuscript.

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Supporting information is available at EMBO Molecular Medicine online.

The authors declare that there is no conflict of interest.

For more information

STOP-TB partnership, Working group on new TB vaccines
http://www.stoptb.org/wg/new_vaccines/
AERAS Global TB Vaccine Foundation
http://www.aeras.org/about-tb/vaccine-science.php

References

Appelmelk BJ, den Dunnen J, Driessen NN, Ummels R, Pak M, Nigou J, Larrouy-Maumus G, Gurcha SS, Movahedzadeh F, Geurtsen J, *et al* (2008) The mannose cap of mycobacterial lipoarabinomannan does not dominate the *Mycobacterium*–host interaction. Cell Microbiol 10: 930-944

Barker LF, Brennan MJ, Rosenstein PK, Sadoff JC (2009) Tuberculosis vaccine research: the impact of immunology. Curr Opin Immunol 21: 331-338

Batni A, Festjens N, Plets E, Gurcha SS, Dewerte I, De Rycke R, Rumbold K, Besra GS, Callewaert N (2011) Characterization of an ordered *Mycobacterium bovis* BCG transposon insertion mutant library. Manuscript submitted for publication

Bonay M, Bouchonnet F, Pelicic V, Lagier B, Grandsaigne M, Lecossier D, Grodet A, Vokurka M, Gicquel B, Hance AJ (1999) Effect of stimulation of human macrophages on intracellular survival of *Mycobacterium bovis* bacillus Calmette-Guerin. Evaluation with a mycobacterial reporter strain. Am J Respir Crit Care Med 159: 1629-1637

Briken V, Porcelli SA, Besra GS, Kremer L (2004) Mycobacterial lipoarabinomannan and related lipoglycans: from biogenesis to modulation of the immune response. Mol Microbiol 53: 391-403

Brosch R, Gordon SV, Garnier T, Eiglmeier K, Frigui W, Valenti P, Dos Santos S, Duthoy S, Lacroix C, Garcia-Pelayo C, *et al* (2007) Genome plasticity of BCG and impact on vaccine efficacy. Proc Natl Acad Sci USA 104: 5596-5601

Castillo-Rodal AI, Castanon-Arreola M, Hernandez-Pando R, Calva JJ, Sada-Diaz E, Lopez-Vidal Y (2006) *Mycobacterium bovis* BCG substrains confer different levels of protection against *Mycobacterium tuberculosis* infection in a BALB/c model of progressive pulmonary tuberculosis. Infect Immun 74: 1718-1724

Colditz GA, Brewer TF, Berkey CS, Wilson ME, Burdick E, Fineberg HV, Mosteller F (1994) Efficacy of BCG vaccine in the prevention of tuberculosis. Meta-analysis of the published literature. JAMA 271: 698-702

Cooper AM, Dalton DK, Stewart TA, Griffin JP, Russell DG, Orme IM (1993) Disseminated tuberculosis in interferon gamma gene-disrupted mice. J Exp Med 178: 2243-2247

Copenhaver RH, Sepulveda E, Armitige LY, Actor JK, Wanger A, Norris SJ, Hunter RL, Jagannath C (2004) A mutant of *Mycobacterium tuberculosis* H37Rv that lacks expression of antigen 85A is attenuated in mice but retains vaccinogenic potential. Infect Immun 72: 7084-7095

Denis O, Tanghe A, Palfliet K, Jurion F, van den Berg TP, Vanonckelen A, Ooms J, Saman E, Ulmer JB, Content J, *et al* (1998) Vaccination with plasmid DNA encoding mycobacterial antigen 85A stimulates a CD4+ and CD8+ T-cell epitopic repertoire broader than that stimulated by *Mycobacterium tuberculosis* H37Rv infection. Infect Immun 66: 1527-1533

Fratti RA, Chua J, Vergne I, Deretic V (2003) *Mycobacterium tuberculosis* glycosylated phosphatidylinositol causes phagosome maturation arrest. Proc Natl Acad Sci USA 100: 5437-5442

Gilboa E (2007) DC-based cancer vaccines. J Clin Invest 117: 1195-1203

Grode L, Seiler P, Baumann S, Hess J, Brinkmann V, Nasser Eddine A, Mann P, Goosmann C, Bandermann S, Smith D, *et al* (2005) Increased vaccine efficacy against tuberculosis of recombinant *Mycobacterium bovis* bacille Calmette-Guerin mutants that secrete listeriolysin. J Clin Invest 115: 2472-2479

Gutierrez MG, Master SS, Singh SB, Taylor GA, Colombo MI, Deretic V (2004) Autophagy is a defense mechanism inhibiting BCG and *Mycobacterium tuberculosis* survival in infected macrophages. Cell 119: 753-766

Harris J, Master SS, De Haro SA, Delgado M, Roberts EA, Hope JC, Keane J, Deretic V (2009) Th1–Th2 polarisation and autophagy in the control of intracellular mycobacteria by macrophages. Vet Immunol Immunopathol 128: 37-43

Hinchey J, Lee S, Jeon BY, Basaraba RJ, Venkataswamy MM, Chen B, Chan J, Braunstein M, Orme IM, Derrick SC, *et al* (2007) Enhanced priming of adaptive immunity by a proapoptotic mutant of *Mycobacterium tuberculosis*. J Clin Invest 117: 2279-2288

Horwitz MA, Harth G (2003) A new vaccine against tuberculosis affords greater survival after challenge than the current vaccine in the guinea pig model of pulmonary tuberculosis. Infect Immun 71: 1672-1679

Jagannath C, Lindsey DR, Dhandayuthapani S, Xu Y, Hunter RL, Jr, Eissa NT (2009) Autophagy enhances the efficacy of BCG vaccine by increasing peptide presentation in mouse dendritic cells. Nat Med 15: 267-276

Jiao X, Lo-Man R, Winter N, Deriaud E, Gicquel B, Leclerc C (2003) The shift of Th1 to Th2 immunodominance associated with the chronicity of *Mycobacterium bovis* bacille Calmette-Guerin infection does not affect the memory response. J Immunol 170: 1392-1398

Kamath AT, Fruth U, Brennan MJ, Dobbelaer R, Hubrechts P, Ho MM, Mayner RE, Thole J, Walker KB, Liu M, *et al* (2005) New live mycobacterial vaccines: the Geneva consensus on essential steps towards clinical development. Vaccine 23: 3753-3761

Kaur D, Berg S, Dinadayala P, Gicquel B, Chatterjee D, McNeil MR, Vissa VD, Crick DC, Jackson M, Brennan PJ (2006) Biosynthesis of mycobacterial lipoarabinomannan: role of a branching mannosyltransferase. Proc Natl Acad Sci USA 103: 13664-13669

Kaur D, Obregon-Henao A, Pham H, Chatterjee D, Brennan PJ, Jackson M (2008) Lipoarabinomannan of *Mycobacterium*: mannose capping by a multifunctional terminal mannosyltransferase. Proc Natl Acad Sci USA 105: 17973-17977

Kernodle DS (2010) Decrease in the effectiveness of Bacille Calmette-Guerin vaccine against pulmonary tuberculosis: a consequence of increased immune suppression by microbial antioxidants, not overattenuation. Clin Infect Dis 51: 177-184

Kindler V, Sappino AP, Grau GE, Piguet PF, Vassalli P (1989) The inducing role of tumor necrosis factor in the development of bactericidal granulomas during BCG infection. Cell 56: 731-740

Korf H, Vander Beken S, Romano M, Steffensen KR, Stijlemans B, Gustafsson JA, Grooten J, Huygen K (2009) Liver X receptors contribute to the protective immune response against *Mycobacterium tuberculosis* in mice. J Clin Invest 119: 1626-1637

Lutz MB, Kukutsch N, Ogilvie AL, Rossner S, Koch F, Romani N, Schuler G (1999) An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. J Immunol Methods 223: 77-92

Manolova V, Flace A, Bauer M, Schwarz K, Saudan P, Bachmann MF (2008) Nanoparticles target distinct dendritic cell populations according to their size. Eur J Immunol 38: 1404-1413

Marino S, Pawar S, Fuller CL, Reinhart TA, Flynn JL, Kirschner DE (2004) Dendritic cell trafficking and antigen presentation in the human immune response to *Mycobacterium tuberculosis*. J Immunol 173: 494-506

Martin C, Williams A, Hernandez-Pando R, Cardona PJ, Gormley E, Bordat Y, Soto CY, Clark SO, Hatch GJ, Aguilar D, *et al* (2006) The live *Mycobacterium tuberculosis* phoP mutant strain is more attenuated than BCG and confers protective immunity against tuberculosis in mice and guinea pigs. Vaccine 24: 3408-3419

Master SS, Rampini SK, Davis AS, Keller C, Ehlers S, Springer B, Timmins GS, Sander P, Deretic V (2008) *Mycobacterium tuberculosis* prevents inflammasome activation. Cell Host Microbe 3: 224-232

Parida SK, Kaufmann SH (2010) Novel tuberculosis vaccines on the horizon. Curr Opin Immunol 22: 374-384

Pym AS, Brodin P, Majlessi L, Brosch R, Demangel C, Williams A, Griffiths KE, Marchal G, Leclerc C, Cole ST (2003) Recombinant BCG exporting ESAT-6 confers enhanced protection against tuberculosis. Nat Med 9: 533-539

Rodrigues LC, Diwan VK, Wheeler JG (1993) Protective effect of BCG against tuberculous meningitis and miliary tuberculosis: a meta-analysis. Int J Epidemiol 22: 1154-1158

Romano M, D'Souza S, Adnet PY, Laali R, Jurion F, Palfliet K, Huygen K (2006) Priming but not boosting with plasmid DNA encoding mycolyl-transferase Ag85A from *Mycobacterium tuberculosis* increases the survival time of *Mycobacterium bovis* BCG vaccinated mice against low dose intravenous challenge with M. tuberculosis H37Rv. Vaccine 24: 3353-3364

Sadagopal S, Braunstein M, Hager CC, Wei J, Daniel AK, Bochan MR, Crozier I, Smith NE, Gates HO, Barnett L, *et al* (2009) Reducing the activity and secretion of microbial antioxidants enhances the immunogenicity of BCG. PLoS One 4: e5531

Sambandamurthy VK, Derrick SC, Jalapathy KV, Chen B, Russell RG, Morris SL, Jacobs WR, Jr (2005) Long-term protection against tuberculosis following vaccination with a severely attenuated double lysine and pantothenate auxotroph of *Mycobacterium tuberculosis*. Infect Immun 73: 1196-1203

Schuler G, Schuler-Thurner B, Steinman RM (2003) The use of dendritic cells in cancer immunotherapy. Curr Opin Immunol 15: 138-147

STOP-TB-Partnership (2009) TB vaccines pipeline

Sun R, Skeiky YA, Izzo A, Dheenadhayalan V, Imam Z, Penn E, Stagliano K, Haddock S, Mueller S, Fulkerson J, *et al* (2009) Novel recombinant BCG expressing perfringolysin O and the over-expression of key immunodominant antigens; pre-clinical characterization, safety and protection against challenge with *Mycobacterium tuberculosis*. Vaccine 27: 4412-4423

van Crevel R, Ottenhoff TH, van der Meer JW (2002) Innate immunity to *Mycobacterium tuberculosis*. Clin Microbiol Rev 15: 294-309

Velmurugan K, Chen B, Miller JL, Azogue S, Gurses S, Hsu T, Glickman M, Jacobs WR, Jr, Porcelli SA, Briken V (2007) *Mycobacterium tuberculosis* nuoG is a virulence gene that inhibits apoptosis of infected host cells. PLoS Pathog 3: e110

Venkataswamy MM, Baena A, Goldberg MF, Bricard G, Im JS, Chan J, Reddington F, Besra GS, Jacobs WR, Jr, Porcelli SA (2009) Incorporation of NKT cell-activating glycolipids enhances immunogenicity and vaccine efficacy of *Mycobacterium bovis* bacillus Calmette-Guerin. J Immunol 183: 1644-1656

Vergne I, Chua J, Deretic V (2003) Tuberculosis toxin blocking phagosome maturation inhibits a novel Ca2+/calmodulin-PI3K hVPS34 cascade. J Exp Med 198: 653-659

Vergne I, Chua J, Lee HH, Lucas M, Belisle J, Deretic V (2005) Mechanism of phagolysosome biogenesis block by viable *Mycobacterium tuberculosis*. Proc Natl Acad Sci USA 102: 4033-4038

Walker KB, Brennan MJ, Ho MM, Eskola J, Thiry G, Sadoff J, Dobbelaer R, Grode L, Liu MA, Fruth U, *et al* (2010) The second Geneva consensus: recommendations for novel live TB vaccines. Vaccine 28: 2259-2270

Wang RF (2001) The role of MHC class II-restricted tumor antigens and CD4+ T cells in antitumor immunity. Trends Immunol 22: 269-276

WHO Report (2009) Global tuberculosis control—surveillance, planning, financing