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SapM mutation to improve the BCG vaccine: Genomic, transcriptomic and preclinical safety characterization

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ABSTRACT

The *Mycobacterium bovis* Bacille Calmette Guérin (BCG) vaccine shows variable efficacy in protection against adult tuberculosis (TB). Earlier, we have described a BCG mutant vaccine with a transposon insertion in the gene coding for the secreted acid phosphatase SapM, which led to enhanced long-term survival of vaccinated mice challenged with TB infection. To facilitate development of this mutation as part of a future improved live attenuated TB vaccine, we have now characterized the genome and transcriptome of this *sapM*::Tn mutant versus parental BCG Pasteur. Furthermore, we show that the *sapM*::Tn mutant had an equal low pathogenicity as WT BCG upon intravenous administration to immunocompromised SCID mice, passing this important safety test. Subsequently, we investigated the clearance of this improved vaccine strain following vaccination and found a more effective innate immune control over the *sapM*::Tn vaccine bacteria as compared to WT BCG. This leads to a fast contraction of IFNγ producing Th1 and Tc1 cells after *sapM*::Tn BCG vaccination. These findings corroborate that a live attenuated vaccine that affords improved long-term survival upon TB infection can be obtained by a mutation that further attenuates BCG. These findings suggest that an analysis of the effectiveness of innate immune control of the vaccine bacteria could be instructive also for other live attenuated TB vaccines that are currently under development, and encourage further studies of SapM mutation as a strategy in developing a more protective live attenuated TB vaccine.

1. Introduction

Today, TB remains one of the major causes of infectious disease and death throughout the world [1]. About 10 million people developed TB in 2017, with an estimated 1.3 million deaths.

Although new and promising anti-tubercular drugs are in or approaching the clinic, full control over this devastating disease will necessarily require a good, preventive anti-TB vaccine [2]. *M. bovis* Bacille-Calmette-Guérin (BCG) is the only licensed vaccine today, and already in use for almost 100 years. Although BCG is protective against TB meningitis and disseminated TB in children, its efficacy against pulmonary TB is highly variable and not sufficient for disease control [3–5].

Currently, 13 vaccine candidates are under active development and in the process of clinical testing. Besides two live recombinant candidates (VPM1002 and MTBVAC) which are being tested to replace BCG as a priming vaccine, the candidates being mainly variations of booster vaccines with recurrent key mycobacterial antigens [2,6,7]. Several of these vaccine candidates yield enhanced interferon γ (IFNγ)-producing cellular immunity and enhanced control over bacterial replication in the first weeks after TB challenge in animal models. However, reports that show extended long-term survival of vaccinated animals upon TB challenge are much rarer. In the category of priming vaccines, it has been shown that rBCG30 (BCG Tice overexpressing the M. tb 30-kDa major secretory protein antigen 85B)-immunized mice survive longer than BCG-immunized mice following TB challenge [8]. SO2 (phoP inactivated in M. tb strain MT103), the MTBVAC (containing a double deletion in phoP and fadD26) prototype, conferred greater efficacy than BCG in a high-dose challenge, long-term survival

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experiment in guinea pigs, while no difference to WT BCG was observed after low-dose challenge [9]. The recombinant BCG strain AFRO-1 (BCG_{1331} ΔureC::Ω$pfoA_{G137Q}$, Ag85A, Ag85B and TB10.4) affords better protection than BCG in mice following aerosol challenge with *Mycobacterium tuberculosis* (*M.tb*) [10]. Additionally, some prime-boost vaccination strategies demonstrated improved long-term survival of *M.tb*-challenged guinea pigs versus BCG alone: BCG priming followed by the M72 recombinant protein booster vaccine (fusion of Mtb32 and Mtb39 antigens of *Mtb*) [11] and ID93 (combines four antigens belonging to families of *M.tb* proteins associated with virulence (Rv2608, Rv3619, Rv3620) or latency (Rv1813)/GLA-SE)) [12].

Earlier, we have demonstrated how a *sapM* transposon mutant of *M. bovis* BCG, hereafter called *sapM*::Tn BCG, resulted in a TB vaccine candidate that provides improved long-term survival in mice upon both systemic and intratracheal challenge with *M. tb* as compared to the parental BCG Pasteur-derived strain (hereafter called WT BCG) [13]. We suggested that the secreted acid phosphatase SapM may have evolved as an important immunomodulatory protein of Mycobacteria. The importance of SapM for mycobacterial immunomodulation was since confirmed by independent laboratories [14–17]. In the present study, we report on the in-depth genomic and transcriptomic analysis of the *sapM*::Tn BCG mutant, to assess which alterations are induced by the transposon insertion in the *sapM* locus.

One of the major impediments to TB vaccine development is the incomplete understanding of the mechanisms of protective immunity against *M.tb*, so far obstructing rational vaccine development [18]. Research on animal models and ongoing clinical trials may reveal markers that correlate with a vaccine's protective potential. However, as long as we lack these correlates, vaccine design will be challenging. One general idea that dominated immunology of infectious diseases over the last decades, is the T helper type 1/-type2 (Th1/Th2) paradigm. Following this model, Th1 cells would protect the host from intracellular pathogens, like *M.tb*. Indeed, the early appearance of Th1 type $CD4^+$ T cells secreting IFNγ is necessary for the orchestration of protective immunity in the infected lung [19]. However, controversy has arisen about whether the quantitative extent of IFNɣ production in recall experiments has value as a correlate of vaccine-induced protection against TB. Indeed, several reports show that other anti-tuberculosis CD4 T-cell effector functions are at play [20–24].

In this evolving context, we also report on the safety in immunocompromised mice as well as rapid innate control over the *sapM*::Tn vaccine bacteria, with intriguing impact on the induced adaptive immunity. Our findings suggest that a live attenuated TB vaccine that behaves more like an acute, rapidly immune-controlled infection, rather than the protracted chronic infection caused by the current BCG vaccine, may yield an immune status that affords more prolonged control of a subsequent TB infection.

2. Results

2.1. In depth characterization of the sapM::Tn BCG versus WT BCG strain

2.1.1. Whole-genome resequencing demonstrated very few polymorphisms compared to the M. bovis BCG Pasteur reference genome

The *M. bovis* BCG Pasteur strain 1721 [25], used to create the *sapM*::Tn BCG mutant strain [13,26], differs from the 1173P2 Pasteur vaccine strain by a K43R point mutation in the *rpsL* gene (we confirmed this by sequencing), conferring streptomycine resistance, which is useful to avoid contaminations during the lengthy and involved procedures in ordered transposon mutant library production. To investigate whether there are any additional variants between the WT BCG Pasteur and the *sapM*::Tn BCG mutant, we performed a whole-genome resequencing analysis on both strains (paired end 2 × 150 bp), mapping to the *M. bovis* BCG Pasteur 1173P reference genome, resulting in ±80x average coverage of mapped reads (Suppl. Fig. 1). We could reconfirm the location of the inserted Himar1 transposon, at the TA dinucleotide 8 bp before the *sapM* start codon (Fig. 1). This was also demonstrated by *de novo* assembly of the sequencing reads from the *sapM*::Tn BCG mutant (data not shown). In addition, we performed a probabilistic variant analysis on both WT BCG and *sapM*::Tn BCG mappings and detected only minor modifications (single amino acid change) with unknown impact. These variants are listed in Table 1 and were verified by Sanger sequencing. All variants versus the Pasteur reference are present in both the parental as well as the *sapM*::Tn strains, with only one exception, i.e. the mutations in the *sugI* gene, coding for sugar-transport integral membrane protein. The frameshift mutation (Indel S13fs) probably arose after the library preparation (in a later passage of the strain), since this frameshift mutation is not present in *sapM*::Tn. The exact function of *sugI* is unknown, however, it shows distant sequence similarity to glucose permease GlcP of *S. coelicolor* and the galactose (GalP) and arabinose (AraE) transporters of *E. coli*. Thus, the system is likely to transport a monosaccharide [27]. It is described to be non-essential in the H37Rv strain [28], which explains the absence of phenotype in WT BCG due to the frameshift mutation. The single nucleotide variant (SNV) in *sapM*::Tn BCG SugI (L44M) also likely arose after library preparation since it is not present in WT BCG.

Fig. 1. Whole genome resequencing of the WT BCG Pasteur 1721 strain and of the *sapM::Tn* BCG mutant. Detailed representation of the read mappings at the *sapM* locus for both strains. The red square marks the TA dinucleotide site where the transposon is inserted in the mutant strain. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Variants detected in WT BCG and the *sapM::Tn* BCG disruption mutant. A probabilistic variant analysis was performed in CLC genome workbench on the reads mappings of both the WT BCG and *sapM*::Tn BCG mutant strain. We observed only very few variations compared to the *M. bovis* BCG Pasteur str. 1173P2 reference genome. (SNV/MNV = single/multiple nucleotide variants; Indel = insertions or deletions). The Tn insertion was picked up using *de novo* assembly of the *sapM::Tn* reads.

					Illumina resequencing			Sanger analysis		
	Position	Mutation Type	Gene	AA change	1173P2 WT (reference)	1721 BCG WT	1721 *SapM*::Tn	1173P2 WT	1721 BCG WT	1721 *SapM*::Tn
Variant 1	813,096	SNV	*rpsL*	K43R	A	G	G	A	G	G
Variant 2	1,344,671	MNV	–	–	CG	GC	GC	GC	GC	GC
	2,764,157	SNV*	BCG_2507c	Synonymous	T	A	A			
Variant 3	3,197,940	InDel	*fadD26*	L85fs	–	A	A	–	A	A
Variant 4	3,833,491	InDel**	BCG_3499c	A433InsA	–	CGC	CGC			
Variant 5	3,854,971	InDel	*cut3*	G261Deletion Insertion GR	–	TCG	TCG	TCG	TCG	TCG
	3,685,759	Tn insertion	SapM promoter	–	–	–	tn	–		
Variant 6a	3,708,425	InDel	*sugI*	S13fs	–	A	–	–	A	–
Variant 6b	3,708,517	SNV	*sugI*	L44M	C	C	A	C	C	A

* Not verified by Sanger sequencing, because the mutation is synonymous.
** Sequencing did not work, due to the presence of repeats in the region of the mutation.

Hence, *sugI* appears to be a highly variable gene in the BCG lineage during *in vitro* cultivation, as the three strains each have a different sequence variant. Interestingly, both the parental as well as the *sapM*::Tn strain contain a frame-shift mutation in the gene coding for FadD26, a key enzyme in the biosynthesis pathway of the phthiocerol dimycocerosate (PDIM) class of virulence lipids. This *fadD26* mutation is interesting from a vaccine engineering point-of-view, as it further attenuates virulence [29,30].

2.1.2. The global transcriptional landscape in the sapM::Tn BCG mutant strain is not significantly altered compared to WT BCG

Transcriptome analysis, by both RNAseq analysis and RT-PCR, demonstrated that the *sapM* transcript is ∼20–50-fold reduced compared to the WT BCG, while the transcription levels of other genes remain largely unchanged (<2-fold change), except for a ∼10-fold upregulation of *upp* (encoding uracil phosphoribosyltransferase) which is immediately upstream of *sapM* in the genome, and a modest 2.4-fold downregulation of its downstream gene (*BCG_3376*) (Fig. 2A–C, Suppl. Fig. 2A-B, Suppl. Table 1). Its orthologue in *M. smegmatis* (msmeg_1684) and *M.tb* (rv3311) has been described as a chaperone for the SecA2 protein export pathway, with SapM being one of its substrates [31]. Six other genes show a differential expression pattern (∼2-fold change) between WT BCG and *sapM*::Tn, however, these expression differences are small compared to the differences in expression of *sapM* and *upp* (Suppl. Table 1). The transposon insertion thus only majorly affects one nearby gene, and the changes in *sapM* and *upp* transcription have almost no secondary effects on overall transcriptional regulation. According to the Operon Correlation Browser on the TB Database [32], the gene coding for SapM in *M. tb* (Rv3310) and the downstream gene Rv3311 potentially form an operon. However, in *M. bovis* BCG we see that transposon disruption of the *sapM* upstream region in *sapM*::Tn BCG very strongly reduces the levels of *sapM* transcript, while having a much more modest effect on the levels of BCG_3376 (Rv3311 ortholog) transcription. These data indicate that both genes are likely differentially regulated, questioning the idea of an operonic structure.

2.1.3. SapM protein is undetectable and secreted total phosphatase activity is strongly reduced

As the SapM protein contains a 43 AA N-terminal signal sequence and thus gets secreted in the culture medium, we collected culture supernatant samples at various time-points.

First, we performed an ELISA on these supernatant samples with a newly generated antibody directed against the recombinant SapM protein produced in *E. coli* (Fig. 2D). While the amount of SapM protein steadily increases over time of culture in the WT BCG, the signal is nearly absent in the *sapM::Tn* BCG mutant samples for all time-points. We further confirmed the absence of full-length SapM protein in the *sapM*::Tn BCG strain by SDS-PAGE and western blotting in an independent experiment (Fig. 2E). We detected a band of ±28 kDa in the WT BCG, but not in the *sapM*::Tn BCG mutant sample. A smear is also detected above the 28 kDa band, which likely represents aspecific binding (as the pattern appears to be identical for all samples). To check for any remaining phosphatase activity in the culture medium, we performed an *in vitro* phosphatase assay with p-Nitrophenyl phosphate (pNPP) as a substrate [33] (Fig. 2F). The measured *in vitro* activity is significantly reduced for the *sapM*::Tn BCG mutant sample compared to WT BCG at early time-points. At later time-points (day 8), we still observe a considerable signal, which is expected, as the assay would also pick up activity of other phosphatases such as the protein tyrosine phosphatases (PtpA and PtpB) [34].

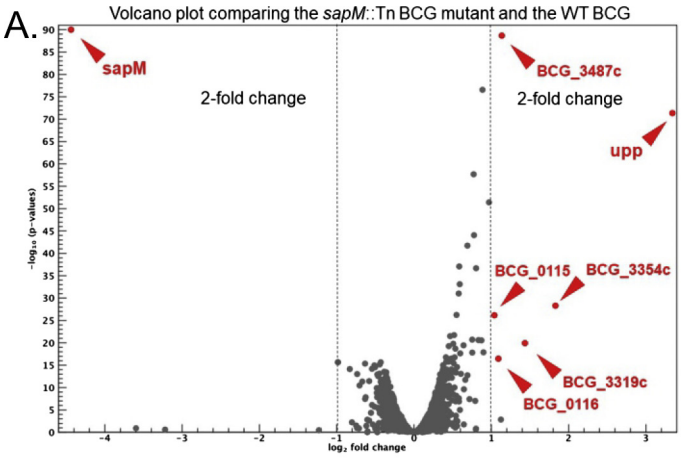
Consequently, based on all of the data on this vaccine strain, we concluded at this point that its improved vaccine efficiency must indeed be due to a loss of function of SapM or the upregulation of *upp* transcription, or both.

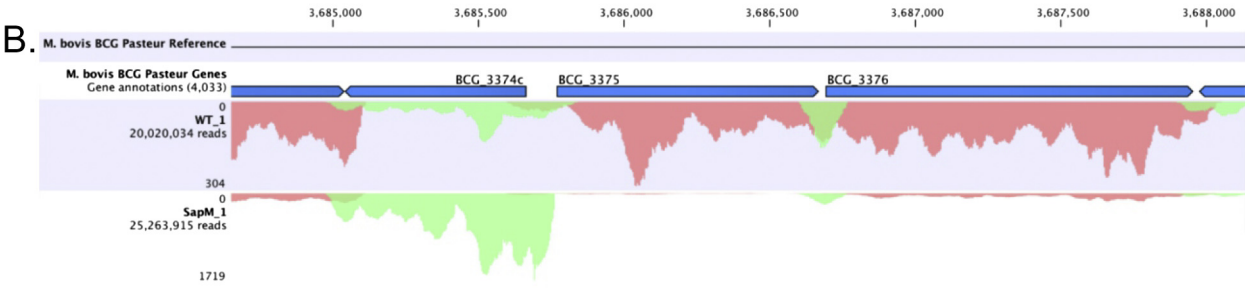
2.2 WT. BCG and sapM::Tn BCG demonstrate comparable in vivo safety

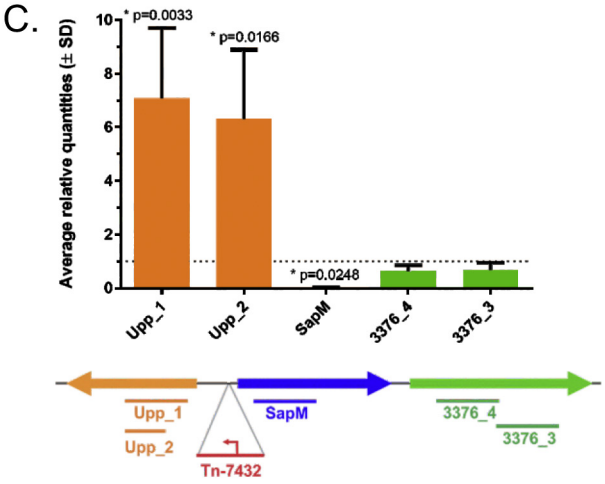
A requirement of live attenuated vaccines is that they should be safe, even in immunocompromised hosts. Our previous analysis of bacterial replication in immunocompetent Balb/c mice, infected intravenously, did not show any difference between WT BCG and *sapM*::Tn BCG [13]. The number of granulomas formed in livers and lungs post-infection was unaffected [13]. To unambiguously demonstrate safety of the *sapM*::Tn BCG mutant compared to WT BCG *in vivo*, we investigated bacterial virulence in the absence of adaptive immunity. Immunocompromised SCID mice, lacking both T and B cells, were infected intravenously (i.v.) with a high ($3x10^7$ CFU) or lower dose ($3x10^6$ CFU) of both strains and survival was monitored (Fig. 3). After both low and high doses, SCID mice infected with *sapM*::Tn BCG mutant showed similar survival to mice infected with WT BCG. Thus, in the SCID model, virulence of *sapM*::Tn BCG is comparable to WT BCG.

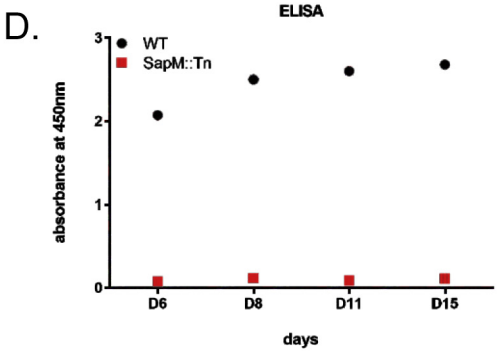
2.3. At the lymph node draining the vaccination site, the sapM::Tn BCG mutant is controlled faster than the WT BCG strain

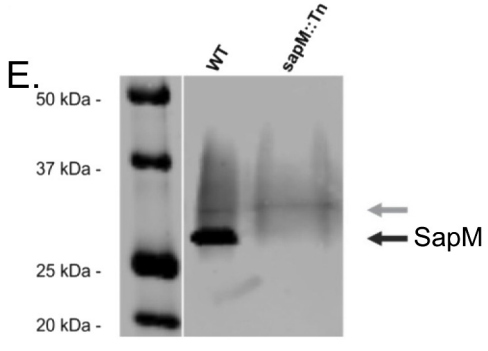
Our previously reported observations indicated that vaccination with the *sapM*::Tn BCG mutant triggered more effective recruitment of iDCs into the draining lymph nodes (LNs) in Balb/c mice

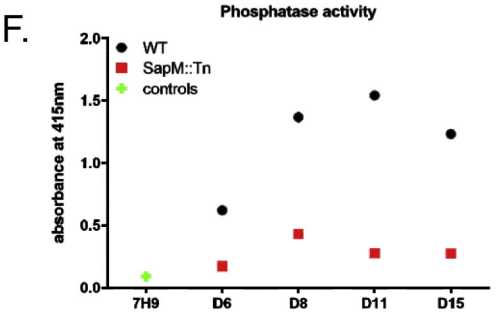

A.
Volcano plot comparing the sapM::Tn BCG mutant and the WT BCG
sapM
2-fold change
BCG_3487c
2-fold change
upp
BCG_0115
BCG_3354c
BCG_3319c
BCG_0116
-log10 (p-values)
log2 fold change


B.
3,685,000
3,685,500
3,686,000
3,686,500
3,687,000
3,687,500
3,688,000
M. bovis BCG Pasteur Reference
M. bovis BCG Pasteur Genes
Gene annotations (4,033)
BCG_3374c
BCG_3375
BCG_3376
WT_1
20,020,034 reads
SapM_1
25,263,915 reads


C.
Average relative quantities (± SD)
* p=0.0033
* p=0.0166
* p=0.0248
Upp_1
Upp_2
SapM
3376_4
3376_3
Tn-7432


D.
ELISA
WT
SapM::Tn
absorbance at 450nm
D6
D8
D11
D15
days


E.
WT
sapM::Tn
50 kDa -
37 kDa -
25 kDa -
20 kDa -
SapM


F.
Phosphatase activity
WT
SapM::Tn
controls
absorbance at 415nm
7H9
D6
D8
D11
D15

[13]. This lead us to study the influence of this more effective induction of the innate immune response at early time points after *sapM*::Tn BCG vaccination on the fate of the vaccine inoculum. To enhance the validity, experiments were performed in F1 mice (C57BL/6J × Balb/c) to assess robustness of the observed effects to a more heterogeneous immunogenetic background [35]. After subcutaneous (s.c) vaccination of F1 mice, less bacteria (∼50%) were recovered from LNs of *sapM*::Tn BCG infected mice as compared to WT BCG-infected mice at all time-points analyzed (i.e. days 8, 14 and 28 post-infection) (Fig. 4A). It is clear that whereas WT BCG could amplify from the inoculum, this was barely the case for the *sapM*::Tn BCG mutant. Since differences are clear already 8 days post-vaccination, this result is most consistent with improved control by innate immune mechanisms. Indeed, an analysis of intracellular survival of WT BCG and *sapM*::Tn BCG upon infection of bone marrow derived macrophages (BM-DMs), showed that WT BCG and *sapM*::Tn BCG are taken up similarly (as shown before [13]) but that growth of the *sapM*::Tn BCG mutant is better controlled compared to the WT BCG strain upon infection (Fig. 4B). In line with these findings, such improved clearance of SapM-mutated *M.tb* by BM-DMs has recently also been described [16], an effect that was attributed to improved phagosomal maturation.

To further evaluate the impact of early innate growth control of the bacteria, we have analyzed survival of *sapM*::Tn BCG and WT BCG after intravenous infection of F1 mice. Twenty-four hours post-infection, the bacterial load in the lungs and spleens from mice that were infected with WT BCG was higher than the loads of those infected with the *sapM::Tn* BCG mutant (∼50%) (Fig. 4C). Three weeks post-infection, the difference was still observed in the lungs, but not in the spleens. Similar results were obtained in the spleen of C57BL/6J mice (Suppl. Fig. 3A), but not in the lungs (data not shown). Cytokine levels were analyzed in the serum of i.v. exposed mice at different time-points (6 h, 24 h, 48 h post-vaccination), and significantly higher levels of IL1-β and IL17 could be measured 24 h post-*sapM*::Tn BCG vaccination compared to WT BCG vaccination (Suppl. Fig. 3B). In view of this early time point post-vaccination, both IL1-β and IL17 are produced by innate immune cells. These particular cytokines are known to be important innate cytokines for bacterial killing, particularly in mycobacterial infection [36,37], which is consistent with the reduced *sapM::*Tn BCG bacterial load.

2.4. Complementation of the sapM::Tn mutation reverses the phenotypes.

As the Tn insertion in the *sapM* locus caused both a loss of function of *sapM* and a strong upregulation of the upstream *upp* gene, we assessed whether the improved early innate growth control of the *sapM*::Tn BCG bacteria was due to the loss of function of SapM or the upregulation of *upp* transcription. For this purpose, we have complemented the *sapM*::Tn BCG mutant strain with the *sapM* gene under control of its own promotor. The *sapM* expression is reverted to wild type levels in this *sapM*::Tn:compl BCG mutant, but *upp* levels are still increased similarly as in the *sapM*::Tn BCG mutant as demonstrated by RT-PCR analysis (Fig. 5A). Protein analysis by ELISA and Western blot (Fig. 5B and C) also demonstrated that SapM protein levels reverted to wild type levels in the *sapM*::Tn:compl BCG mutant. Phosphatase activity was not completely restored in the supernatant of the *sapM*::Tn:compl BCG mutant (Fig. 5D), but was majorly increased compared to the phosphatase activity measured in the *sapM*::Tn BCG mutant. Analysis of bacterial load in the draining LNs from mice that were subcutaneously infected with either *sapM*::Tn:compl BCG mutant, *sapM*::Tn BCG or WT BCG, showed that complementation of *sapM* expression partly restored WT phenotype (Fig. 6A). By determining bacterial load in the lungs and spleens from mice that were intravenously infected with either of the three strains, we could also show that complementation of *sapM* expression could obliterate the *sapM*::Tn BCG mutant phenotype of reduced bacterial load early post-infection (Fig. 6B, upper panels). Since bacterial loads reach similarly lower levels at 3 weeks post-infection (i.v.), the results show that SapM is mostly important for control of bacterial growth at early time points post-infection. These data also confirm that the observed phenotypes are due to the reduced expression of *sapM* and not because of upregulation of *upp* because *upp* expression is the same in *sapM*::Tn BCG and the *sapM*::Tn:compl BCG mutant.

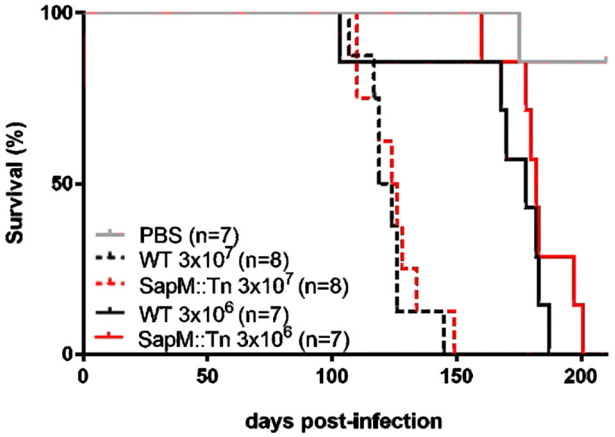


Fig. 3. | *SapM*::Tn BCG safety study in SCID mice. Immunocompromised SCID mice were infected intravenously with 3×10^6 versus 3×10^7 cfu of the WT BCG or *SapM*::Tn BCG mutant and survival was monitored. (Log-rank test; WT BCG vs. *sapM::Tn*; P = not significant). The control groups represent animals vaccinated with PBS.

Fig. 2. (A) Volcano plot comparing the *sapM*::Tn BCG mutant and the WT BCG. Genes with a > 2-fold change and a p-value <0.001 are depicted in red. **(B) Mapped (sense) reads at the SapM locus in the WT BCG vs *sapM*::Tn BCG condition.** Only one replicate of both WT BCG and *sapM*::Tn BCG samples is depicted. BCG_3374c = *upp*, BCG_3375 = *sapM*. **(C) RT-PCR analysis of the *sapM* locus.** RNA was prepared of cultures of biological triplicates of the *sapM*::Tn BCG disruption mutant, as well as of the WT BCG. An RT-PCR on the cDNA using primer sets directed against the sapM gene (blue bar) and the directly up- and downstream genes (*upp* and *BCG_3376*, orange and green bars, respectively). The data presented here are averages (±SD) of the three biological replicates. For each mutant, the targets are individually normalized to the transcription levels in the WT BCG strain (grey dotted line set at an average relative quantity of 1). The * indicates significance ($p < 0.05$) by a two tailed *t*-test comparing the transcription levels of each target in the mutant strain to the same target in the WT BCG. Exact p-values are given in the figure. (D-F) **SapM protein and activity analysis.** The *sapM*::Tn BCG mutant and WT BCG 1721 were grown in 7H9 medium and supernatant samples were collected on various time points (D6 (Day 6) – D15). **(D)** ELISA using an anti-SapM polyclonal antibody. **(E)** SDS-PAGE and western blotting of WT BCG and *sapM*::Tn BCG mutant supernatant samples collected on day 15 and grown in 7H9 medium without OADC and supplemented with glucose only. The blots were developed with the anti-SapM antibody. The processed SapM protein (=removal of the N-terminal signal sequence) is ±28 kDa in size and is only present in the WT BCG strain (black arrow). **(F)** *In vitro* phosphatase assay using p-Nitrophenyl phosphate (pNPP) as a substrate to check the activity of the SapM enzyme. The plotted data for both ELISA and phosphatase assay are averaged over 2 technical replicates and corrected for the background signal induced by sterile 7H9 medium supplemented with 10% OADC. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

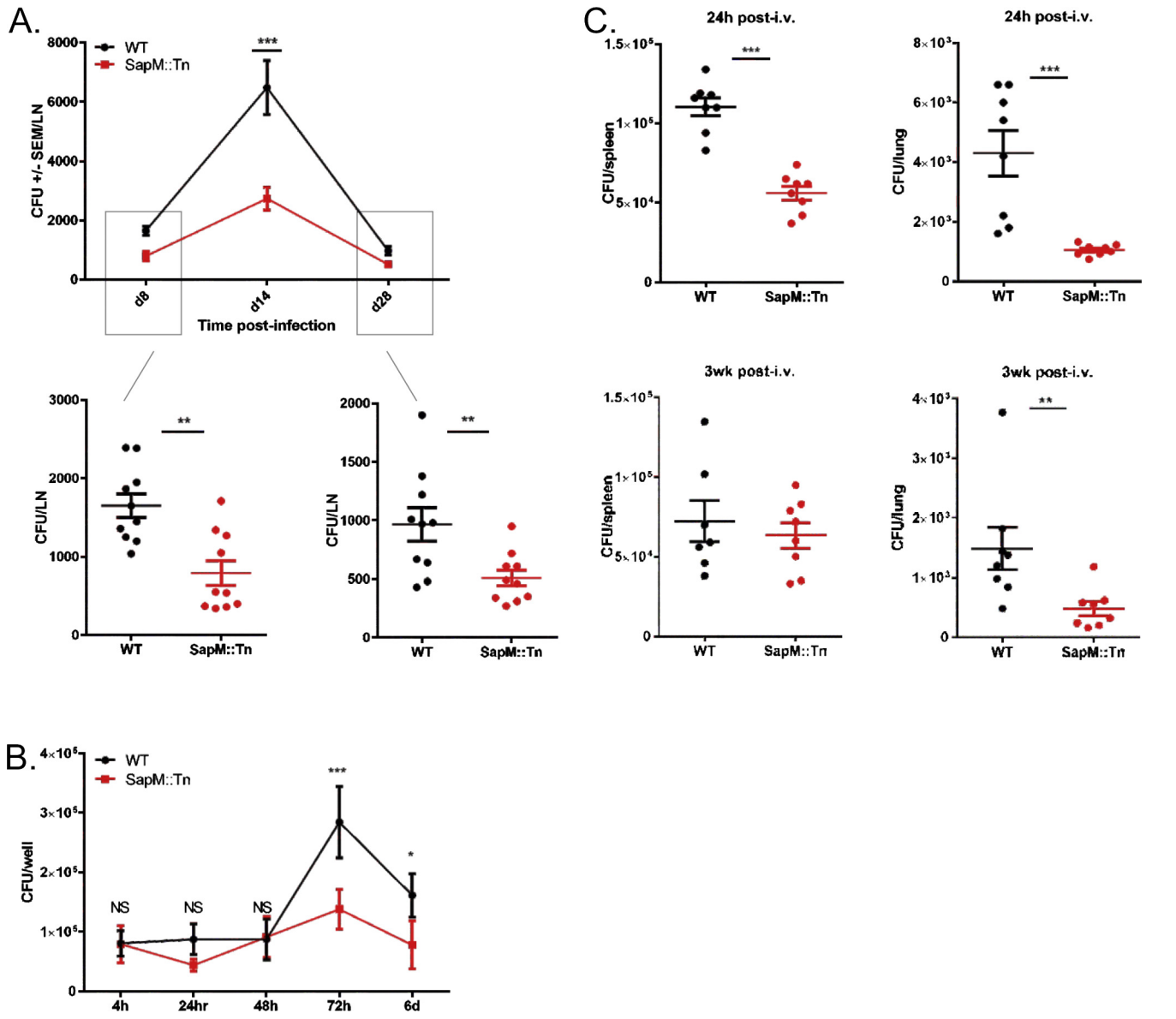


Fig. 4. ***In vivo* and *in vitro* replication analysis: *sapM*::Tn BCG versus WT BCG. (A)** F1 mice (Balb/c × C57BL/6J) were vaccinated s.c. with the WT BCG or *sapM*::Tn BCG (2×10^6 cfu, 10 mice/group). At day 8, 14 and 28 post-infection, mice were sacrificed and the number of bacteria in the draining LNs was determined by cfu plating (Mann-Whitney test; ** $P < 0.01$). **(B)** BM-DMs were infected with WT BCG or *sapM*::Tn BCG (MOI 10:1) for 4 h. Cells were washed and bacterial uptake was determined 4 h post-infection. Bacterial replication was analyzed by cfu plating 24, 48 and 72 h, 6 days post-infection. **(C)** F1 mice were vaccinated i.v. with the WT BCG or *sapM*::Tn BCG (2×10^6 cfu, 8 mice/group). At 24 h and 3 weeks post-infection, mice were sacrificed and the number of bacteria in the spleens and lungs was determined by cfu plating (Mann-Whitney test; ** $P < 0.01$).

2.5. Faster kinetics of iDC recruitment to lymphoid organs and decrease in IFNγ-producing CD4⁺ and CD8⁺ T recall response in sapM::Tn BCG vaccinated mice compared to WT BCG vaccinated mice

We then further analyzed how improved innate control over vaccine bacteria changes the immune response to the vaccine. Hereto, we have vaccinated C57BL/6J mice s.c. with WT BCG or *sapM*::Tn BCG mutant. At different time points, mice were sacrificed and LNs and spleens were isolated. DC phenotyping was performed. Significant differences were observed in total leukocyte numbers and iDC recruitment kinetics after *sapM*::Tn BCG versus WT BCG vaccination. In general, we observe a faster induction of the innate immune response, reflected by a faster recruitment of innate inflammatory cells to the secondary lymphoid organs (dLN-spleen), and by a faster contraction of the innate immune response, seen by lower leukocyte cell numbers at day 14 post-vaccination with *sapM*::Tn BCG (Fig. 7). Next, T cell immunity was assessed by restimulation of splenocytes or LN cells with either the mycobacterial antigen Ag85A, the *M.tb* extract PPD or by anti-CD3/28, a stimulus that activates all T cells, as a control. Vaccination with *sapM*::Tn BCG leads to a lower number of lymph node and spleen IFNγ-producing $CD4^+$ (Th1) and $CD8^+$ (Tc1) T cells 2 weeks post-vaccination compared to WT BCG (Fig. 8A). After vaccination with a lower dose, this observation is only seen for Tc1 cells, following polyclonal stimulation in the LNs and spleen (Suppl. Fig. 4). IFNγ measurements in the supernatant following stimulation with the different antigens also demonstrated lower levels of IFNγ in the *sapM*::Tn BCG condition (Fig. 8B). These lower numbers of IFNγ-producing $CD4^+$ and $CD8^+$ T cells were seen at early time points (2 weeks post-infection), but the difference became non-significant at later time points (Suppl. Fig. 5), consistent with the hypothesis that a fast innate control of bacterial load

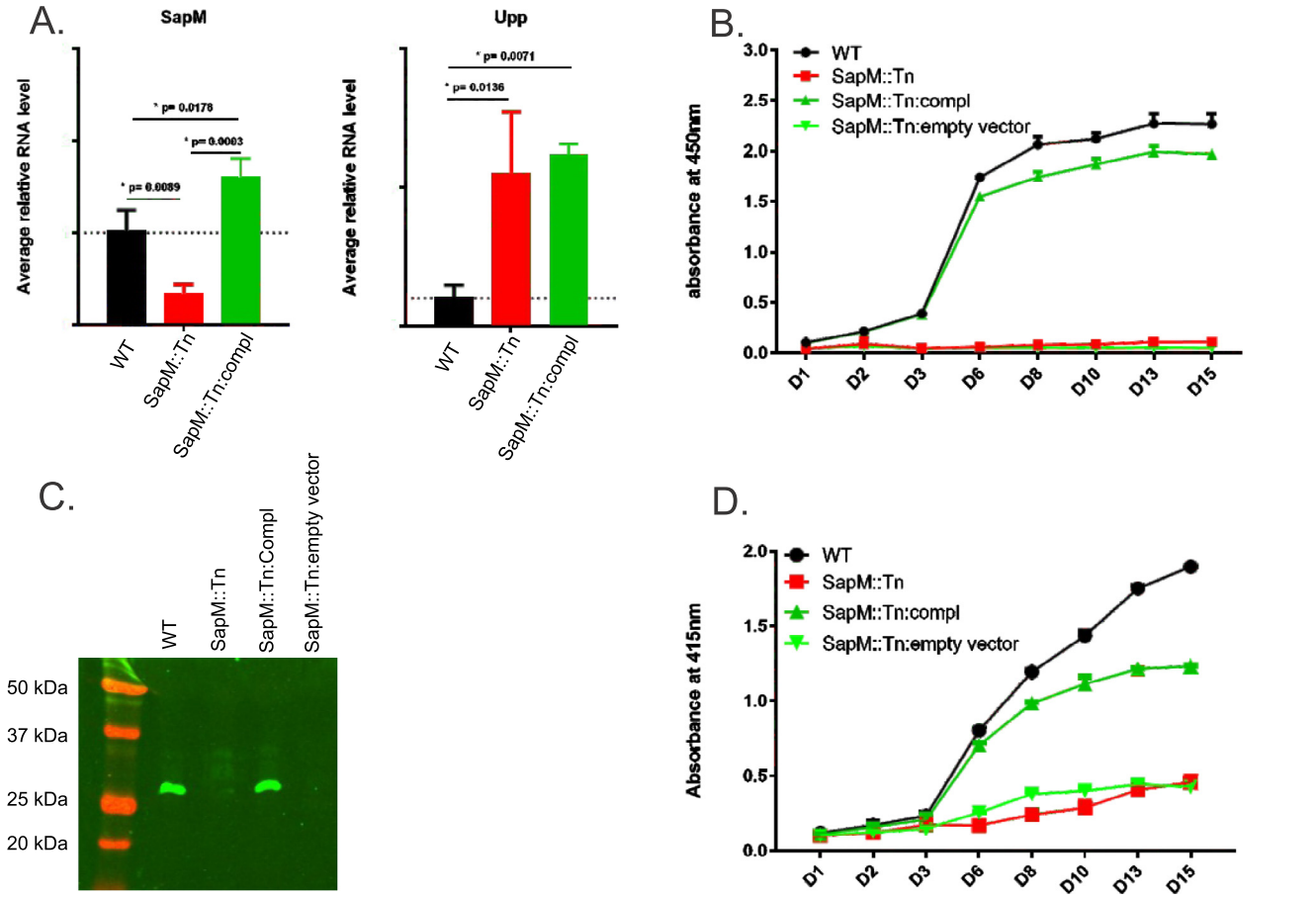


Fig. 5. RT-PCR analysis of the *sapM* locus and SapM protein and activity analysis in the *sapM*::Tn:complementation mutant versus WT BCG. (A) RNA was prepared of cultures of biological triplicates of the *sapM*::Tn BCG disruption mutant (red), the WT BCG (black) and the sapM::Tn:complementation BCG mutant (green). An RT-PCR on the cDNA used primer sets directed against the sapM gene (sapM b primers - left panel) and the directly upstream gene upp (upp2 primers - right panel). The data presented here are averages (±SD) of three technical replicates. For each mutant, the targets are individually normalized to the transcription levels in the WT BCG strain (grey dotted line set at an average relative quantity of 1). The * indicates significance ($p < 0.05$) by an ANOVA test (Tukey post-hoc), comparing the transcription levels of each target in the 3 different strains. Exact p-values are given in the figure. **(B-D)** The *sapM*::Tn BCG mutant, WT BCG 1721, sapM::Tn:complementation BCG mutant and sapM::Tn:empty vector were grown in 7H9 medium and supernatant samples were collected on various time points (D1 (Day 1) – D15). **(B)** ELISA using an anti-SapM polyclonal antibody. The plotted data for both ELISA and phosphatase assay is averaged over 2 technical replicates and corrected for the background signal induced by sterile 7H9 medium supplemented with 10% OADC. **(C)** SDS-PAGE and western blotting of indicated supernatant samples collected on day 10 and grown in 7H9 medium without OADC and supplemented with glucose only. The blots were developed with the anti-SapM antibody. The processed SapM protein (=removal of the N-terminal signal sequence) is ±28 kDa in size and is only present in the WT BCG strain and *sapM*::Tn:complementation mutant. **(D)** *In vitro* phosphatase assay using p-Nitrophenyl phosphate (pNPP) as a substrate to check the activity of the SapM enzyme. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

results in a swift contraction of the TB-specific Th1 and Tc1 cellular response after *sapM*::Tn BCG vaccination. The *sapM*::Tn BCG vaccine thus behaves like a rapidly (though not completely) controlled bacterial infection, contrary to WT BCG, which expands and causes a more protracted infection at the vaccination site.

3. Discussion

Our former research demonstrated that an *M. bovis* BCG *sapM*::Tn mutant led to enhanced long-term survival of vaccinated mice challenged with TB [13]. Further development of this TB vaccine candidate requires further characterization of the mutant and its safety, which was the main purpose of the extensive work presented in this study.

We have performed a whole genome resequencing-based variant analysis of this *sapM*::Tn BCG mutant, as well as of its parent BCG strain [25], using a shotgun Illumina sequencing approach. Very few polymorphisms could be detected compared to the *M. bovis* BCG Pasteur reference genome, most of which were already present in the streptomycin-resistant Pasteur derivative in which we designed our transposon mutant. A frame-shift mutation had inactivated the gene coding for FadD26, a key enzyme in the biosynthesis pathway of the PDIM class of virulence lipids that are involved in hiding *M. tb*'s own PAMPs from the host's innate immune system [29]. Knocking out this gene in *M. tb* severely impairs the pathogen's ability to survive *in vivo* [29,30]. For this reason, the *fadD26* gene is deleted in MTBVAC, the first live-attenuated *M. tb* vaccine [38]. Chen *et al.* compared PDIM/PGL production in 12 different *M. bovis* BCG substrains and found that while most were PDIM-positive (including BCG Pasteur), three of these strains, BCG Japan (or Tokyo), Moreau and Glaxo, do not produce this class of lipids [39]. The authors further reported that there was a correlation between the virulence that was associated with the BCG substrains and their lipid profile: the on average more virulent, PDIMs/PGLs-producers and the on average less virulent, PDIMs/PGLs non-producers. In a later study it was shown that the PDIM-defect in BCG Moreau was due to a deletion in the FadD26 and the directly downstream *ppsA* gene in the same operon, which is also involved in the PDIM/PGL lipid biosynthesis pathway [40]. The authors reported that this locus was intact in BCG Japan and Glaxo. Naka *et al* later showed that BCG Tokyo 172 is actually divided into two subpopulations, in which type II,

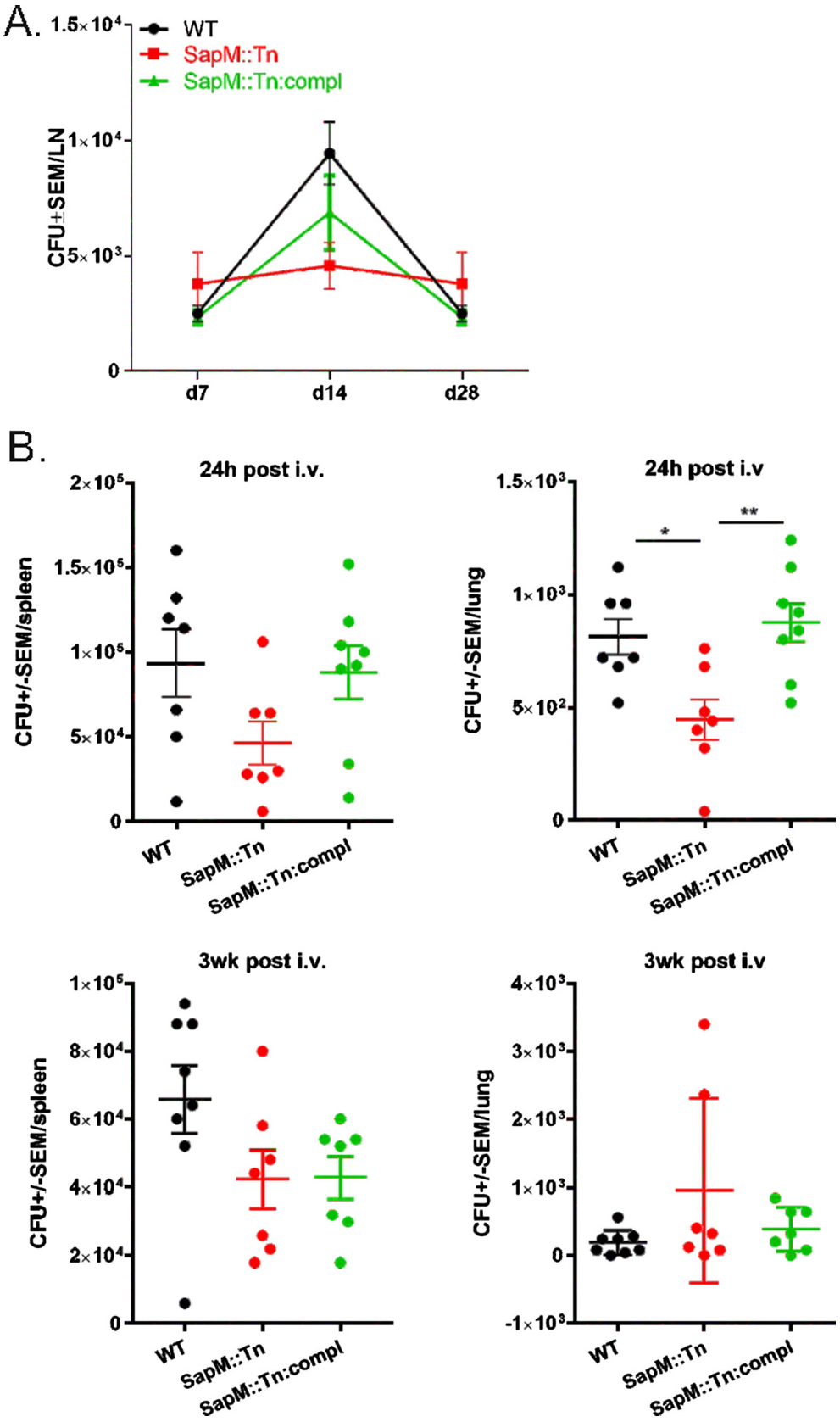


Fig. 6. ***In vivo*** **replication analysis:** ***sapM*****::Tn BCG versus** ***sapM*****::Tn:complementation BCG versus WT BCG. (A)** C57BL/6J mice were vaccinated s.c. with the WT BCG, *sapM*::Tn BCG or *sapM*::Tn:complementation BCG (2×10^6 cfu, 6 mice/group). At day 8, 14 and 28 post-infection, mice were sacrificed and the number of bacteria in the draining LNs was determined by cfu plating (Mann-Whitney test; ** $P < 0.01$). **(B)** C57BL/6J mice were vaccinated i.v. with the WT BCG, *sapM*::Tn BCG or *sapM*::Tn:complementation BCG (2×10^6 cfu, 7–8 mice/group). At 24 h and 3 weeks post-infection, mice were sacrificed and the number of bacteria in the spleens and lungs was determined by cfu plating (Mann-Whitney test; ** $P < 0.01$).

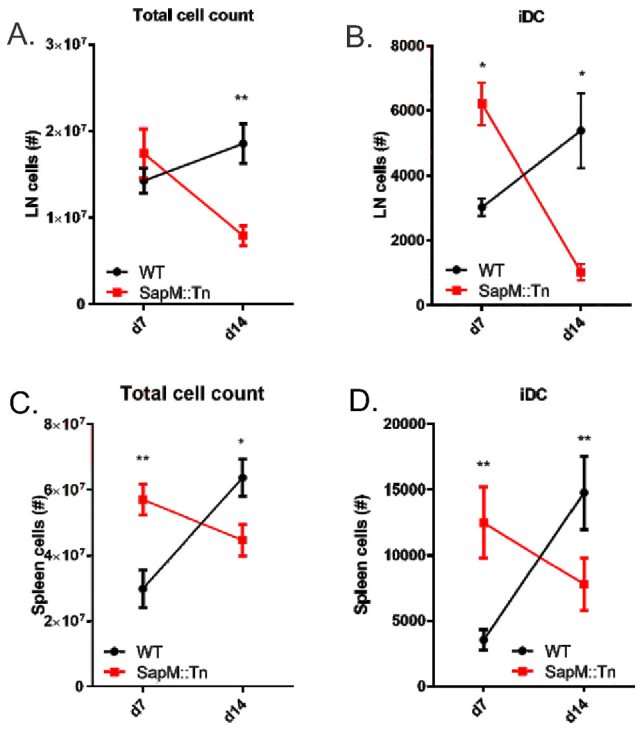


Fig. 7. iDC recruitment to lymphoid organs starts earlier when mice are vaccinated with the BCG *sapM*::Tn BCG strain. C57BL/6J mice were immunized s.c. at the base of the tail (2×10^6 cfu, 5–10 mice/group (A-B); 2.10^5 cfu, 10 mice/group (C-D)) with WT BCG or *sapM*::Tn BCG mutant. Seven and 14 days later, the mice were sacrificed, and inguinal LNs, brachial LNs and spleens were isolated. Cells were prepared, labelled with different antibodies staining DCs and analyzed by flow cytometry. Total cell numbers **(A, C)** and the kinetics of iDC ($CD103^+MHCII^{hi}$ $CD11b^+$ $Ly6C^+$) recruitment **(B, D)** differ upon BCG *sapM*::Tn BCG and WT BCG vaccination. (Mann-Whitney; $^*P < 0.05$, $^{**}P < 0.01$).

but not type I, has a frameshift mutation in the *ppsA* gene and thus does not produce PDIM lipids [41]. It is unclear whether this strain is the same as the Japan strain that was earlier reported to lack PDIMs [39]. As the Japan/Tokyo, Moreau and Glaxo substrains are not derived from one another [42], and as we have here detected a *fadD26* frameshift in a substrain derived from BCG Pasteur, these independent mutations in genes coding for key enzymes in the PDIM/PGL lipid biosynthesis pathway indicate that there may be a selective force in favor of losing this class of lipids during *in vitro* BCG cultivation. We also conclude that, as our strain has this background *fadD26* mutation, the *sapM*::Tn vaccine strain in our studies is in fact a *fadD26/sapM*::Tn double mutant, which is important knowledge for further development, as it shows that the *sapM* mutation improves vaccine efficacy even in this *fadD26* background, as is currently being used for safety enhancement of the MTBVAC lead clinical TB vaccine candidate. In addition to our *sapM*::Tn BCG mutant, this FadD26 mutation will almost certainly also be present in the Zmp1 BCG mutant candidate vaccine that was described in [43], since it is also derived from the *M. bovis* BCG Pasteur 1721 strain.

By comparative transcriptome profiling between WT BCG and *sapM*::Tn BCG, we observed an almost complete absence of *sapM* transcript in the mutant strain, together with a significant upregulation of *sapM*'s upstream gene *upp*. By RNAseq analysis, we further showed that the global transcriptional landscape in the *sapM*::Tn BCG mutant strain is not significantly altered compared to the WT BCG.

The most promising strategy for improved TB vaccines currently is the development of an improved attenuated live vaccine, either used alone or in combination with subunit booster vaccines [6]. Furthermore, re-vaccination with BCG in adolescence has recently shown promise as well [44]. An improved version of the BCG vaccine could thus be valuable in this context too. However, working with live vaccines encompasses potential safety issues, especially since a high incidence of HIV is found in areas where TB is endemic. A novel vaccine candidate should therefore be at least as safe as BCG. Safety of the *sapM*::Tn BCG strain was tested in immunocompromised SCID mice, in comparison with the WT BCG. Survival time upon infection of the mice was similar for both strains, at both high and low infectious doses, demonstrating comparable safety of the *sapM*::Tn BCG strain and WT BCG.

Due to the lack of natural infection-induced protection, the type of immune response that is crucial in preventing *M.tb* infection and TB, and which therefore should be induced by vaccination, remains largely unknown. At present, it is believed that a particular balance between differentially polarized adaptive immune responses is crucial to overcome a TB infection. *M. tb* as well as *M. bovis*, from which BCG derives, have evolved immunomodulatory mechanisms to perturb this balance to its own benefit [45,46]. We and others propose that an improved, live attenuated vaccine will need to be engineered to take down these immunomodulatory virulence factors from the pathogenic parental Mycobacteria from which these vaccines (incl. BCG) have been derived, and we propose that the SapM secreted phosphatase is such a factor. Our results demonstrate a better innate control of *sapM*::Tn BCG vaccine bacteria as compared to WT BCG upon vaccination, correlating with a faster influx of iDCs in lymphoid organs draining the vaccination site, and more modest primary expansion of TB-antigen-specific IFNγ-producing $CD4^+$ and $CD8^+$ T cells. Since we did not further define subsets of responding cells (such as expressing markers associated with antigen exposure, e.g. KLRG-1, PD-1, CD62L, CD44, CCR7 [47] or expressing other cytokines, e.g. TNFα, IL-17, FoxP3,...), we cannot further subtype the T cells responding to the specific antigens used, limiting our conclusions to the effect on Th1/Tc1 cells. Further study will be performed to investigate adaptive immune responses more in depth. Both WT BCG and *sapM*::Tn BCG have equivalent growth characteristics during *in vitro* cultivation (Suppl. Fig. 6), excluding that mere growth rate differences could have caused any difference in abovementioned phenotypes. Since complementation of *sapM* expression abolished the improved growth control of the *sapM*::Tn BCG mutant upon infection, the observed phenotypes are specifically due to the reduced expression of *sapM*. To conclude, all of this shows that the *sapM* mutated BCG vaccine behaves like a more immediately effectively controlled bacterial infection as compared to WT BCG. Importantly, in the context of viral infections, it has been well established that a protracted fight of the immune system to a chronic infection yields poorly efficacious memory due to poor generation of central memory T cells [48]. Possibly, the same holds true for chronic bacterial intracellular infections, of which BCG vaccination is an example, a hypothesis which will need to be proved by future research. Together with the comparable safety of the SapM-mutated BCG and WT BCG in immunodeficient mice, and the fact that the *sapM*::Tn BCG vaccine is presently one of the few priming vaccines for which enhanced long-term survival of TB-infected animals has been demonstrated [13], these results warrant inclusion of the SapM inactivation strategy in future generations of live attenuated TB vaccines.

4. Materials and methods

4.1. Mycobacterial strains and media

The streptomycin resistant *M. bovis* BCG Pasteur strain 1721 [25] (*RpsL*, K43R; a gift of Dr. P. Sander, Institute for Medical

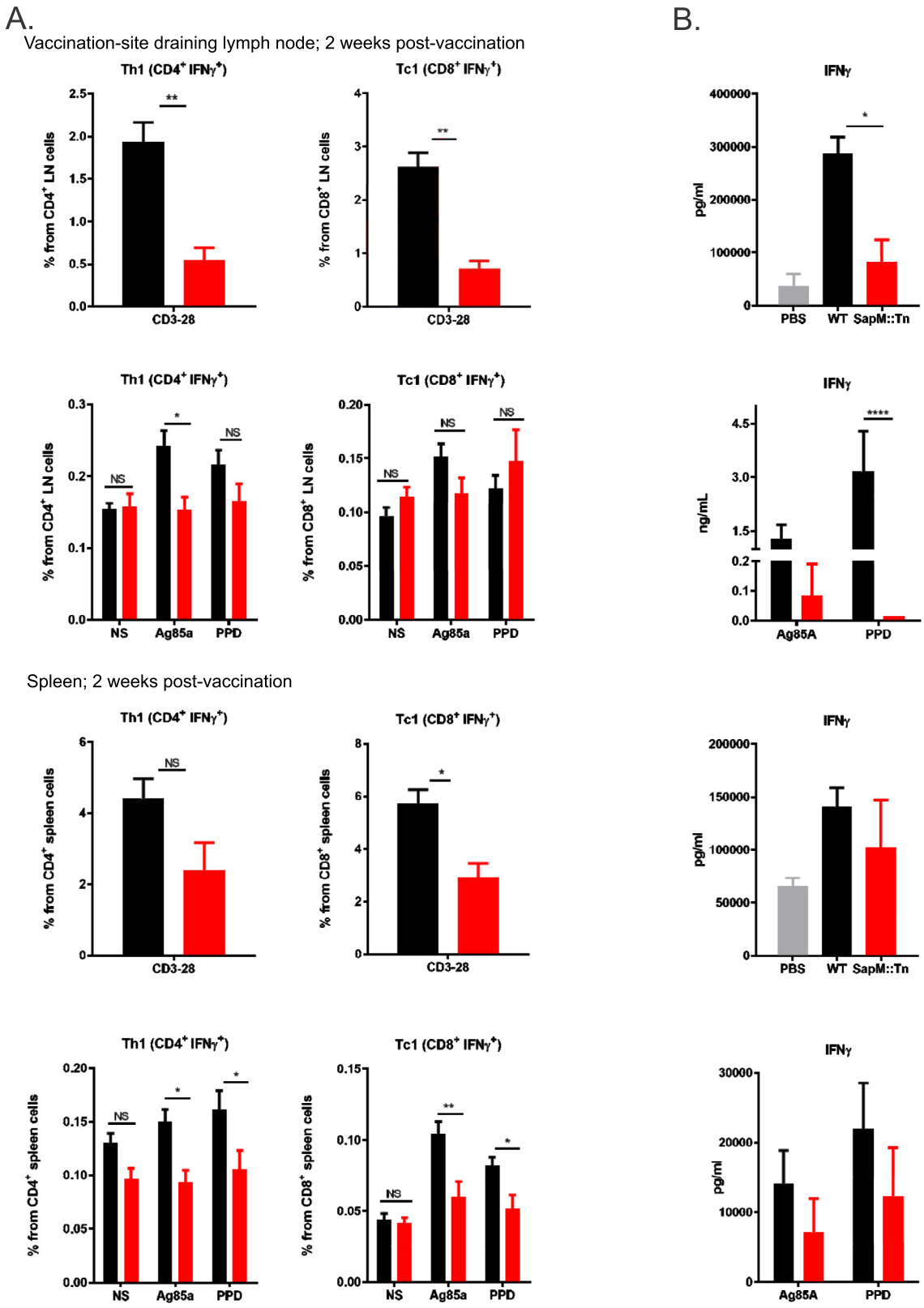


Fig. 8. Vaccination with *sapM*::Tn BCG induces reduced frequencies of IFNγ-producing CD4$^+$ and CD8$^+$ T cells compared to WT BCG. C57BL/6J mice were immunized s.c. at the base of the tail with a high dose of WT BCG (black bars) or *sapM*::Tn BCG mutant (red bars) (2×10^6 cfu, 5 mice/group). 14 days later, the mice were sacrificed, and inguinal LNs, brachial LNs and spleens were isolated. Cells were prepared and the T cell response was analyzed by intracellular cytokine staining followed by flow cytometry **(A)**. Additionally, supernatants of stimulated spleen cells and controls were harvested 48 h post-stimulation and IFNγ concentration was determined by bioplex **(B)**. (Mann-Whitney or 2way ANOVA; *P < 0.05, **P < 0.01). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Microbiology, Zürich) and its *sapM* transposon insertion mutant [13] were grown in shaking culture flasks in Middlebrook 7H9 broth (Difco) supplemented with 0.05% Tween80 and Middlebrook Oleic Albumin Dextrose Catalase (OADC, Becton Dickinson) when grown in liquid culture. Difco Middlebrook 7H10 agar was used for growth on solid culture. For the SDS-PAGE and western blotting experiment, *M. bovis* BCG cultures were grown in 7H9 medium without OADC, but supplemented with 0.2% glucose, 0.2% glycerol and 0.05% of Tween80. All strains were frozen as 1 mL cultures at OD_{600} 1 in 20% glycerol (final concentration). For all *in vitro* and *in vivo* infections described in this paper, cells were started from a glycerol stock, grown in shaking culture flasks (OD_{600} always ≤ 1) as described above, subcultured once and further grown until $OD_{600} \sim 0.8$ after which cells were collected and prepared for infection. For each experiment, we confirmed that we immunized with the same number of viable CFU by CFU plating of the inoculum.

4.2. Whole genome Illumina resequencing and data analysis

Genomic DNA of the *M. bovis* BCG strain 1721 and its *sapM*::Tn BCG mutant (Tn-7432) was prepared [49] and used for an Illumina library prep (Nextera XT DNA Library Preparation kit). The libraries were sequenced on an Illumina MiSeq instrument (2×150 bp reads), with an average 80x coverage per genome. The library prep and sequencing was performed by the VIB Nucleomics Core facility (www.nucleomics.be). The data was analyzed by the VIB Nucleomics Core facility and the VIB Bioinformatics Core facility (www.bits.vib.be) using CLC Genomics Workbench [50]. All reads were processed using the standard CLC-GWB settings (Details in Suppl. Methods).

4.3. RT-qPCR analysis

10 mL of *M. bovis* BCG cultures (grown in standard 7H9 medium until an OD_{600} of 0.8 – 1.0) were centrifuged and the pellets were washed once with 0.5% Tween80 solution in PBS. RNA was prepared as described in [51] with minor changes (Suppl. Methods). cDNA was prepared from 1 μg of DNase-treated RNA using the iScript Synthesis Kit (BioRad) and a control reaction lacking reverse transcriptase was included for each sample. The RT-PCR program was as follows: 10 min at 25 °C, 30 min at 42 °C, 5 min at 85 °C, and cooling down to 12 °C. Real time quantitative PCR was done on a LightCycler 480 (Roche Diagnostics) using the SensiFast SYBR-NoRox kit (BioLine), in triplicate for each cDNA sample, on a 384-multiwell plate, with 1 ng of cDNA in a total volume of 10 μL. Details in Suppl. Methods.

4.4. RNA-Seq analysis

M. bovis BCG cultures were grown in standard 7H9 medium in triplicates until an OD_{600} of 0.8 – 1.0 and RNA was prepared as described for the RT-qPCR analysis. This RNA was then depleted of ribosomal RNA (Ribo-Zero rRNA removal kit for gram-positive bacteria) and the remaining RNA was prepared for Illumina sequencing (TruSeq stranded total RNA preparation kit). This library preparation takes into account the strandedness of the RNA and is able to distinguish between sense and antisense transcripts. The libraries were sequenced on an Illumina NextSeq500 (75 bp single-end). The library preparation and sequencing was performed by the VIB Nucleomics Core facility. The data was analyzed using the standard RNA-seq analysis workflow in CLC Genomics Workbench v7 [50] (Details in Suppl. Methods).

4.5. Laboratory animals

Female C57BL/6J mice (Janvier), C57BL/6J × Balb/c mice (F1, Harlan) and CB-17 SCID mice (Charles River) were housed under specific pathogen-free conditions in micro-isolator 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 ethical committee of Ghent University, Belgium.

4.6. SCID safety study

Eight-week-old female CB-17 SCID mice (Charles River Laboratories, 7 or 8 mice/group as indicated) were immunized intravenously via tail vein injection with 100 μL (3×10^6 versus 3×10^7 cfu) of WT BCG Pasteur or *sapM*::Tn BCG mutant (diluted in PBS). The control group received PBS (pH 7.5). The animals were observed and weighed initially on a ~2-weekly basis and from day 82 post-infection on, ~every 2 days (until day 201 post-infection). The differences of time to survival between the different groups were compared using log-rank test. Weight loss of 20% of initial body weight was set as the ethical endpoint.

4.7. Vaccination with M. bovis BCG

At the age of 8 weeks, female mice were infected s.c. or i.v. with WT BCG or *sapM*::Tn BCG mutant in PBS. At different time points post-infection, mice were sacrificed and organs removed aseptically for further processing and either analysis of CFU counts or DC and T cell phenotyping and antigen-specific cytokine determination (details in Suppl. Methods).

4.8. Statistical analysis

Results are presented as means ± standard error of the mean (SEM) unless otherwise stated and groups were compared using statistical tests as mentioned in the text, using Prism Software (GraphPad Software, San Diego, CA).

Declaration of Competing Interest

The authors declared that there is no conflict of interest

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Complex, Purified Native Protein from *Mycobacterium tuberculosis*, Strain H37Rv, NR-14855.

N.C and N.F. are named inventors on a patent covering the use of SapM mutation as a vaccine improvement strategy.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.05.022.

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