Lipoarabinomannan mannose caps do not affect mycobacterial virulence or the induction of protective immunity in experimental animal models of infection and have minimal impact on in vitro inflammatory responses.

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Running title: Lipoarabinomannan and mycobacterial virulence

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/cmi.12065

Abstract

Mannose-capped lipoarabinomannan (ManLAM) is considered an important virulence factor of *Mycobacterium tuberculosis*. However, while mannose caps have been reported to be responsible for various immunosuppressive activities of ManLAM observed *in vitro*, there is conflicting evidence about their contribution to mycobacterial virulence *in vivo*. Therefore, we used *M. bovis* BCG and *M. tuberculosis* mutants that lack the mannose cap of LAM to assess the role of ManLAM in the interaction of mycobacteria with the host cells, to evaluate vaccine-induced protection and to determine its importance in *M. tuberculosis* virulence. Deletion of the mannose cap did not affect BCG survival and replication in macrophages, although the capless mutant induced a somewhat higher production of TNF. In dendritic cells, the capless mutant was able to induce the up-regulation of co-stimulatory molecules and the only difference we detected was the secretion of slightly higher amounts of IL-10 as compared to the wild type strain. In mice, capless BCG survived equally well and induced an immune response similar to the parental strain. Furthermore, the efficacy of vaccination against a *M. tuberculosis* challenge in low-dose aerosol infection models in mice and guinea pigs was not affected by the absence of the mannose caps in the BCG. Finally, the lack of the mannose cap in *M. tuberculosis* did not affect its virulence in mice nor its interaction with macrophages in vitro. Thus, these results do not support a major role for the mannose caps of LAM in determining mycobacterial virulence and immunogenicity *in vivo* in experimental animal models of infection, possibly due to redundancy of function.

Introduction

The cell envelope of *M. tuberculosis* is considered a major determinant of virulence in this global pathogen. A major component of the cell envelope of all mycobacteria is lipoarabinomannan (LAM) (Briken *et al*., 2004; Chatterjee *et al*., 1998; Gilleron et al., 2008; Nigou, 2003), which appears to play a key role in the interaction with the host, and modulation by the bacterium of the host response (Briken *et al*., 2004; Gilleron *et al*., 2008; Mishra *et al*., 2011; Nigou *et al*., 2002; Nigou *et al*., 2003). This complex molecule of approximately 17 kDa is the largest member of a series of lipoglycans of varying size based on a conserved mannosyl-phosphatidyl-*myo*-inositol anchor. In slow growing mycobacteria, such as *M. tuberculosis*, the presence of one to three mannopyranosyl residues linked to the non-reducing ends of the arabinan domain constitute the mannose cap of LAM (ManLAM), while in fast-growing mycobacteria, such as *Mycobacterium smegmatis*, this latter domain is capped with phospho-inositol residues (PILAM) (Nigou, 2003) and in some species like *M. chelonae*, no such capping motif is present (Guerardel *et al*., 2002). The proportion of LAM non-reducing termini that are capped with mannose varies among different species of slow-growing mycobacteria and among strains of *M. tuberculosis*, with fully virulent *M. tuberculosis* laboratory strains having up to 70% capping (Chatterjee et al. 1992, Khoo et al., 1995). The number of mannose residues per cap is also variable even within LAM from the same species or strain (Nigou, 2003).

A large number of studies have assigned a role in virulence to LAM. Initial studies aiming at determining the role of LAM in mycobacterial virulence addressed the *in vitro* effects of the purified molecules. These studies showed that LAM from *M. tuberculosis* is able to alter macrophage functions associated with protective immunity (Chan *et al*., 1991; Fratti *et al*.,

2003; Kang *et al*., 2005; Pathak *et al*., 2005; Sibley e*t al*., 1988). It was additionally shown that LAM from mycobacteria of different virulences have different immuno-modulatory activities (Adams *et al*., 1993; Bradbury *et al*., 1993; Chatterjee *et al*., 1992; Knutson *et al*., 1998; Roach *et al*., 1993; Vergne *et al*., 2003; Yoshida *et al*., 1997). Numerous studies have so far suggested a role of ManLAM in binding to and in modulating the function of macrophages and dendritic cells (Chan *et al*., 1991; Fratti *et al*., 2003; Geijtenbeek *et al*., 2003, Kang *et al*., 2005; Maeda *et al*., 2003; Nigou *et al*., 2001; Schlesinger, 1993; Schlesinger *et al*., 1994; Sibley e*t al*., 1988; Tailleux *et al*., 2003; Torrelles *et al*., 2006; Vergne *et al*., 2003; Welin *et al*., 2008).

The studies above have led to the conclusion that ManLAM is an important virulence factor in tuberculosis, and that mannose capping plays an essential role. However, interpretation of these experiments is complex, with the experiments comparing ManLAM from fully virulent *M. tuberculosis* either with ManLAM from strains with a lower proportion of mannose capping, or with PILAM (Khoo *et al*., 1995). It is therefore important to carry out experiments directly comparing LAM produced by isogenic strains differing only in their terminal mannosyl decoration.

Enzymatic removal of mannose residues with α-mannosidase revealed that the inhibition of IL-12 secretion by human dendritic cells caused by BCG ManLAM is strictly dependent on an intact molecule (Nigou *et al*., 2001). However, to unambiguously study the role of mannose capping of LAM in an infectious setting, we have been using genetically engineered mutants in which the cap is not added. A mannosyltransferase encoded by the *M. tuberculosis capA* gene (*rv1635c*) is responsible for the addition of the first mannose residue of the mannose cap in an α(1→5) linkage (Dinadayala *et al*., 2006). Previously, we identified homologous enzymes in *M. marinum* and *M. bovis* BCG (Appelmelk *et al*., 2008). Disruption of the *capA* gene resulted

in bacteria deficient in the biosynthesis of the mannose cap of LAM (Appelmelk *et al*., 2008; Dinadayala *et al*., 2006).

When we tested capless mutants of *M. marinum* and *M. bovis* BCG *in vitro* and *in vivo* (Appelmelk *et al*., 2008), we obtained the surprising result that there was no evidence for an altered host-pathogen interaction, and that capless *M. marinum* and BCG mutants were not less virulent than their respective parent strains. Thus, the data obtained with live isogenic strains were discrepant with the mass of data obtained earlier with purified ManLAM.

Here we follow up on our previous study of the role of the mannose cap further using isogenic strains differing only at the *capA* locus: we (1) look at the role of the mannose cap in the protective efficacy of BCG in vaccination studies, and (2) extend our studies on virulence to look at *M. tuberculosis* itself.

M. bovis BCG, the vaccine for tuberculosis protects well against disseminated TB, including meningitis in childhood, but protection against adult pulmonary TB is highly variable, ranging from 80% to no protection at all (reviewed by Colditz *et al*., 1994), and a more effective vaccine is therefore required. As ManLAM has been reported as blocking phagolysosome fusion, and/or inducing IL-10, and LAM lacking mannose caps does not exhibit these activities, we hypothesized both that a capless mycobacterium would induce a different type of immune response, being less immunosuppressive and thus more protective. We have therefore tested a capless BCG as a vaccine both in a low-dose challenge model in mice, in contrast to the high-dose challenge model used by Festjens *et al*. (2011) and in the guinea pig model.

To date, *in vivo* virulence studies have been carried out with capless mutants of BCG and *M. marinum*, but not *M. tuberculosis* itself. Here we test the virulence of capless *M. tuberculosis* in a low dose aerosol model.

Results:

We first studied a capless mutant of BCG. This strain has already been used in an earlier publication where it was fully characterized and shown to synthesize LAM devoid of mannose caps (see supplementary figure 4 from Appelmelk *et al*., 2008). Here we extended the analysis of its interaction with the host cells initially in *in vitro* assays using cultured macrophages and dendritic cells and subsequently in *in vivo* experiments.

Response of mouse macrophages to wild type BCG and capless BCG mutant.

Macrophages are in the first line of cellular defense against mycobacterial infection. However, as intracellular pathogens, mycobacteria have evolved strategies to manipulate the host cell mechanisms responsible for their killing. ManLAM inhibits the maturation of phagosomes (Fratti *et al*., 2003; Vergne et al., 2003) and prevents macrophage activation by IFNγ (Chan *et al*., 1991; Sibley e*t al*., 1988). To assess the importance of the mannose caps in BCG survival and macrophage activation, we infected bone marrow-derived macrophages (BMDM) from Balb/c mice with either the parental BCG strain or the capless mutant strain and measured mycobacterial growth and the production of TNF and reactive nitrogen species by the macrophages. No differences in phagocytosis were observed between the mutant and parental BCG, with both BCG strains being internalized to the same extent following exposure of the macrophage monolayers to similar multiplicity of infection (MOI=2) leading to similar CFU counts at time 0 of infection, i.e. after 4 hours of contact of the macrophages with the inocula **(Fig 1A).** Both strains replicated in macrophages with similar growth rates **(Fig 1A).** Activation of the macrophages with IFNγ or IFNγ plus TNF caused macrophages to kill BCG but again both BCG strains behaved in the same manner **(Fig 1B)**. Despite the same

degree of replication in resting macrophages and the similar bacterial loads found at the end of the experiment, the mutant induced higher amounts of TNF secretion at day 7 of infection **(Fig 1C)**. The production of reactive nitrogen species was also assessed, with both BCG strains inducing the same amount **(Fig 1D).** These data show that the lack of mannose caps in LAM leads to different signaling in infected macrophages but has no consequences on the ability of BCG to proliferate or survive intracellularly.

Response of mouse dendritic cells to wild type BCG and capless BCG mutant

Dendritic cells (DCs) are key players in the induction of cellular immune responses against mycobacteria (Murray, 1999). It has been shown that ManLAM (but not non-capped LAM) prevents human DC maturation, inhibits the production of the pro-inflammatory cytokines IL-12 and TNF (Nigou *et al.*, 2001; Nigou *et al.*, 2002) and triggers the production of the immunosuppressive cytokine IL-10 in lipopolysaccharide (LPS)-activated DCs (Geijtenbeek *et al*., 2003). To evaluate the effect of the mannose capping of LAM in the activation and modulation of DC function, we infected bone marrow-derived dendritic cells (BMDC) from Balb/c mice with the parental BCG or the mutant strain and measured the induction of co-stimulatory molecules and the production of cytokines released into the culture supernatants. Both wild type and mutant BCG activated DC and induced the same extent of up-regulation of co-stimulatory molecules (MHC-II, CD40 and CD86) **(Fig. 2A)**. They also induced the secretion of the same amount of IL12p70 and TNF but, interestingly, the mutant strain induced higher amounts of IL-10 **(Fig. 2B)**. Thus both in macrophages and DC, mannose capping of LAM affects cytokine induction.

Capless BCG mutant replicates and induces identical immune responses *in vivo* as compared to the wild type strain.

As described above, ManLAM is proposed to be a key molecule in mycobacterial virulence (reviewed by Nigou, 2003; Briken *et al*., 2004) and the mannose capping of LAM may be related to an immunosuppressive activity of this lipoglycan impacting on vaccine efficacy of BCG. We therefore wished to determine the protective efficacy of the capless BCG strain.

Before testing the protective abilities of the parent BCG and its capless mutant we compared their replication *in vivo*, and the immune response they induced. In order to compare the growth of wild type and capless BCG, Balb/c mice were intravenously infected with $5x10^4$ CFU of either the parental strain or the capless mutant and groups of five mice were sacrificed at days 1, 10, 20, 30 and 60 post-infection. No differences in mycobacterial loads were observed for the two strains in either the spleen or the liver **(Fig.3A).** In the spleen, both BCG strains proliferated until around day 20, with mice reducing the bacterial load afterwards, while in the liver, the growth of the parent and mutant strains was slowly controlled after day 10, with bacterial numbers decreasing over time, as has been classically described (Gheorghiou et al., 1985).

We next took spleen cells from infected mice, and stimulated them *in vitro* with BCG antigens to assess cytokine responses. Cells from capless mutant-infected animals released the same amount of IFNγ, TNF and IL-10 as cells from mice infected with the wild type strain **(Fig 3B)**. No differences were found in the number of several different immune cell populations **(table 1).**

These data do not substantiate our hypothesis that a capless BCG would induce a different type of immune response.

Wild type and capless BCG induce the same level of protection against a *M. tuberculosis* challenge in a murine low dose aerosol infection model.

We proceeded to carry out a protection study comparing BCG and its capless mutant. Festjens *et al*. (2011) used a high dose ($5x10^4$ CFU intravenous or $2x10^5$ CFU intratracheal) virulent *M. tuberculosis* challenge to assess the protection afforded by wild type or capless BCG. We reasoned that this high dose might overwhelm host immunity and hence mask protective efficacy, and therefore used a low dose exposure aerosol challenge with virulent *M. tuberculosis* to compare the protection afforded by either wild type BCG or its capless mutant.

Balb/c mice were intra-dermally inoculated with $5x10^4$ CFU of the parental or the mutant strain and the dissemination of BCG to the lung and spleen was determined 70 days post-vaccination. Very low numbers of bacteria were detected in the spleen and no CFUs could be detected in the lung of both vaccinated groups (**Fig 4A**), indicating a poor dissemination of BCG after intradermal inoculation. We then determined if a specific immune response could be observed in the lung of these vaccinated mice. Seventy days post-vaccination, leukocytes isolated from the lungs of both immunized groups produced the same amounts of the protective cytokines IFNγ, TNF and IL-17 after *in vitro* re-stimulation with BCG antigens (**Fig 4B**). Furthermore, both BCG strains induced the same number of $CD4^+IFN\gamma^+$ producing cells (**Fig 4C**), showing that both the parental strain and the capless

mutant induce a similar type of immune response in the lung. No differences in cytokine production by spleen cells from either group were detected (data not shown).

Seventy days post-vaccination, the two groups of immunized mice and a control group of non-immune animals were subjected to a low dose aerosol challenge with a virulent strain *M. tuberculosis* (strain H37Rv) leading to the implantation of <100 CFU in the lung. The lungs and spleens were harvested at different time points and the numbers of CFU were determined. As expected, 30 days after the challenge with *M. tuberculosis*, a significantly reduced bacterial growth was observed in the lungs and spleens of vaccinated mice when compared to unvaccinated controls (**Fig 4D, $p<0.001$**). The capless mutant induced the same level of protection as the wild type strain.

Wild type and capless BCG induce the same level of protection against a *M. tuberculosis* challenge in a low dose guinea pig aerosol model.

Guinea pigs are a key TB vaccine model, as they present pathological features resembling the human disease and, like humans, express CD1b an antigen presenting molecule which is not expressed by mice and that is known to present LAM to antigen-specific T cells (Prigozy *et al*., 1997; Sieling *et al*., 1995). Thus, potential LAM-mediated immunity may not be evident in mice, making tests in the guinea pig model prone to show differences in protection not seen in mice. We thus tested capless vs. WT BCG in a low dose aerosol challenge (10-50 CFU in the lung upon infection) in the guinea pig model. **Figure 5** shows that the capless BCG is not more protective as a capped BCG.

Isolation of a capless *M. tuberculosis* mutant.

The results obtained here as well as the already published information (Appelmelk *et al*., 2008; Festjens *et al*., 2011) point to a redundancy of the mannose cap in BCG and *M. marinum* virulence. However, BCG and *M. tuberculosis* differ drastically in terms of virulence and one could argue that the mannose cap in *M. tuberculosis* may have a more essential role for its virulence than in BCG. We thus studied a *M. tuberculosis* mutant lacking the ability to synthesize the mannose cap to evaluate the role of mannose-capped LAM in *M. tuberculosis* virulence. A capless mutant of *M. tuberculosis* H37Rv was constructed using the pGOAL-pNIL procedure (Parish *et al*., 2000) exactly as described for *M. bovis* BCG, including isolation of the complementant (Appelmelk *et al,* 2008). Supplementary Figure 1 provides genetic evidence that indeed *Rv1635c* was disrupted in the mutant and that in the complementant we succeeded in the reintroduction of an intact copy of this gene.

Mild acid hydrolysis of purified LAM followed by analysis of liberated oligosaccharides by capillary electrophoresis (Nigou *et al*., 2000) provided evidence that all cap motifs were missing (see Supplementary Figure 2). We investigated if differences in glycosylation, other than the absence of the cap, were present in the knockout by two independent approaches, i.e. SDS-PAGE followed by immunoblotting with glycosylation-specific probes (Supplementary Figure 3) and, secondly, mass spectrometric analysis of isolated PIMs (Supplementary Figure 4). No evidence for differences in glycosylation other than in the cap was found.

Wild type and capless *M. tuberculosis* show similar survival in murine macrophages and induce similar amounts of TNF and NO in vitro.

We tested the interaction of wild type and capless *M. tuberculosis* with Balb/c macrophages. As shown in **Figure 6A** the phagocytosis and growth rate of the two strains in resting BMDM was similar. The restriction of their growth in macrophages following activation by cytokines was also of the same extent (**Figure 6B**). The induction of TNF secretion and nitrite production did not differ significantly between the two strains (**Figure 6C and D**). Hence, the difference in TNF production observed in capless BCG (see Figure 1 C) was not found for capless *M. tuberculosis*.

Wild type and capless *M. tuberculosis* behave similarly in vivo with regards to replication and cytokine induction.

The *M. tuberculosis* wild type strain, capless mutant, as well as a complemented strain were then used for *in vivo* infection studies. Balb/c mice were infected by the low-dose aerosol route. Infection resulted in the implantation of 10 to 25 bacilli in the lungs of each animal. Mice were sacrificed at days 1, 60 and 120 post-infection and the bacterial loads in the lung and spleen determined. The course of the infection is presented in **Figure 7A** and shows that all strains replicated to similar extents in both organs, with mice stabilizing the infection after day 60 post-infection in both the lung and the spleen at similar bacterial loads. In addition, an identical immune response was observed for all the strains, with the production of similar amounts of IFNγ, TNF and IL-17 by spleen cells of mice with 60 days of infection **(Fig. 7B)**. No IL-10 was detected in culture supernatants (data not shown). We extended this study to the widely used C57/BL6 mouse strain. Mice were infected as previously described and sacrificed at days 1 and 120 post-infection. Results are expressed as

"log CFU increase" corresponding to the difference of growth, in log CFU between day 1 and day 120**.** Like in the Balb/c mouse model, there were no significant differences in growth between the parental and the capless strain **(Fig. 7C)**.

Discussion:

The cell envelope of *M. tuberculosis* plays an important role in the pathogenesis of tuberculosis. For years, the mannose-capped lipoarabinomannan (ManLAM) has been considered a key factor in host cell recognition and immunomodulation. The present work questions the importance of mannose capping of LAM as a requirement for virulence by showing that the *in vivo* growth in mice of mycobacteria (*M. bovis* BCG and *M. tuberculosis*) that are deficient in the biosynthesis of the mannose cap is not affected, and that a BCG mutant lacking mannose caps is as effective as a vaccine as its capped parent strain in low-dose (<100 CFU) aerosol models in mice and guinea pigs. These data are surprising since mannose capping of LAM is found in slow growing mycobacteria, amongst which are many pathogenic species, but is (almost completely) lacking in fast growing, environmental non-pathogenic species.

It has been shown *in vitro* that, at the level of the macrophage, purified ManLAM interferes with phagosome maturation (Fratti *et al*., 2003; Vergne *et al*., 2003) and IFNγ-dependent activation (Chan *et al*., 1991; Sibley e*t al*., 1988). ManLAM inhibits IL-12 secretion by LPS-stimulated human dendritic cells (Nigou *et al*., 2001). In addition, ManLAM also prevents DC activation as measured by CD80, CD83 and CD86 expression and triggers secretion of IL-10 by LPS primed human DCs through DC-SIGN binding (Geijtenbeek *et al*., 2003). Together these data demonstrate an immunosuppressive activity of

this lipoglycan and suggest that a capless *Mycobacterium* would be attenuated and would replicate less well in macrophages. Importantly, these studies were all done with purified ManLAM, not live bacteria.

Our *in vitro* findings comparing BCG with isogenic capless BCG showed that mannose capping of LAM is not crucial for mycobacteria survival in macrophages (Fig. 1A,B), with the capless BCG mutant growing exactly as the wild type strain both in non-activated and in activated bone marrow-derived primary murine macrophages (Fig. 1B) Our data are in agreement with two other studies where capless BCG replicated as well as its parent in either human THP-1 macrophages (Appelmelk *et al*., 2008) or in the murine macrophage cell line Mf4/4 (Festjens *et al*., 2011). Hence, the prediction that the mannose cap of LAM would influence mycobacterial survival does not come true in mice. With regard to cytokine induction *in vitro*, no difference was seen for IL-12p70 (Fig. 2B); for TNF, a difference was seen after 7 days only (Fig. 1C and 2B); in contrast to data obtained with purified ManLAM and non-capped LAM (Geijtenbeek *et al*., 2003), BCG with capless LAM induced more IL-10 than parent BCG (Fig. 2B). Also *in vivo*, after intravenous injection in C57/Bl6 mice (Fig. 3), capless BCG was not attenuated as compared to parent strains, confirming earlier data following intranasal challenge in C57/Bl6 mice (Appelmelk *et al*., 2008) or intravenous injection in Balb/c mice (Festjens *et al*., 2011). Altogether these data show that studies with live capless LAM mutant bacteria yield data conflicting with those obtained with purified LAM. This could be due to several reasons. First, to show enhanced IL-10 (Geijtenbeek *et al.,* 2003) or decreased IL-12 (Nigou *et al.*, 2001; Pathak *et al.,* 2005) production by purified ManLAM as compared to LAM without mannose caps, DC and macrophages were primed by the TLR4 ligand LPS; however, live mycobacteria are poor triggers of TLR4 signaling,

(Reiling *et al*., 2008) and hence, not surprisingly, live mycobacteria do not recapitulate the behavior of purified ManLAM. Second, we assume there is redundancy for the role of ManLAM. For example, binding of BCG to DC is dominated by DC-SIGN ligand interactions (Appelmelk *et al*., 2008; Geijtenbeek *et al*., 2003, Geurtsen *et al*., 2009) and when the cap, i.e. one of the ligands, is removed, binding to DC stays fully intact as a sufficient number of back-up ligands remains available e.g., PIMs (Driessen *et al*., 2009), lipomannan and glycoproteins (Pitarque *et al*., 2005). We conclude that ligands other than the mannose cap determine binding to DC, and assume that a similar redundancy exists for other effects of ManLAM, such as the inhibition of phagolysosome fusion.

A major goal of our studies was to evaluate the potency of capless BCG as a vaccine in a low dose *M. tuberculosis* aerosol challenge model. Based on the ability of ManLAM to induce immunosuppressive IL-10 (Geijtenbeek *et al*., 2003) one could expect that a capless BCG might be a more effective vaccine than parent BCG. Using a high dose murine *M. tuberculosis* challenge models ($5x10^4$ CFU intravenously or $2x10^5$ CFU intratracheally), Festjens *et al.* (2011) showed that prior immunization with a capless BCG (10^5 CFU subcutaneously) indeed appeared to be more protective than parent BCG: mean survival time increased from 26.5 to 27.5 wk (intravenous challenge) and from 48 to 52 wk (intratracheal challenge); also after capless BCG immunization and i.v *M. tuberculosis* challenge, the weight loss was delayed as compared to immunization with parent BCG. However, the differences in protection between capless BCG and parent strain were small and not statistically significant (N. Festjens, pers. communication). We reasoned, based on early experience, that differences in immune protection could become more evident in a low dose challenge model (Appelmelk *et al*., 1986). The low dose aerosol *M. tuberculosis* infection

model is currently considered to be the golden standard to evaluate protective efficacy of tuberculosis vaccines. In the aerogenic model, infectious doses are as low as 10-25 CFU. The outcome of our studies (Fig. 4A) is that BCG and capless BCG hardly disseminate to the spleen or lung after intradermal injection, that (Figs. 4B and C) immune parameters following immunization were similar for both vaccines, and most importantly, the capless BCG had a protective efficacy identical to its parent strain (Fig. 4D).

A second goal was to evaluate capless BCG in a particularly susceptible host, the guinea pig, an animal species which is also able to present the glycolipid LAM to T cells. Specialized CD1b lipid antigen presenting molecules are present in humans, but are lacking in mice. ManLAM has long been known to be presented by CD1b to antigen-specific T cells (Sieling *et al*., 1995), and hence in mice protection mediated by LAM-specific CD1b-restricted T cells will not be evident. Guinea pigs in contrast express CD1b. In addition, unlike in mice, experimental tuberculosis in the guinea pigs causes caseating granulomas. Hence, as compared to mice the guinea pig model is seen as more representative of human disease and is an essential step in human tuberculosis vaccine development. To mimic natural disease, the aerogenic challenge route was again chosen. Fig. 5 shows that no differences in protection to *M. tuberculosis* infection were seen in this model following vaccination with the capless BCG or the parent strain.

A final goal was to test the role in virulence of the mannose cap of *M. tuberculosis*, a species not tested hitherto. In vitro, we found no differences between wild type and capless *M. tuberculosis* with regards to replication rate in mouse macrophages, susceptibility to cytokine activated macrophages, and induction of TNF and nitrite secretion. To maximize the possibility to observe potential LAM-mediated immunomodulatory effects, we challenged

via the aerogenic route two immunologically contrasting mouse strains, i.e., Balb/c (a Th2-skewed strain) and C57Bl/6 (a Th1-skewed strain) (see Fig. 7A and 7B, respectively). However, in both mouse strains capless *M. tuberculosis* and parent strain proliferated equally well.

Altogether our data suggest that the dominant role attributed to the mannose cap of LAM, which was predominantly based on *in vitro* studies with purified ManLAM (Mishra *et al*., 2011) cannot be confirmed by in vivo studies in mice with isogenic pairs of mutants. The cap does not affect immunoprotection by BCG, nor does it affect virulence of pathogenic mycobacteria in mice. One explanation for this lack of effect is that the role of the cap might be redundant as discussed above. Still, the outcome of our studies is puzzling: we investigated, within the taxonomic tree of the genus *Mycobacterium*, which species expressed a mannose-capped LAM and we found it almost exclusively in slow growing species, many of which are pathogens; in fast growing, often environmental species, the cap was mostly lacking (Driessen and Appelmelk, unpublished). This suggests evolutionary pressure to preserve the cap. It cannot be excluded that the cap albeit not relevant in the biological assay systems tested so far with live mycobacteria, may still be important for example for transmission of *M. tuberculosis* from one person to another. As the DC-SIGN system in humans differs strongly from that of mice (Park *et al*., 2001), this aspect of tuberculosis is not accessible to animal experimentation; possibly, non-human primates mimic the human DC-SIGN system more accurately.

In short, together with two earlier studies, our novel data provide overwhelming evidence that the mannose cap of lipoarabinomannan does not dominate the interaction of mycobacteria with the three experimental animal hosts (zebrafish, mice and guinea pigs)

tested.

Experimental procedures:

Mycobacterial strains and growth conditions:

The BCG *capA* mutant, lacking the mannose cap of LAM, has been described and characterized before (Appelmelk *et al*., 2008). The *capA* mutant in *M. tuberculosis* H37Rv has not been described before and was constructed in a similar way via a two-step p1NIL-pGOAL19 approach developed by Parish and Stoker (Parish *et al*., 2000), leading to a markerless deletion in the gene of interest (*rv1635c*). In fact, exactly the same plasmids were used to genetically modify BCG and *M. tuberculosis*: the DNA sequences of Rv1635c and its BCG homolog are 100% identical. Briefly, after the second step (sucrose selection) of the p1NIL-pGOAL procedure fourteen colonies were picked. The presence of this deletion was investigated by PCR. Phenotypically, the lack of mannose caps was investigated in immunoblot. In this colony dot blot, eleven of the colonies were non-reactive with the cap-specific Mab 55.921A (Appelmelk *et al*., 2008) and three were reactive. In PCR those three yielded a *rv1635c* product, which was absent in the other eleven colonies. We concluded that of the fourteen colonies picked, three were revertants and eleven were (markerless) mutants lacking Rv1635c. These three mutants were investigated in SDS-PAGE-Immunoblot with monoclonal antibodies (MAbs) F30-5, 183-24, and 55.92.1A1, concanavalinA, and DC-SIGN-Fc and further evidence was obtained they missed the cap (Supplementary Figure 3). Finally, one of the capless colonies was further investigated in capillary electrophoresis again providing evidence the cap is missing (Supplementary Figure 2). A complementant was also made: *rv1635c* was cloned into the shuttle vector pSMT3 and the construct electroporated in

the *capA* mutant of *M. tuberculosis* H37Rv. *M. bovis* BCG Copenhagen and mutant CapA were grown in liquid Middlebrook 7H9 broth (Difco) supplemented with 0.05% Tween-80, until the log phase. The H37Rv *M. tuberculosis* wild type, capless mutant and complemented strain were grown in Proskauer-Beck medium (PB) containing 0.05% Tween 80 to mid–log phase. Cultures were aliquoted and frozen at –80°C until the day of use.

Chemical analysis of the mannose caps:

LAM from *M. tuberculosis* wild-type and *M. tuberculosis* capless mutant were analyzed for presence of the mannose cap by the capillary electrophoresis technique described earlier (Nigou et al, 2000). Briefly, purified LAM was partially degraded by controlled acid hydrolysis (0.1M HCl for 20 min. at 110°C), and the oligosaccharides liberated tagged with the fluorescent label 8-aminopyrene-1,3,6-trisulfonate (APTS). During CE, the labeled oligosaccharides are separated and peaks are detected by laser-induced fluorescence and migration times compared with the appropriate controls.

Analysis of purified PIMs by Mass Spectrometry.

Lipids were obtained by chloroform/methanol extraction of bacteria and subjected to MALDI-TOF MS analysis in the negative ion mode as previously described (Gilleron *et al.*, 2003).

Laboratory mice:

8 week-old female BALB/c and C57/B6 mice were purchased from Charles River (L'Arbresle, France) and housed under specific pathogen-free conditions in our facilities. Sterile chow and tap water ad libitum was given. All experiments were approved by and performed according to the guidelines of the animal ethical committee of IBMC. Five to seven animals were used per experimental group for each time-point.

BCG antigens for cell stimulation:

Mycobacteria antigens were prepared as described elsewhere (Pais *et al*., 2000). BCG was grown until log phase, at 37°C, in Middlebrook 7H9 medium (Difco) supplemented with 10% albumin/dextrose/catalase (ADC) and 0,05% Tween 80. The culture was centrifuged (10,000 x g, 40 minutes, 4°C) and the remaining pellet washed and re-suspended with phosphate-buffered saline (PBS), containing 0.1% Tween 80 (Sigma), 1 mM $MgCl_2$ (Merck, Darmstadt, Germany) and 1 mM benzamidine (Sigma). The bacteria in suspension were disrupted through sonication with pulses of 1 min at maximum power, with the sample kept in ice during the whole procedure. The sonicate was centrifuged, to discard intact mycobacteria (30 min at 2,700 x g), and the supernatant was dialyzed against PBS (molecular weight cut-off of 12,000), followed by ultra-centrifugation for 2 h at 150,000 x g. The remaining pellet, containing the envelope proteins, was re-suspended in PBS, and the supernatant, enriched in cytosolic proteins, was precipitated with 80% ammonium sulfate and dialyzed against PBS. Aliquots were quantified and stored at -80°C until the day of use.

Generation of bone marrow-derived macrophages (BMMØ) and dendritic cells (BMDC):

Bone marrow cells were flushed from the femurs of mice with 5 ml of cold Hanks' balanced salt solution (HBSS; Gibco, Paisley, United Kingdom) using a 26-gauge needle. For macrophage generation, the resulting cell suspension was centrifuged for 10min at 1200rpm, 4ºC; resuspended in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10 mM HEPES (Sigma, St. Louis, MO), 1 mM sodium pyruvate (Gibco), 10 mM glutamine (Gibco), 10% heat-inactivated fetal bovine serum (Sigma), 10% L929 cell conditioned medium (LCCM, as a source of M-CSF) and cultured for a period of 4 h on cell

culture dishes (Nunc, Naperville, IL) in order to remove already differentiated cells. Non adherent cells were then collected with warm HBSS, counted, distributed in 24-well plates at a density of $5x10^5$ cells/well and incubated in 1 ml of similar media at 37°C in a 5% CO2 atmosphere. On day 4 after seeding, 100µL of LCCM was added and on day 7 the medium was renewed. Macrophages were infected at day 10. For dendritic cell differentiation, the cell suspension was cultured at a density of 10^6 cells/ml in RPMI 1640 containing GlutaMAX-I, supplemented with 5% v/v fetal calf serum (FCS), 50 mM β-mercaptoethanol and 10% GM-CSF-containing culture supernatant from transformed J558 cells. Every 2 days, one-half of the media was removed and supplemented with complete medium with GM-CSF. On day 9, the non-adherent cells were cultured at 2 x 10^5 cells/ml in 96-well plate (100 µl/well), and infected at day 10. The purity of the population was determined by FACS analysis of specific surface markers and ranged from 85 to 95%.

Macrophage infection:

A bacterial suspension containing $5x10^6$ CFU/mL was prepared and 200 µl was added to each well to obtain a multiplicity of infection (MOI) of 2 bacteria per macrophage. After 4 h of incubation at 37°C in a 5% CO2 atmosphere, cells were washed with warm HBSS to remove the non-internalized bacteria, and re-incubated in DMEM with 10% LCCM. All treatments (100U IFNγ; 50U TNF) were applied from day zero until day 4. At different time points 100µl of supernatant was collected for subsequent cytokine measurements. For the CFUs assay, the infected cells were lysed with a 10% saponin solution, in sterilized water with 0,05% Tween 80, and serial dilutions of triplicate wells were performed. The number of viable bacteria was assessed by counting the colonies 3–4 weeks after plating on 7H11 Agar medium (Difco) supplemented with 10% oleic acid/ albumin/dextrose/catalase (OADC) and

incubated at 37°C.

Dendritic cell infection:

A bacterial suspension containing $5x10^6$ CFU/mL was prepared and 100 µl was added to each well in order to obtain a multiplicity of infection (MOI) of 2 bacteria per DC. Supernatants were collected at different time points for IL-12p70, TNF and IL-10 cytokine measurements and the levels of expression of co-stimulatory molecules in cells were assessed by flow cytometry.

Cytokine measurement:

Cytokine detection in the supernatants was performed by ELISA. For IFN-γ quantification, affinity-purified monoclonal antibodies (R4-6A2 as capture and biotinylated AN-18 as detecting antibody) were used, while commercial kits were used according to the manufacturer's instructions for the detection of TNF, IL-10 (R&D Systems), IL-12p70, and IL-17A (Biolegend).

Replication *of M. bovis* BCG in mice:

Mice were infected intravenously through the lateral tail vein, with $5x10^4$ CFU *M. bovis* BCG, wild type or mutant, in 200 µL of PBS. Bacterial loads in the organs of infected mice were evaluated at different time points post infection. Organs were homogenized in sterile water with 0.05% Tween 80 and ten-fold serial dilutions of organ homogenates were plated in duplicate onto Middlebrook 7H11 agar plates containing OADC. Plates where incubated at 37°C and colonies were counted 21 days later. Results are expressed as log CFU per organ.

Immunization and TB challenge:

Balb/c mice were immunized by a single intradermal injection with $5x10^4$ CFUs of *M. bovis* BCG, wild type or mutant. Two months later, mice were aerogenically infected with *M. tuberculosis* H37Rv using a Glass-col aerosol generation device chamber (Terre Haute, IN, USA). Briefly, mice were exposed for 30min to an aerosol produced by nebulizing 10 mL of PBS-Tween80 containing 10^6 CFU/ml that resulted in the implantation of 10-25 bacilli in the lung of each animal. Bacterial loads in the organs of infected mice were assessed by plating organ homogenates onto Middlebrook 7H11 agar plates, and counting the colonies formed 14 to 21 days after incubation at 37ºC. Results are expressed as log CFU per organ.

Guinea pig immunization and infection:

Groups of 8 Dunkin–Hartley guinea pigs, weighing between 250 and 300 g (and free of infection), obtained from a commercial supplier (Harlan, UK), were used to evaluate the efficacy of capless BCG compared with BCG Danish 1331 (Statens Serum Institute, Copenhagen, Denmark), both delivered subcutaneously in a single dose at a concentration of 5×10^4 CFU and a negative control (PBS vaccinated) group. Individual animals were identified using subcutaneously implanted microchips (PLEXX BV, The Netherlands). Guinea pig experimental work was conducted according to UK Home Office legislation for animal experimentation and was approved by the local ethics committee.

Animals were infected with a low aerosol dose (10–50 CFU retained dose in the lung) of *M. tuberculosis* H37Rv (Williams *et al*., 2001) 12 weeks after vaccination. Aerosol challenge was performed using a fully contained Henderson apparatus as previously described (Chambers *et al*., 2000; Clark *et al*., 2011; Lever *et al*., 2000) in conjunction with the AeroMP (Biaera) control unit (Hartings *et al*., 2004). Fine particle aerosols of *M.*

tuberculosis H37Rv, with a mean diameter of 2 µm (diameter range, 0.5–7 µm) (Hartings *et al*., 2004) were generated using a Collison nebulizer and delivered directly to the animal snout. The aerosol was generated from a water suspension containing 5 x 10^6 CFU/ml in order to obtain an estimated retained, inhaled dose of approximately 10–50 CFU/lung. The Henderson apparatus allows controlled delivery of aerosols to the animals and the reproducibility of the system and relationship between inhaled CFU and the concentration of organisms in the nebulizer has been described previously (Chambers *et al*., 2000; Clark *et al*., 2011). The challenge system is controlled by an AeroMP: the aerosol management platform controls, monitors, and records all relevant parameters during an aerosol procedure including air flow rate, temperature and relative humidity (Hartings *et al*., 2004). At 4 weeks post-challenge, guinea pigs were killed humanely by intraperitoneal injection of pentabarbitone (Euthatal). Post-mortem, tissues were aseptically removed for bacteriology analysis. Tissues were homogenized in 5 ml of sterile distilled water using a rotating blade macerator system (Ystral, UK). Viable counts were performed on the macerate by preparing serial dilutions in sterile deionized water and plating 100 µl aliquots onto Middlebrook 7H11 + OADC agar (BioMerieux, UK). Plates were incubated at 37 °C for 3 weeks before counting the number of *M. tuberculosis* colonies (CFU).

Cell preparation and *in vitro* stimulation:

Spleens were gently disrupted with the help of a cell glass homogenizer. The resulting cell suspension was passed through a 70 µm nylon cell strainer, in order to remove large pieces and debris. Lung cell suspensions were obtained as follows: thoracic cavities were opened, and sterile PBS was gently injected into the right heart ventricles to perfuse lungs. Lungs were excised, sectioned, and incubated with digestion media (DMEM supplemented

with Collagenase IX (0.7 mg/ml; Sigma) for 30 min at 37°C. The final cell suspension was obtained by passing the digested lung tissue through a 70 µm nylon cell strainer. In all cell suspensions the red blood cells were lysed with a hemolytic solution (155 mM NH_4Cl, 10 mM $KHCO_3$ (pH 7.2)) during 5 min at room temperature. Cells were then distributed into 96-well plates (2.5 x 10^5 cells/well) and incubated in triplicate with different stimuli: DMEM culture medium and 4 µg/ml of ConA (Sigma-Aldrich) as negative and positive controls, respectively, and depending on the experiment, 4µg/ml of BCG extract or $1.25x10^5$ of live *M. tuberculosis* H37Rv bacilli. After 72h of incubation at 37°C in a 5% CO2 atmosphere, the supernatants were collected for cytokine measurement. For intracellular staining, 1 x 10^6 cells/ml were incubated for 4 h at 37°C in the presence of PMA (Sigma-Aldrich) plus ionomycin (Calbiochem) at a final concentration of 25 µg/ml each, followed by an incubation of 2 h in the presence of 0.01 mg/ml brefeldin A (Sigma-Aldrich). Then, cells were fixed, permeabilized and stained with IFNγ-specific antibodies.

Flow cytometry:

Cells were labeled with specific antibodies for CD3 (clone 145-2C11), CD4 (clone RM4-5), CD8 (clone 53-6.7), CD11b (clone M1/70), CD19 (clone 6D5), DX5 (clone HMα2), CD25 (clone PC61), CD86 (clone GL-1), CD40 (clone 5C3), MHC-II (clone M5/114.15.2) from BioLegend, San Diego, CA) and FOXP3 (e-bioscience). Cell populations were acquired in a FACS Calibur instrument equipped with CellQuest software. Data were analyzed using FlowJo software (Tree Star, Ashland, OR).

Statistical analysis:

Data were analyzed by using Student's *t* test. Statistical analyses of guinea pig data were performed using Minitab (version 13.32). The CFU data were analyzed by non-

parametric Mann–Whitney test comparisons to compare median values of the vaccine group with either the saline or BCG control groups.

Acknowledgments:

This work was supported by grant ImmunovacTB, ref. 37388 of the FP6 from the European Union, the NEWTBVAC project, ref. 241745 of the FP7 from the E.U. and by a grant from the Gulbenkian Foundation and TBVI. AAB, GTR, SSG, CN, and SVC were supported by fellowships from Fundação para a Ciência e a Tecnologia (FCT) from the Portuguese Government. F.M. was supported by Wellcome Trust grant 073237. JG is financially supported by the Netherlands Organization for Scientific Research (NWO) through a VENI research grant (016.101.001). AAB is enrolled in the PhD Program in Experimental Biology and Biomedicine (PDBEB), Center for Neuroscience and Cell Biology, University of Coimbra, Portugal. We thank Marion Sparrius, Amsterdam, for technical assistance.

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Figure legends

Figure 1: In vitro interaction of BCG with macrophages. A- Growth of wild type (WT) or capless (CapA) mutant BCG in bone marrow derived macrophages (BMDM) from Balb/c mice. **B-** Survival of WT and capless (CapA) mutant BCG in untreated BMDM or BMDM treated with 50U TNF, 100U IFNγ or both (results expressed as "log CFU increase", that corresponds to the difference of growth, in log CFU, between day 7 and day 0). **C**- Secretion of TNF into the culture medium by BMDM infected with the BCG strains. **D-** Release of nitrite into the culture supernatants by BMDM infected with BCG and treated with IFNγ. Data represent the mean ± 1SD of a representative experiment out of a total of 3 experiments. Statistically significant differences are labeled with an asterisk.

Figure 2: In vitro interaction of BCG with dendritic cells. A- Expression of co-stimulatory molecules in bone marrow-derived dendritic cells infected with WT or capless (CapA) mutant BCG as evaluated by flow cytometry. **B-** Secretion of IL-12p70, IL-10 and TNF by dendritic cells infected with WT or capless (CapA) mutant BCG. Data represent the mean ± 1SD of a representative experiment out of a total of 3 experiments. Statistically significant differences are labeled with an asterisk.

Figure 3: Capless *M. bovis* BCG replicates similarly and induces identical immune responses as compared to the WT strain. A- Proliferation of WT or capless (CapA) mutant BCG in the liver and spleen of BALB/c mice intravenously infected with $5x10^4$ CFUs. **B-** Secretion of IFNγ, TNF and IL-10 into the culture supernatants by splenocytes from the infected animals following in vitro re-stimulation with 4µg/ml of *M. bovis* BCG extract for

72h. A total of 5 to 7 mice per time point were used, and all results are representative of at least two independent experiment.

Figure 4: Capless and wild type *M. bovis* BCG induce the same level of protection to a *M. tuberculosis* challenge in the low dose aerosol mouse model. A- Dissemination of WT and capless (CapA) mutant BCG, after subcutaneous immunization with $5x10^4$ CFU. The number of CFU in the spleen and lung were assessed 70 days after immunization. **B-** Quantification of cytokine responses in the lungs of vaccinated mice. 70 days post immunization, lung cell suspensions were re-stimulated in vitro with 4µg/ml of BCG extract for 72h and cytokines measured in the supernatants. **C-** Number of $CD4^+IFN\gamma^+$ cells in the lungs of vaccinated mice at day 70 post immunization, determined by intracellular cytokine staining of lung cell suspensions re-stimulated in vitro with PMA and Ionomycin. **D-** Protective efficacy of WT versus capless (CapA) mutant BCG in a *M. tuberculosis* challenge. Balb/c mice were immunized with $5x10^4$ CFU of parental or mutant CapA BCG. Two months later, mice were aerogenically infected with approximately 100 CFU of *M. tuberculosis* H37Rv. Mice were sacrificed at days 1, 30, and 90 post-infection and the number of bacteria in lungs and spleens determined. In all experiments a total of 5 to 7 mice per time point were used and all results are representative of at least two independent experiments. Significant values are labeled by an asterisk.

Figure 5: Capless and wild type *M. bovis* BCG induce the same level of protection against a *M. tuberculosis* challenge in the low dose aerosol guinea pig model. Bacterial load of viable *M. tuberculosis* in spleen and lung of guinea pigs was determined. Adult guinea pigs

were infected, sacrificed (8 per group) after 4 weeks and lung and spleen homogenates were plated for enumeration of bacilli (total CFU). Horizontal bars indicate medians after log transformation; error bars indicate range and *p* values represent statistical comparisons (Mann-Whitney test) between BCG WT(closed circles) and PBS (closed squares) control groups and capless BCG (open circles).

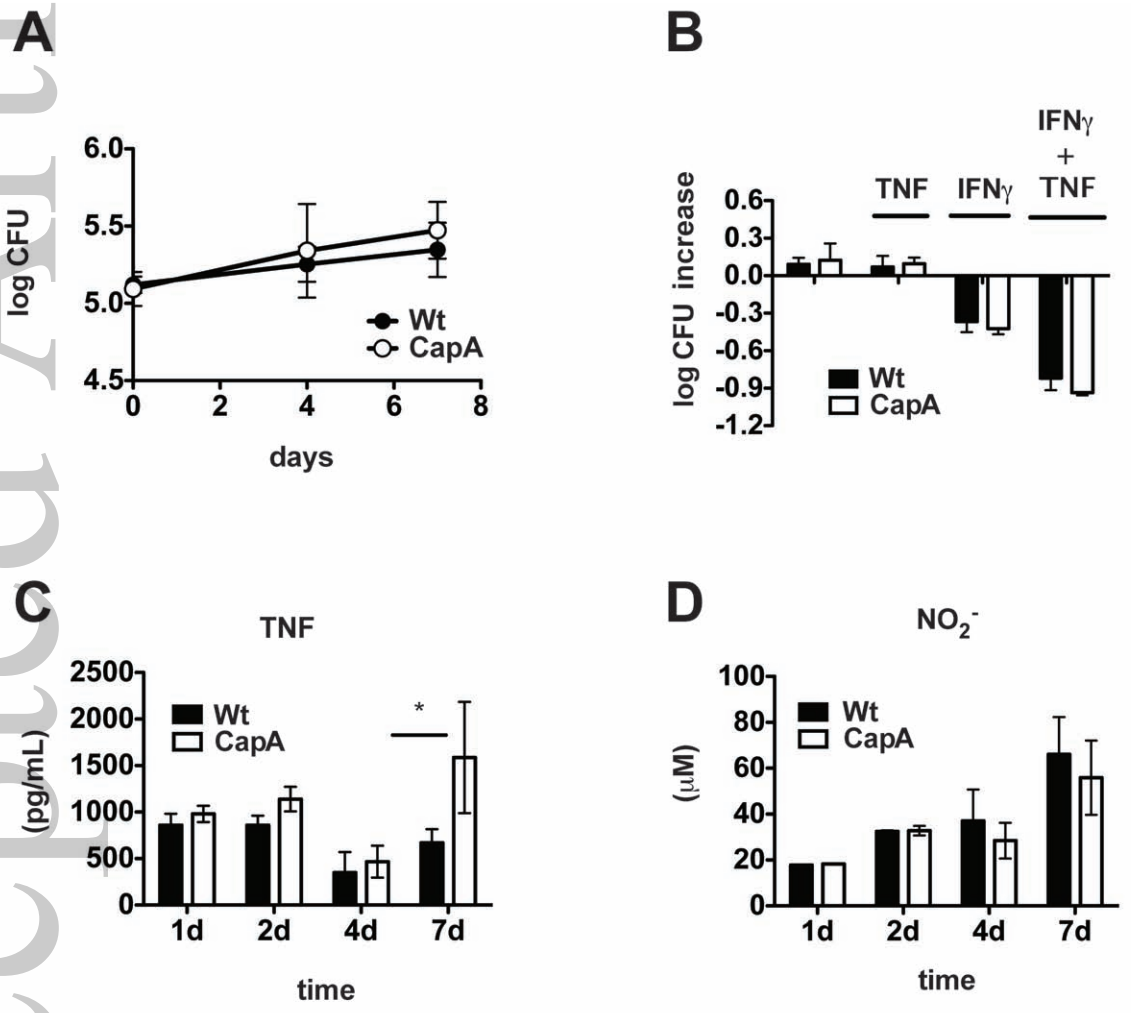
Figure 6: In vitro interaction of *M. tuberculosis* with macrophages. A- Growth of wild type (WT) or capless (CapA) mutant *M. tuberculosis* in bone marrow derived macrophages (BMDM) from Balb/c mice. **B-** Survival of WT and capless (CapA) mutant *M. tuberculosis* in untreated BMDM or BMDM treated with 50U TNF, 100U IFNγ or both (results expressed as "log CFU increase", that corresponds to the difference of growth, in log CFU, between day 7 and day 0). **C**- Secretion of TNF into the culture medium by BMDM infected with the *M. tuberculosis* strains. **D-** Release of nitrite into the culture supernatants by BMDM infected with *M. tuberculosis* and treated with IFNγ. Data represent the mean ± 1SD of a representative experiment out of a total of 3 experiments. No statistically significant differences were found.

Figure 7: Capless and wild type *M. tuberculosis* have the same virulence. A- Proliferation of WT, capless (CapA) mutant and Complementant strains of *M. tuberculosis* in the lung and spleen of Balb/c mice following an aerogenic infection leading to the implantation of 10 to 25 bacilli in the lung of each animal. **B-** Secretion of IFNγ, TNF and IL-17 into the culture supernatants by lung leukocytes from Balb/c mice infected for 60 days and following in vitro re-stimulation with live *M. tuberculosis* bacilli for 72h. **C-** Proliferation of *M. tuberculosis*

WT or capless (CapA) mutant in C57BL/6 mice following an aerogenic infection. Mice were sacrificed at days 1 and 120 post-infection and the bacterial loads in the lung and spleen determined. Results are expressed as "log CFU increase", that corresponds to the difference of growth, in log CFU, between day 1 and day 120**.** A total of 5 to 7 mice per time point were used.

Table 1: *M. bovis* BCG CapA mutant induces identical immune responses as compared to the wild type strain.
Balb/c mice were intravenously infected with $5x10^4$ CFUs of *M. bovis* BCG WT or CapA mutant. The animals were sacrificed at various time points and spleen cells were labeled with specific antibodies for flow cytometric analysis of the splenic cell population. A total of 5 to 7 mice per time point were used, and all results are representative of at least two independent experiments

	Average cells x 10^7 ± SD													
	CD4+		**CD8+**		**CD4+Foxp3+**		**CD19+**		**CD3+DX5+**		**DX5+**		**CD11b+**	
Days	**WT**	**CapA**	**WT**	**CapA**	**WT**	**CapA**	**WT**	**CapA**	**WT**	**CapA**	**WT**	**CapA**	**WT**	**CapA**
10	1.2±0.2	1.2±0.2	0.6±0.1	0.6±0.1	0.2±0.02	0.2±0.02	2.4±0.6	2.1±0.9	0.1±0.01	0.1±0.02	0.2±0.08	0.2±0.07	0.3±0.1	0.3± 0.1
20	1.5±0.3	1.5±0.4	0.7±0.1	0.6±0.1	0.2±0.03	0.2±0.05	3.6±0.7	3.6±0.9	0.1±0.02	0.1±0.03	0.3±0.08	0.3±0.09	0.6±0.2	0.6± 0.2
30	1.8±0.4	1.5±0.1	0.6±0.2	0.5±0.1	0.3±0.06	0.2±0.03	3.9±0.6	3.3±0.7	0.1±0.03	0.1±0.02	0.4±0.04	0.4±0.07	0.7±0.2	0.6± 0.1
60	1.6±0.2	1.4±0.3	0.6±0.1	0.5±0.1	0.2±0.03	0.2±0.03	4.1±0.6	3.4±0.5	0.1±0.04	0.1±0.03	0.3±0.07	0.3±0.03	0.8±0.1	0.6± 0.1



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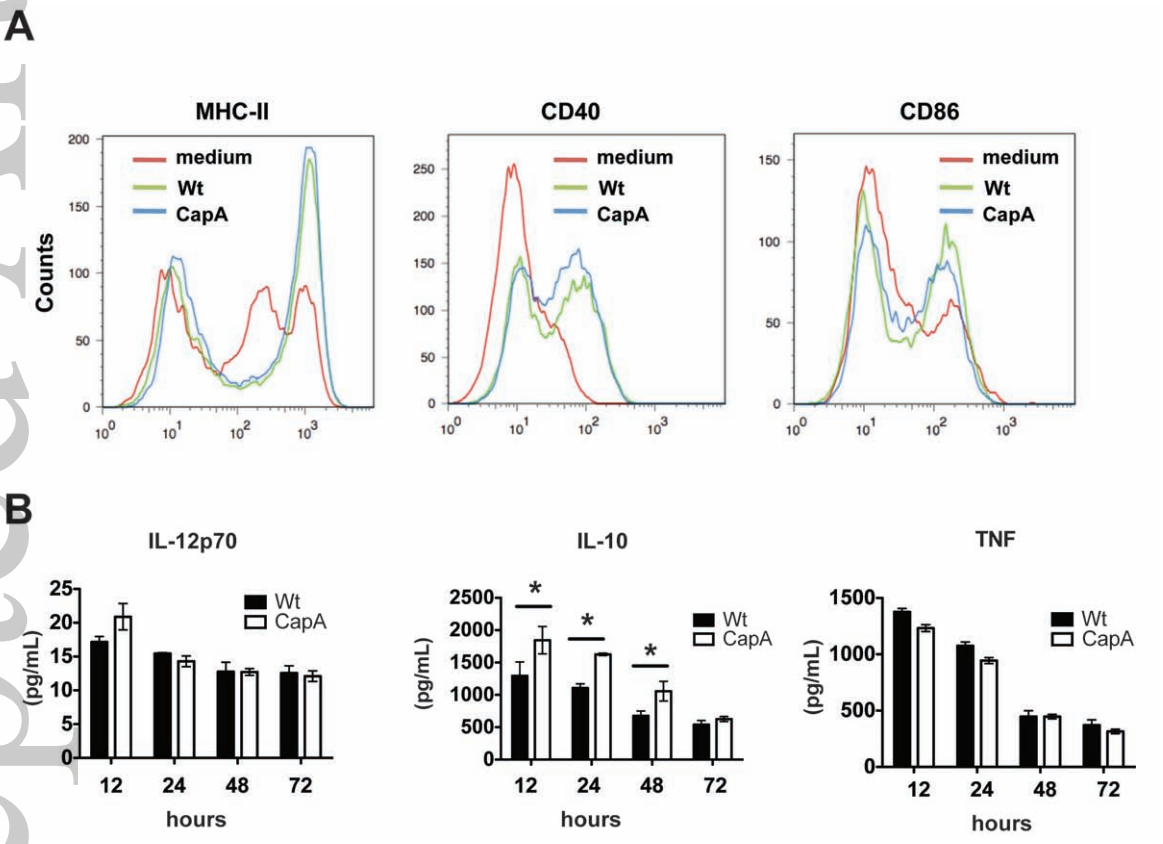

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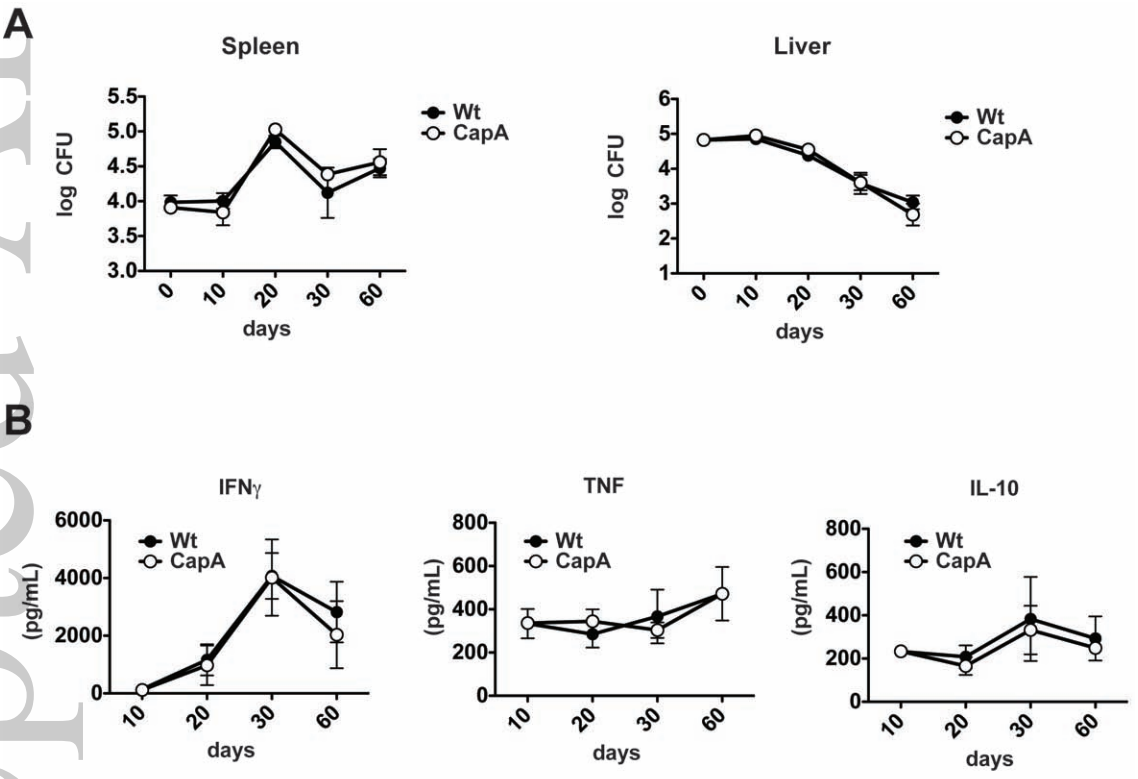

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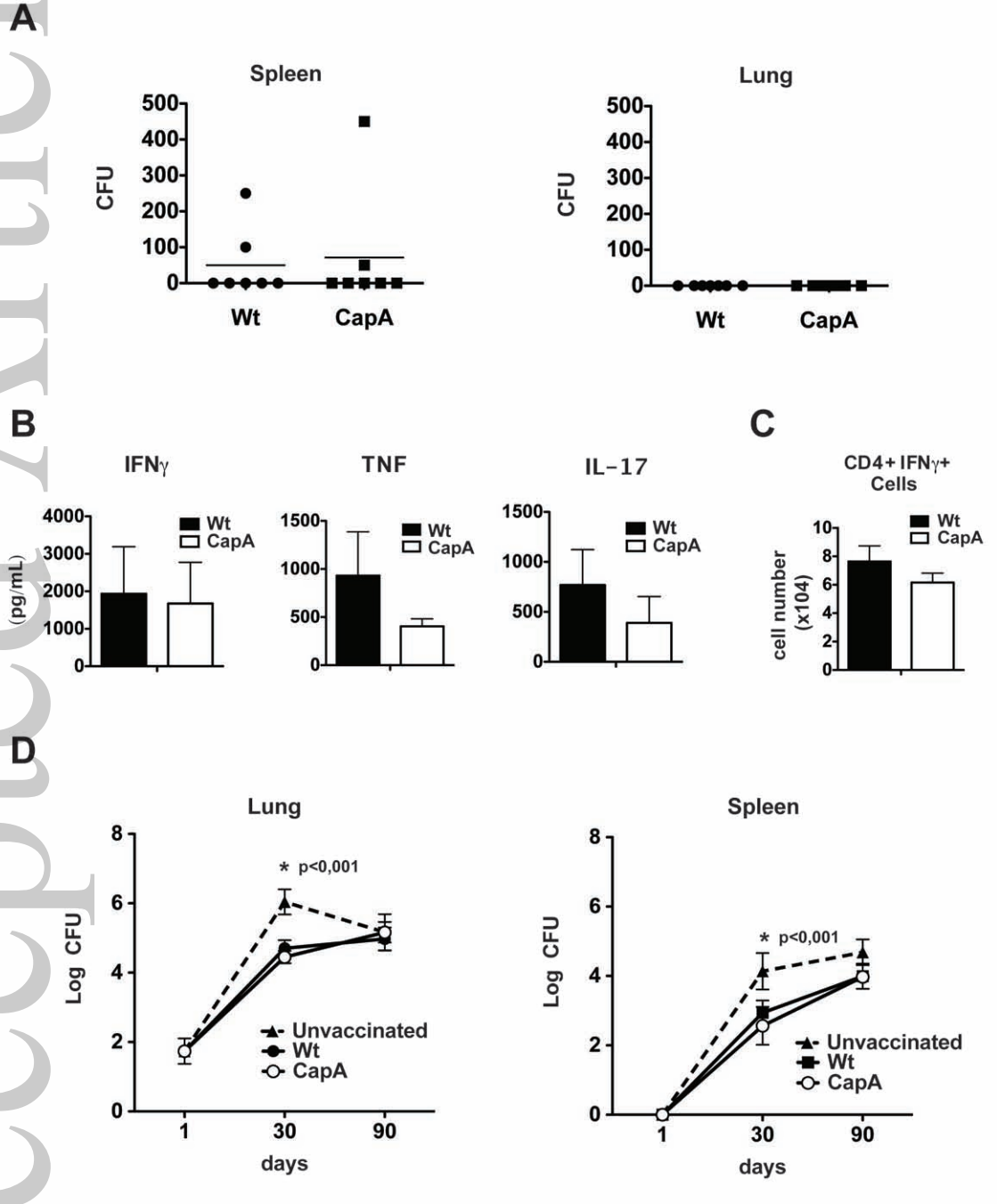

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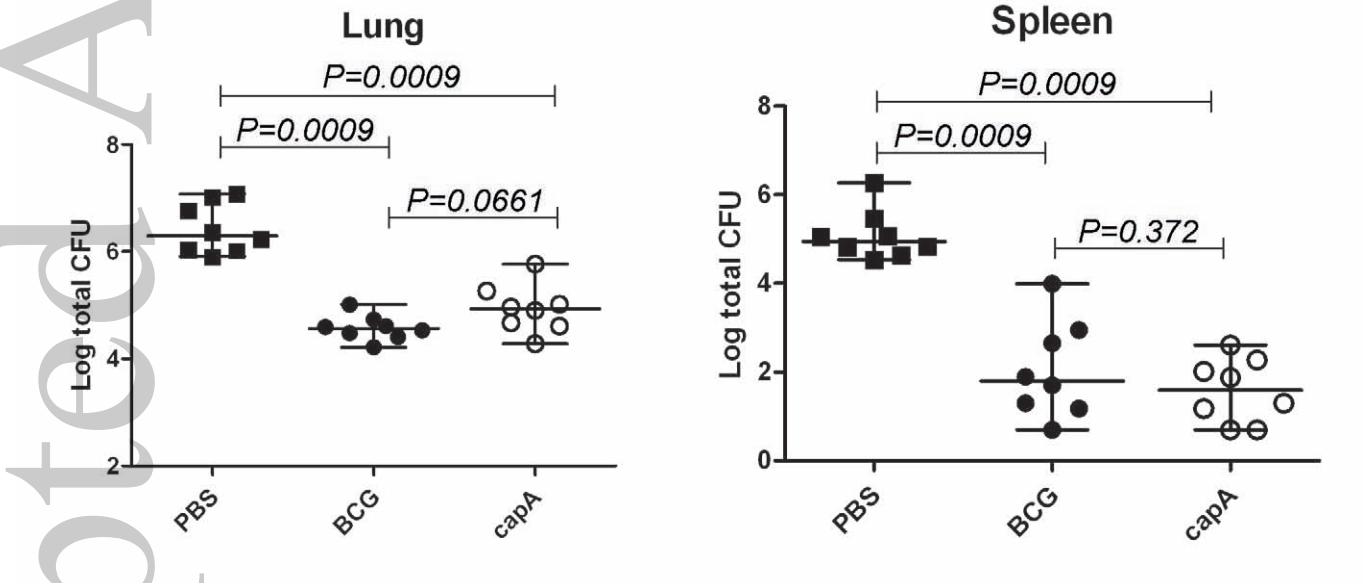

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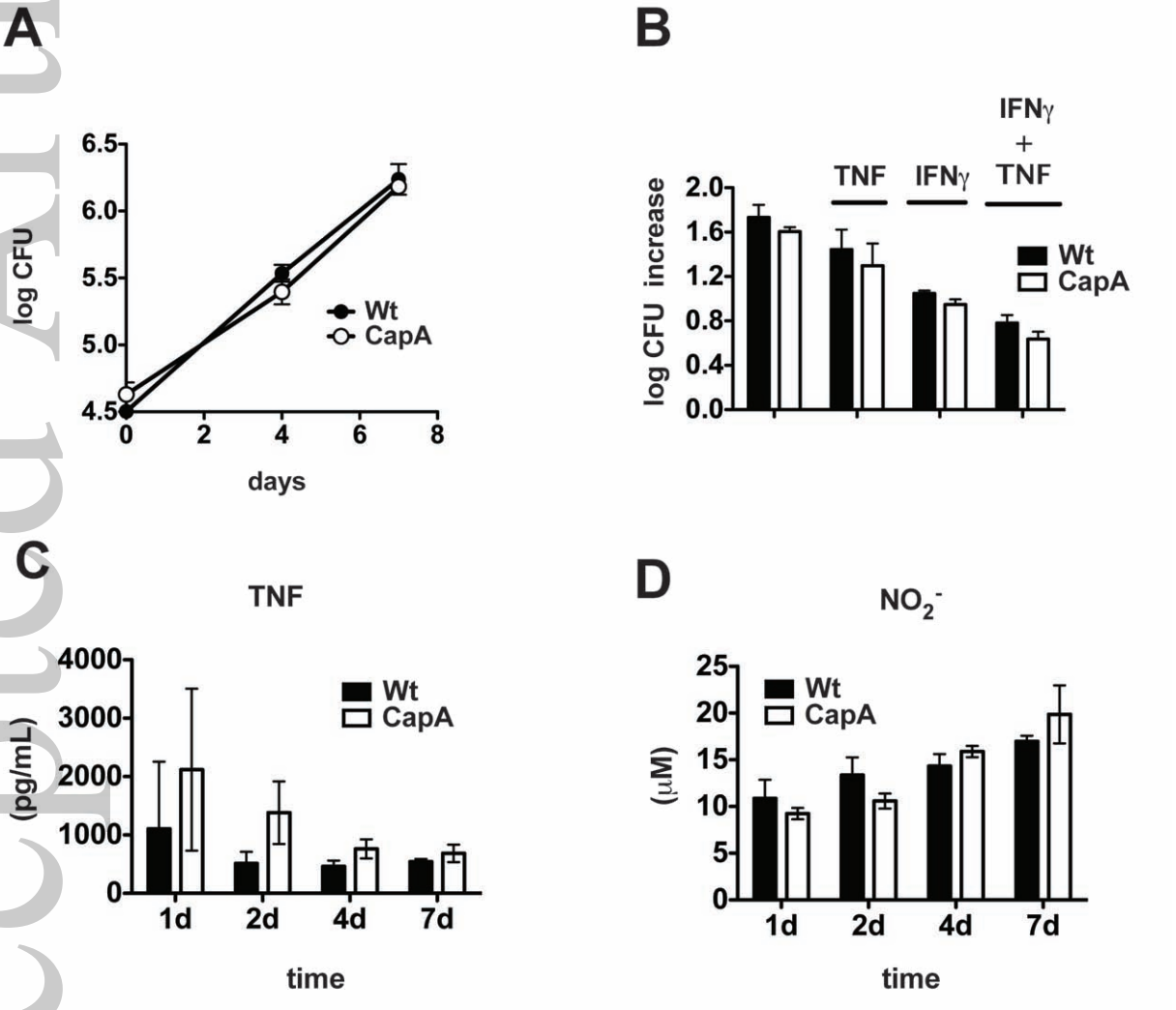

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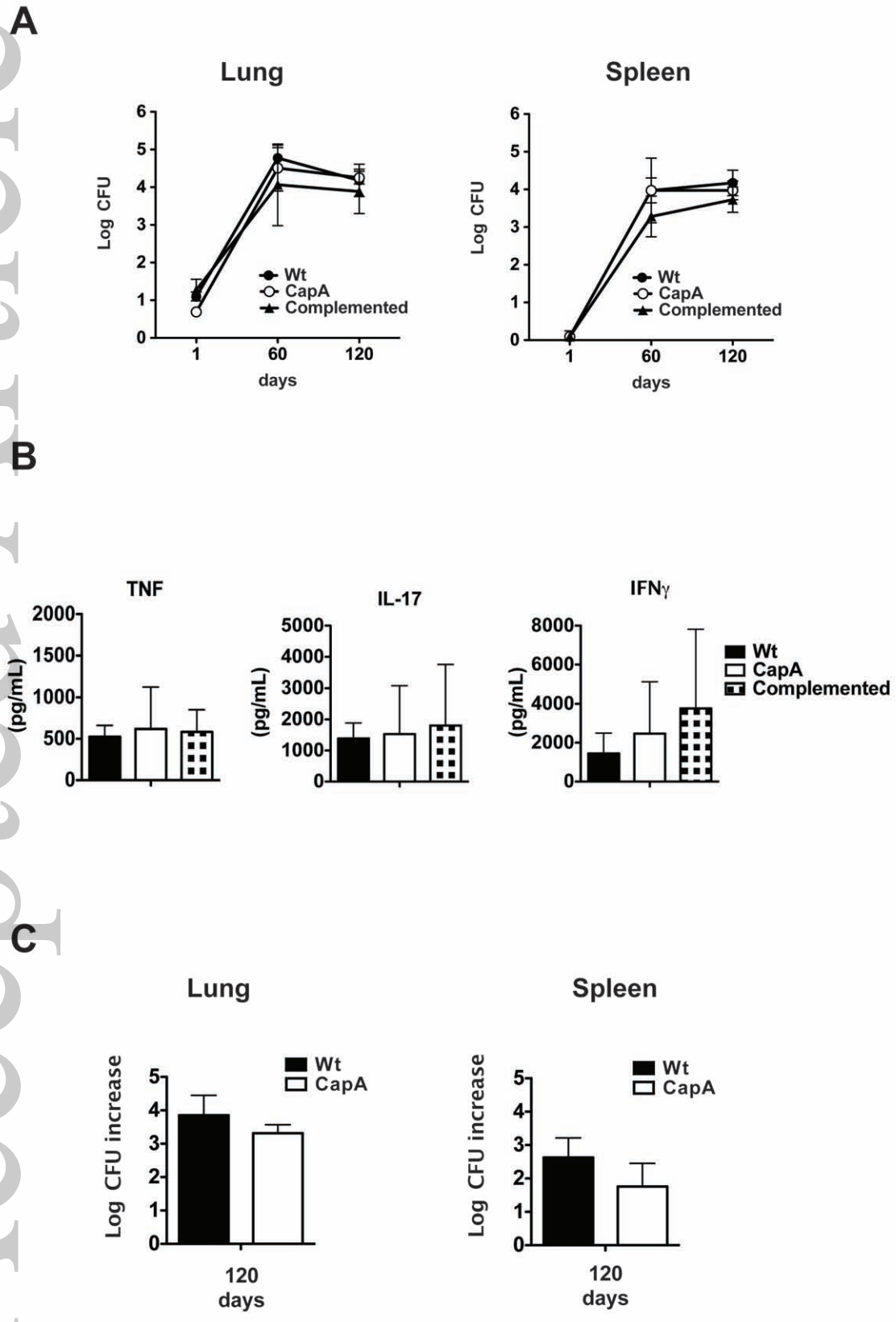

Figure 7.tif