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TITLE: Effect of Variations in BCG Vaccine Capsule Polysaccharides on Immunogenicity and Protective Efficacy

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14. ABSTRACT This project sought to address 3 questions: 1) If genetically distinct BCG vaccine sub-strains, of which several are in use in different parts of the world, produce variable amounts of capsular polysaccharides. 2) If these variations correlate with differential activation of host innate and adaptive immune responses. 3) If the judicious changes to the growth culture media might impact the synthesis of these immune-active polysaccharides by BCG and might represent a simple way to enhance the immunogenic potential and protective efficacy of BCG vaccines. Our subsequent analyses revealed AM and DG production does indeed vary among different BCG sub-strains. We found non-treatment of BCG with detergents prior to interaction with macrophages interferes with invasion but is essential for the induction of inflammation. We observed that growth of BCG in 7H9, modified Sautons medium with low glycerol and its derivatives results in the increased production of AM in all BCG sub-strains while DG production is variably increased in most but not all BCG sub-strains. Interestingly, growth of BCG in 7H9 and low glycerol Sautons medium also enhances their ability to induce inflammation in macrophages although this varies greatly among different BCG sub-strains. However, we found no clear association between media-derived changes to AM and/or DG levels among BCG sub-strains with their capacity to induce inflammation in macrophages. These results suggest BCG cell-surface molecules other than CPs may be involved. In conclusion, the collective results from our work in this project indicate the relationship between BCG CP composition/abundance and the induction of host immune responses is far more complex than previously appreciated.									
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1. INTRODUCTION:

Tuberculosis (TB) is an airborne bacterial infection caused by members of the *M. tuberculosis*-complex (MTBC) that typically affects the lungs¹. The live attenuated *Mycobacterium bovis* bacillus Calmette-Guerin (BCG) is the only vaccine available to protect against TB. Although it has been in use for almost 100 years to protect new-born infants against serious forms of TB disease, its performance is not consistent². Moreover, BCG is not effective at protecting adults against pulmonary TB. Of the several different reasons provided to explain its shortcomings as a TB vaccine, is the fact that there are many genetically distinct BCG sub-strains in existence today, including the ones in clinical use in different parts of the world². Given that the major capsular polysaccharides (CP), arabino-mannan (AM) and α -D-glucan (DG), are implicated in mycobacteria-host interactions and immune activation³, we hypothesized genetic heterogeneity might impact the production of AM and DG in BCG and in turn partially explain its imperfect record as TB vaccines. As such, we set out to determine if AM and DG do indeed vary among different BCG sub-strains and if this might correlate with differential activation of host innate and adaptive immunity. Furthermore, it has been shown that growth conditions can strongly influence BCG immunobiology. Therefore, we also decided to interrogate whether changes in growth culture media might influence production of AM and DG. As such, this project had 3 main objectives:

1. Determine if genetically distinct BCG sub-strains produce different amounts of AM and DG.
2. Determine if variations in AM and DG correlate with differential BCG activation of host innate and adaptive immunity.
3. Determine if changes to the composition of the culture media affects AM and DG in BCG, and in turn impact their immunogenicity.

The information obtained in this project was intended to provide an explanation as to why some BCG sub-strains are more immunogenic and protective than others. And by examining if judicious changes in the composition of BCG growth culture media can modify AM and DG levels and enhance the live TB vaccine's immunogenicity, we hope to provide is a simple way to produce more protective BCG vaccines.

2. KEYWORDS:

BCG, capsular polysaccharides, BCG-macrophage interactions, innate immune response

3. ACCOMPLISHMENTS:

Major goals of the project.

Aim 1. Examination of CP (AM and DG) produced by BCG sub-strains.

Major task 1: Optimization of BCG CP extraction and analyses.

Milestones achieved: Preparation and analysis of CP samples produced by BCG grown in Sautons vs 7H9 media. 100% completed by month 6.

Major task 2: LC-MS analyses of CP.

Milestone: LC-MS experiments of BCG CP samples 10% of proposed work achieved by month 19. Further optimisation and data analysis as planned in revised NCE SOW could not be completed due to COVID-19 pandemic related shutdowns and public health department-mandated home-isolation of lab personnel due to diagnoses of a positive case of COVID-19 in 2020.

Aim 2. Assessment of CP-mediated macrophage-BCG interactions.

Major task 1: Preparation of 7H9- and Sautons-grown BCG^{+CP} and BCG^{-CP}, and assessment of their interactions with macrophages.

Milestone: Assessment of CP-mediated macrophage-BCG interactions 100% complete by month 25. This aim was expanded and completed by month 30 to compensate for the inability to proceed with mouse studies in aim 3 and 4 (see below).

Aim 3. Assessment of CP-mediated immune-stimulation in mice by BCG^{-CP} vs BCG^{+CP}.

Major task 1 and 2: Preparation of 7H9- and Sautons-grown BCG^{+CP} and BCG^{-CP}, inoculate mice and assess cell-mediated immunity.

Milestone: Assessment of CP-mediated immune-stimulation in mice could not be performed due to COVID-19 pandemic related shutdowns and public health department-mandated home-isolation of lab personnel due to diagnoses of a positive case of COVID-19.

Aim 4. Identification of exogenous factors that might enhance CP (AM and DG) production.

Major task 1: Assess effects of culturing in modified Sautons on BCG CP.

Milestone: Preparation and analysis of CP from BCG grown in modified Sautons media 100% completed by month 30.

Major task 2: Assess growth in Sautons with serine hydroxamate (SHX) on BCG CP and cell-mediated immunity in mice.

Milestone: Preparation and analysis of CP from BCG grown in modified Sautons media containing SHX 100% completed by month 30. Assessment of BCG grown in presence of SHX in mice could not be performed due to COVID-19 pandemic related shutdowns and public health department-mandated home-isolation of lab personnel due to diagnoses of a positive case of COVID-19.

Accomplishments to date.

Aim 1. Examination of CP (AM and DG) produced by BCG sub-strains.

Major task 1: Optimize BCG CP extraction and analyses.

Initially, six BCG sub-strains - BCG-Russia, Japan, Moreau, Danish, Tice and Pasteur - were analyzed. Briefly, these live BCG vaccine sub-strains were grown in 7H9 and Sautons liquid culture media, both of which are commonly used to grow mycobacteria including during large-scale preparations of clinical grade BCG. Typically, detergents like Tween-80 and tyloxapol are also added to liquid growth media to prevent clinical mycobacterial cell clumping. This practice is known however to cause the passive sloughing off and significant loss of mycobacterial cell surface glycans and glycolipids which interferes with their analysis. As such, the BCG cultures used here for CP analysis were not grown in the presence of detergents, and once grown to mid-logarithmic phase of growth, were centrifuged and the resulting cell pellets washed with phosphate-buffered saline (PBS) before lyophilization. 20 mg dry biomass of the BCG sub-strains were carefully weighed out, resuspended in 1mL PBS with 0.5% of the non-ionic detergent tyloxapol and vigorously agitated in a bead-beater set at maximum strength. Agitation over various intervals of time were tested for maximal extraction before 3 cycles of 1 minute each was found to be optimal. The mixture was centrifuged and the supernatant containing extracted CP filtered through 0.45-micron filters. The filtrates containing extracted CP were serially diluted 2-fold in PBS. 3 μ L each of undiluted, 2, 4, 8 and 16-fold dilutions of CP extract from BCG sub-strains grown in either Sautons or 7H9 media were spotted on nitrocellulose membranes before probing with mouse monoclonal antibodies specific for either AM (clone F30.5) or DG (clone IV58B6)⁴. After much

trial and error, anti-AM and anti-DG antibodies at 1:1000 and 1:100 dilutions respectively were found to yield the best results. The results from experiments focused on BCG-Russia, Japan, Moreau, Danish, Tice and Pasteur revealed the amounts of AM did not vary among these six BCG sub-strains (Figure 1A). Nevertheless, AM levels produced by the six BCG sub-strains when grown in 7H9 medium were approximately 2 to 3-fold higher than when they were grown in Sautons medium (Figure 1A). With respect to DG, BCG-Moreau was found to produce more DG than the other 5 BCG sub-strains regardless of growth in 7H9 or Sautons medium (Figure 1B). Furthermore, DG produced by BCG-Russia, Japan, Moreau and Tice appeared to be increased after growth in 7H9 medium compared to Sautons medium (Figure 1B). Exceptionally, DG of BCG-Danish grown in 7H9 and Sautons media appeared to be similar while BCG-Pasteur appeared to produce more DG when it was grown in Sautons media compared to when it was grown in 7H9 (Figure 1B).

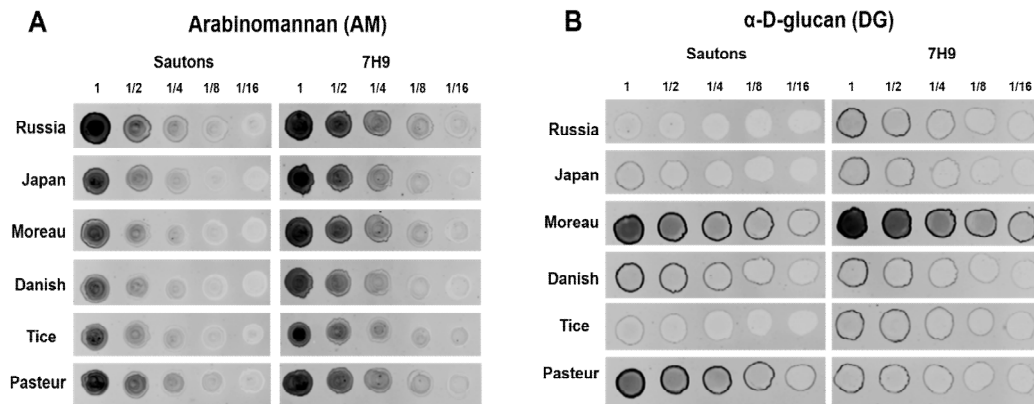


Figure 1. Dot-blots of AM (A) and DG (B) produced by BCG-Russia, Japan, Moreau, Danish, Tice, and Pasteur grown in Sautons and 7H9 media. The first undiluted spot is 3 μ L of an extract of freeze-dried BCG cells PBS with 0.5% tyloxapol at 20 mg/mL. Subsequent spots are 3 μ L of 2-fold serial dilutions. Each dot-blot is representative of 3 independent experiments.

Differential growth rates of the different BCG sub-strains in the two media types was ruled out as the underlying cause of the differences in AM and DG abundance since the total dry biomass of a given BCG sub-strain was found to be similar regardless of growth in Sautons or 7H9. Measurement of BCG growth over time in both Sautons or 7H9 by turbidometry (absorbance reading at OD_{600nm}) also supports this conclusion (Figure 2A and B).

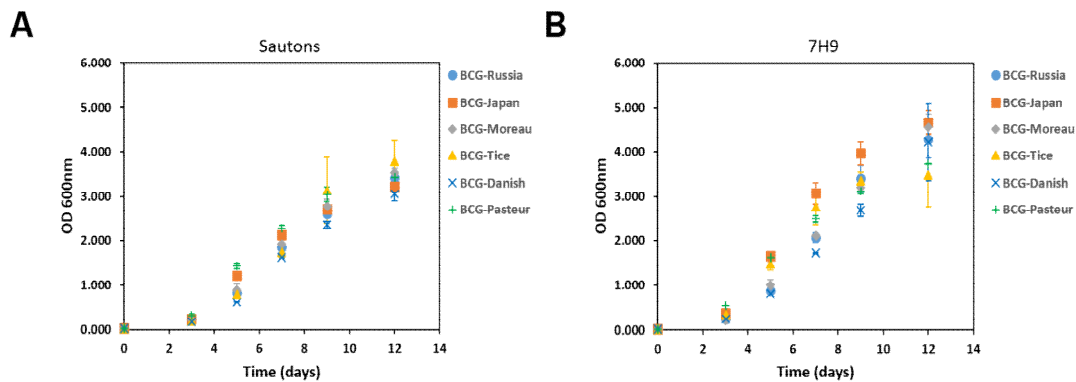


Figure 2. Growth rate measurements of BCG-Russia, Japan, Moreau, Danish, Tice, and Pasteur in Sautons (A) and 7H9 (B) media. Each data-point is the mean of 3 independent experiments and error bars represent standard deviations.

Given the absence of significant variations in AM abundance among the six BCG sub-strains, additional BCG sub-strains in our collection were also included for assessment and characterization. Accordingly, BCG-Sweden, Birkhaug, Prague, Glaxo, Frappier and Phipps in addition to the six sub-strains were analyzed further. Indeed, we were able to replicate and confirm our previous results with BCG-Russia, Japan, Moreau, Danish, Tice and Pasteur but were now able to identify BCG sub-strains with more noticeable differences in CP composition regardless of growth in Sautons or 7H9 medium. Most notably, BCG-Prague was found to produce little to no AM compared to the other BCG strains grown in Sautons medium (Figure 3A). Most BCG sub-strains including BCG-Prague, when grown in 7H9 medium increased their production of AM (Figure 3A). With respect to DG, BCG-Russia, Birkhaug, Prague, and Glaxo were found to produce extremely low amounts of this CP when grown in Sautons medium (Figure 3B). And with the exception of BCG-Danish and Pasteur, most BCG sub-strains cultured in 7H9 medium appeared to increase their production of DG albeit to varying degrees (Figure 3B).

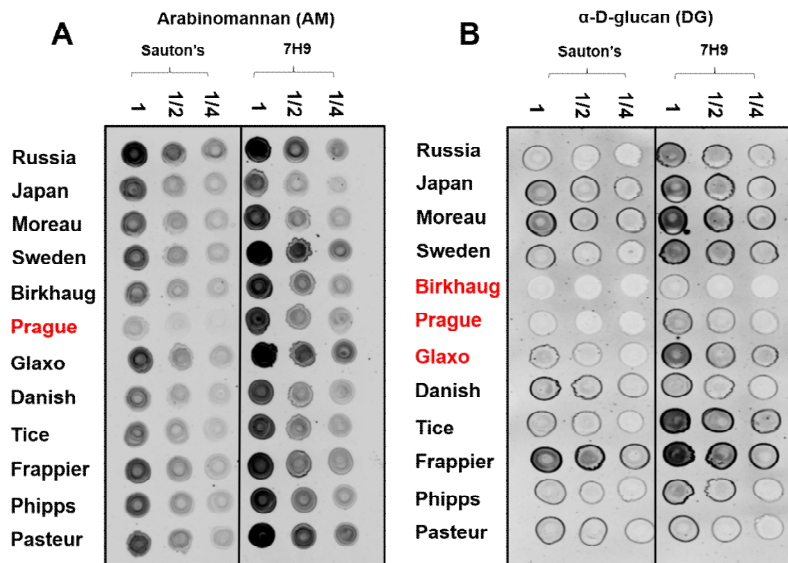


Figure 3. Dot-blots of AM (A) and DG (B) produced by 12 different BCG strains grown in Sautons and 7H9 medium without detergent. The first undiluted spot is 3 μ L of PBS with 0.5% tyloxapol extracts of the freeze-dried BCG cells at 20 mg/mL. Subsequent spots are 3 μ L of 2-fold serial dilutions. Representative of 4 independent experiments.

Attempts to quantitatively assess AM and DG by ELISA was technically challenging and depleting on the limited amounts of AM and DG-specific monoclonal antibodies available. Furthermore, due to the COVID-19 related shut-down of our lab in 2020, and limited operational capacity since then, attempts to refine ELISA-based methods to quantify CPs was abandoned and the semi-quantitative dot-blot approach was used exclusively going forward. Nevertheless, the results from these experiments reveal:

- 1) AM and DG production is highly variable among the twelve BCG sub-strains assessed in this project – this addresses objective 1 and confirms our hypothesis that genetically distinct BCG produce variable amounts of AM and DG.
- 2) The variation in AM and DG among different BCG sub-strains is growth medium dependent.
- 3) Compared to Sautons medium, growth in 7H9 medium leads to increased production of AM in all BCG sub-strains while DG production is variably increased in most but not all BCG sub-strains.

Major task 2: LC-MS analyses of CP.

LC-MS analysis of BCG CP extracts was attempted but due to the complexity of the extract the results were undecipherable. Due to COVID-19 related shutdowns and limited operational capacity of our lab, a re-prioritization of efforts was required and no further attempts to optimize and develop this approach could be made.

Aim 2. Assessment of CP-mediated macrophage-BCG interactions.

Major task 1: Preparation of 7H9 and Sautons-grown BCG^{+CP} and BCG^{-CP}, and assessment of their interactions with macrophages.

Much testing and optimisation was required to generate BCG lacking CP (BCG^{-CP}) to compare with BCG possessing their full complement of CP (BCG^{+CP}) in interactions with macrophages and the induction of innate immune responses. Consistent with what has been reported in the literature⁵, we discovered that vortexing and/or sonication followed by washing of BCG cells with PBS does not remove CP from the cell surface (Figure 4A). In contrast, incubation of BCG cells for 10 minutes at 37°C in PBS supplemented with 0.5% tyloxapol followed by washing with PBS removed AM and DG from the BCG cell surface without affecting its viability (Figure 4B). This detergent-mediated effect on CP is consistent with what has been reported previously in the research literature⁶.

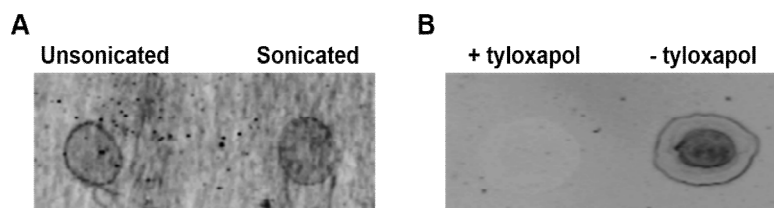


Figure 4. Dot-blot of undiluted AM from extracts of unsonicated and sonicated (A), and tyloxapol treated and untreated (B) cells of BCG-Pasteur. The same results were obtained with DG and was similar for all other BCG sub-strains used in this study. Representative of 3 independent experiments.

To compare host-cell invasion by BCG^{-CP} and BCG^{+CP}, the human THP-1 macrophage cell-line was employed as reported previously⁷. Briefly, 3×10^5 THP-1 cells seeded in micro-well plates were infected for 4 hours with either BCG^{-CP} or BCG^{+CP} cells at a multiplicity of infection (MOI) of 1. Thereafter, the adherent THP-1 cells were washed to remove uninternalized BCG and lysed. The lysates were serially diluted and plated out on Middlebrook 7H11 agar plates to enumerate internalized BCG. Percent invasion was defined as: colony forming units (CFU) recovered from lysis of the 3×10^5 THP-1 cells after 4 hours of infection/ 3×10^5 CFU of BCG added to the macrophages at the start of infection $\times 100$. Consistent with published reports⁵, BCG^{-CP} were found to invade THP-1 cells approximately 1.5 to 2-fold more than BCG^{+CP}. However, among BCG^{+CP} BCG-Moreau, Birkhaug and Sweden appeared to be less invasive than other sub-strains regardless of the type of media they had been grown in prior to infection (Figure 5). The results of these experiments reveal:

- 1) While BCG CPs are highly resistant to mechanical stress, we found that detergents can effectively strip CPs off the mycobacterial cell surface.
- 2) While BCG^{-CP} are more efficient at entering macrophages, different sub-strains enter at different rates regardless of their CP status.
- 3) Different BCG sub-strains enter macrophages at significantly different rates, regardless of growth in Sautons or 7H9 media.

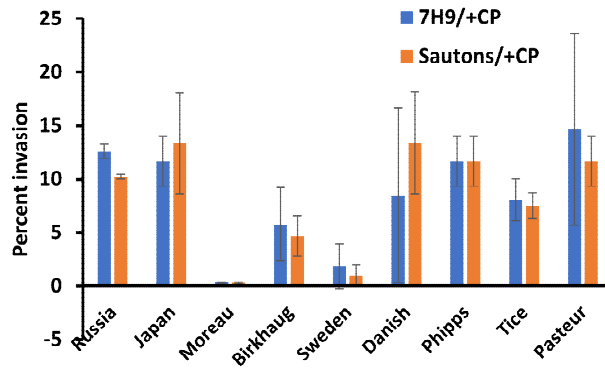


Figure 5. Invasiveness of different BCG^{+CP} grown in Sautons or 7H9 media. Data shown is the average of 2 independent experiments with error bars signifying standard deviations of the values.

To assess the induction of pro-inflammatory responses which is usually predictive of TB vaccine protective efficacy^{6,8,9}, 3×10^5 human THP-1 macrophage cells/well in micro-well plates were infected for 24 hours with either BCG^{-CP} or BCG^{+CP} cells at a MOI of 10. The levels of pro-inflammatory cytokines TNF- α and IL-1 β produced and released by THP-1 cells into the culture supernatants were quantified by ELISA as described previously⁷. BCG^{+CP} were consistently found to induce higher TNF- α production by THP-1 cells than BCG^{-CP}, regardless of growth in Sautons or 7H9 media (Figure 6).

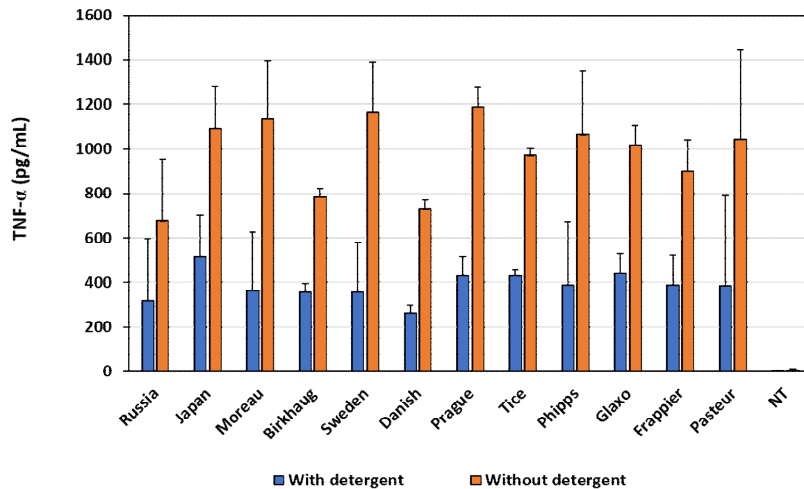


Figure 6. TNF- α production in THP-1 macrophages induced by BCG grown in 7H9 but subsequently treated with or without detergent. Values are averages of two independent experiments with each biological replicate consisting of two technical replicates. Error bars indicate standard error of means.

Further analysis revealed that some but not all BCG sub-strains induce higher TNF- α production in THP-1 cells when they are grown in 7H9 media (Figure 7). Of these BCG-Prague and Pasteur were notable examples that we decided to earmark for further study in aim 4.

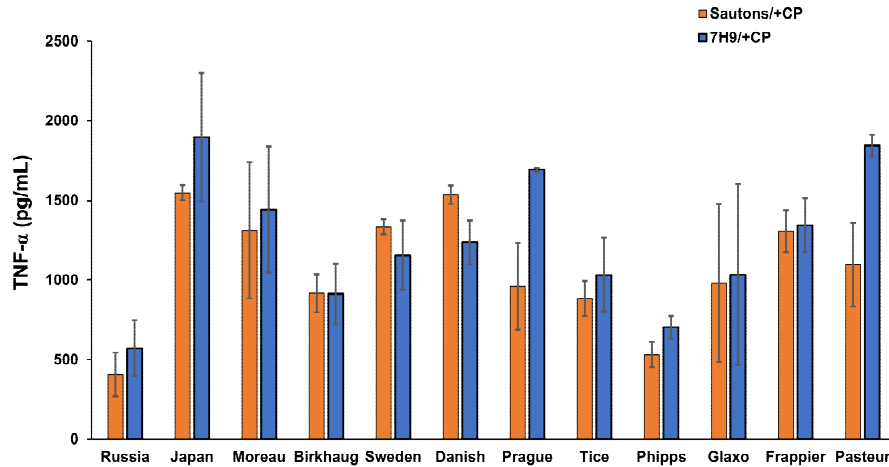


Figure 7. TNF- α production in THP-1 cells induced by BCG^{+CP} grown prior in Sautons vs 7H9. Values are averages of two independent experiments with each biological replicate consisting of two technical replicates. Error bars indicate the standard error of means.

As with TNF- α , higher IL-1 β production tended to be induced only by BCG^{+CP} regardless of growth in Sautons or 7H9 media (Figure 8A and B). Notable exceptions were observed with BCG-Japan and Moreau grown in 7H9 media (Figure 8B).

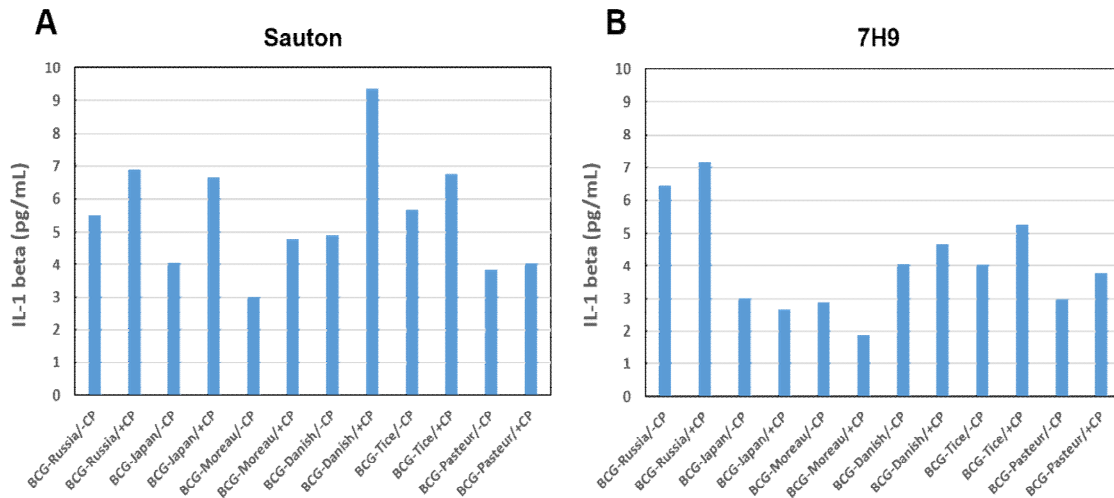


Figure 8. IL-1 β production in THP-1 cells induced by BCG^{-CP} vs BCG^{+CP} grown in Sautons (A) and 7H9 (B) media. Histogram is the mean of duplicate wells and representative of 2 independent experiments.

Although clear associations between AM and DG abundance and the ability of different BCG sub-strains to invade and induce pro-inflammatory responses in THP-1 macrophages were not observed, the results of these experiments clearly indicate:

1) CP retention on the BCG cell-surface by not treating with detergent is required to induce higher pro-inflammatory responses in macrophages by BCG. However, given that several other cell-surface components - proteins, glycolipids and other glycans - can also be removed as a result of detergent action, we cannot conclude that it is BCG CP alone that is mediating inflammation.

2) Induction of pro-inflammatory responses varies significantly between different BCG sub-strains, even between those with very similar AM and DG profiles and in some cases appear to be growth media dependent. This again suggests other BCG cell surface-associated proteins, glycolipids and other glycans are also involved in immune-stimulation.

Aim 3. Assessment of CP-mediated immune-stimulation in mice by BCG^{-CP} vs BCG^{+CP}.

While associations between AM or DG abundance in BCG sub-strains and their ability to induce pro-inflammatory responses in macrophages was not observed, an association with the ability to induce cell-mediated immunity, particularly T-cell mediated adaptive immunity cannot be ruled out. Although BCG^{-CP} or BCG^{+CP} were prepared and immunization trials in C57BL/6 mice were about to start, COVID-19 related shutdowns and the limited operational capacity of our lab and animal care personnel forced its termination.

Aim 4. Identification of exogenous factors that might enhance CP (AM and DG) production.

Major task 1: Assess growth in modified Sautons on BCG CP, prepare CP and analyze.

The results of experiments from aim 1 indicate that some BCG sub-strains when grown in Sautons medium tend to produce lower amounts of both AM and DG compared to growth in 7H9 medium. A key difference between the two types of liquid media is the significantly higher concentrations of glycerol present in Sautons¹⁰. Sautons medium contains 60mL/L glycerol while 7H9 medium contains 2mL/L glycerol. We therefore hypothesized that glycerol might negatively regulate CP synthesis. To test this, all twelve BCG strains were cultured in standard Sautons medium and modified Sautons medium with 2mL/L glycerol (hereafter referred to as Sauton LG). CP analysis performed as described in aim 1 revealed that with the exception of BCG-Russia and possibly BCG-Danish, most BCG sub-strains when cultured in Sautons LG produced more AM, albeit to varying degrees than in regular Sautons medium (Figure 9).

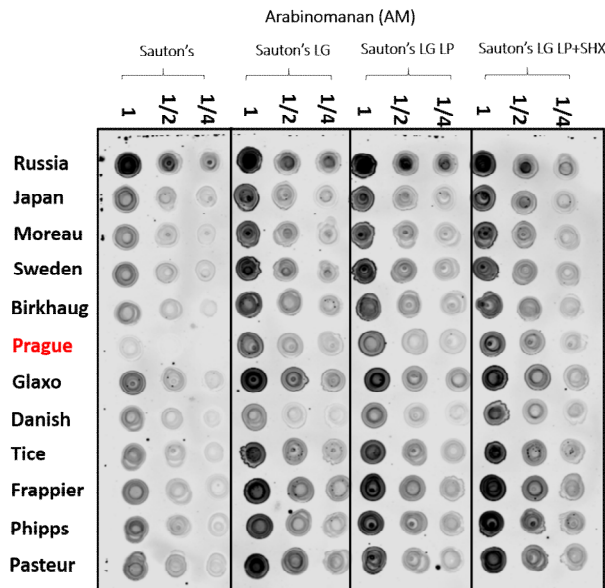


Figure 9. Dot-blots of AM produced by 12 different BCG sub-strains grown in Sauton's medium and modified versions (without detergent). The first undiluted spot is 3 μ L PBS with 0.5% tyloxapol extracts of the freeze-dried BCG^{+CP} cells at 20 mg/mL. Subsequent spots are 3 μ L of 2-fold serial dilutions. Representative of 3 independent experiments.

The regulatory effect of reduced glycerol on the production of DG in BCG on the other hand appeared to be even less consistent (Figure 10). With the exception of BCG-Russia, Danish, Phipps and Pasteur, most BCG sub-strains were found to produce more DG albeit to varying degrees when cultured in Sautons LG. These results suggest that increased DG production seen in BCG-Russia and Phipps cultured in 7H9 relative to Sauton's medium (Figure 1) may be due to factors other than glycerol. Moreover, the ability of BCG-Russia to respond specifically to glycerol levels in the growth medium may be impaired as its production of AM is unaffected. In contrast, the lack of changes in DG production by BCG-Danish and Pasteur, regardless of their growth in regular Sauton's, Sauton's LG or 7H9 media suggests their ability to respond to exogenous cues in the growth medium may be compromised.

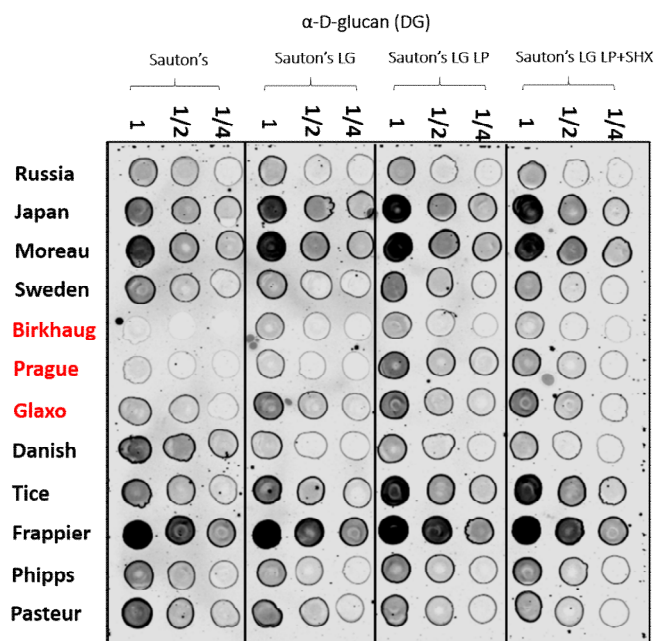


Figure 10. Dot-blots of DG produced by 12 different BCG strains grown in Sauton's medium and modified versions (without detergent). The first undiluted spot is 3 μ L PBS with 0.5% tyloxapol extracts of the freeze-dried BCG^{CP} cells at 20 mg/mL. Subsequent spots are 3 μ L of 2-fold serial dilutions. Representative of 3 independent experiments.

Reducing phosphate levels in culture media or addition of serine hydroxamate (SHX) have been shown previously to trigger the bacterial stringent or starvation response and upregulate CP production in mycobacteria⁴. As such, we wanted to determine if the presence of these two conditions might synergize with the lowered glycerol in Sautons LG medium to substantially boost AM and DG production by the BCG sub-strains (Figures 9 and 10). Thus, Sautons LG was modified by reducing the phosphate content to yield Sautons LG LP. This was modified further with the addition of SHX to yield Sautons LG LP+SHX. The different media types were then inoculated with BCG and the CP produced analyzed. Reduced phosphate and addition of SHX to Sautons LG induced very little to no change in the production of AM by **all** BCG sub-strains compared to Sautons LG alone (Figure 9). This suggests that under pre-existing conditions of low glycerol, the regulatory effects of reduced phosphate and SHX on AM production is negligible. In contrast, lowered phosphate and addition of SHX to Sautons LG appeared to marginally increase DG production in BCG-Japan, Moreau, Prague and Tice only. This suggests that under conditions

of low glycerol, the regulatory effects of reduced phosphate and SHX on DG production may be inactive in some BCG sub-strains but not in others. Taken together, these results indicate:

1) Reduced glycerol in growth media consistently increases AM production albeit to different levels by most BCG sub-strains.

2) However, reduction in glycerol does not uniformly affect DG production by different BCG sub-strains.

3) Further modification to low glycerol media such as reductions in phosphate levels and/or addition of SHX, which are known to induce the stringent/starvation response, has no further effect on AM and DG levels.

Major task 2: Assess growth in Sautons supplemented with serine hydroxamate (SHX) on BCG CP and cell-mediated immunity in mice.

Given that with BCG-Prague and BCG-Pasteur, increases in AM levels and their abilities to induce TNF- α production by THP-1 cells were noticeably higher when these two BCG sub-strains were grown in 7H9 media, we decided to examine the effects of growth in Sautons LG, Sautons LG LP and Sautons LG LP+SHX on their ability to induce inflammation in macrophages instead of T-cell activation *in vivo*. Consistent with what was observed previously (Figure 7), both BCG-Prague and Pasteur induced higher production of TNF- α when grown in 7H9 compared to Sautons (Figure 11). Interestingly, growth in modified Sautons LG also enhanced their capacities to induce higher production of TNF- α compared to Sautons (Figure 11).

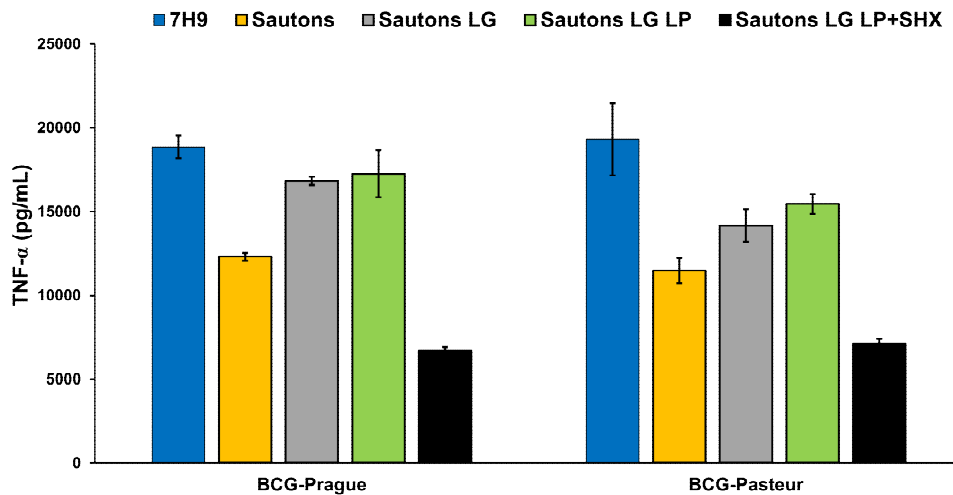


Figure 11. TNF- α production in THP-1 cells induced by BCG^{CP} grown in 7H9, Sautons, Sautons LG, Sautons LG LP and Sautons LG LP+SHX. Values are averages of two independent experiments with each biological replicate consisting of two technical replicates. Error bars indicate the standard error of means.

Further modification of low glycerol media by lowering phosphate had no appreciable effect on induction of TNF- α in THP-1 cells (Figure 11). Most strikingly, growth in Sautons LG LP+SHX actually reduced the ability of both BCG sub-strains to induce pro-inflammatory responses in THP-1 cells (Figure 11), despite the fact AM levels were equally higher when both BCG sub-strains were grown in 7H9, Sautons LG, Sautons LG LP and Sautons LG LP+SHX compared to growth in Sautons media (Figure 9). These results collectively indicate that other cell-surface molecules, presumably regulated by changes in media composition, can also modulate the host immune response.

Main Findings and Conclusions:

In this project, we sought to analyze the CPs of twelve different BCG sub-strains that reflect the global diversity of existing BCG vaccines. We also compared their abilities to enter and induce pro-inflammatory innate immune responses in macrophages in an effort to identify an association between BCG CP and immunostimulatory capacity. Finally, we examined if changes to growth media can affect CP composition and abundance in BCG and if any changes so mediated associate with changes in immunostimulatory capacity. Some of our most salient findings are reiterated below:

- AM and DG production varies among the twelve BCG sub-strains assessed and is growth media dependent.
- BCG CPs (AM and DG) are resistant to removal due to mechanical stress but not to the action of detergents.
- Growth in lower glycerol-containing media like 7H9, modified Sautons with low glycerol and its derivatives results in the increased production of AM in all BCG sub-strains while DG production is variably increased in most but not all BCG sub-strains.
- While retention of CP by not treating BCG cells with detergent interferes with the invasion of host-cells like macrophages, it is essential for the induction of higher levels of inflammation in this innate immune cell. However, as there are potentially other cell-surface components - proteins, glycolipids and other glycans – that can also be removed as a result of detergent action, it cannot be concluded BCG CP alone that is responsible for mediating both these effects.
- Different BCG sub-strains invade macrophages and induce inflammation at significantly different rates, regardless of CP status.
- There is no clear association between AM and/or DG levels among BCG sub-strains with the capacity to induce inflammation.
- Reducing glycerol levels in the culture media also enhances the capacity to induce inflammation, however a clear association between the changes in AM and/or DG with respect to immunogenicity was not observed.

Collectively, our investigations have revealed the relationship between BCG CP composition and abundance with the induction of host immune responses is not straight forward and far more complex than previously appreciated.

What opportunities for training and professional development has the project provided?

Nothing to report.

How were the results disseminated to communities of interest?

Nothing to report.

What do you plan to do during the next reporting period to accomplish the goals?

Nothing to report.

4. IMPACT:

Impact on the development of the principal discipline(s) of the project.

Non-treatment of BCG with detergent prior to its interaction with macrophages is critical for induction of higher pro-inflammatory responses in these innate immune cells - a key predictor of vaccine efficacy. As such growth of BCG in detergent-free media may be a valuable approach to enhance its inflammatory capacity. Likewise, growth of BCG in media with low glycerol but not with SHX enhances the vaccine's ability to induce inflammation – as such these two particular growth conditions may be a valuable approach to boost its protective efficacy and will be of significance to commercial TB vaccine producers.

What was the impact on other disciplines?

Nothing to report.

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

Nothing to report.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Our initial plan to focus on only six BCG strains has been revised to include additional strains – bringing the total analyzed to twelve. The reason for this change was the observation that the six initial BCG strains assessed did not exhibit significant variations in CP. As such the remaining experiments performed involved all twelve BCG strains.

Actual or anticipated problems or delays and actions or plans to resolve them.

The COVID-19 pandemic, particularly the shutdown of our laboratory from March to July 2020 significantly hampered progress. Partial resumption of operations with strict public health restrictions in place, allowed us to conduct some of the proposed experiments indicated in the NCE SOW but at a much slower pace. Unfortunately, *in vivo* mouse work to assess the impact of CP on activation of adaptive immunity could not be performed. Nevertheless, this investigation will be performed in conjunction with other research projects that are ongoing in our lab.

Changes that had a significant impact on expenditures.

Nothing to report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents.

No mouse experiments were conducted.

Significant changes in use or care of human subjects.

Not applicable.

Significant changes in use or care of vertebrate animals.

No mouse experiments were conducted, as such no animals were used.

Significant changes in use of biohazards and/or select agents.
Not applicable.

6. PRODUCTS:

- **Publications, conference papers, and presentations**
Nothing to report.
- **Website(s) or other Internet site(s)**
Nothing to report.
- **Technologies or techniques**
Nothing to report.
- **Inventions, patent applications, and/or licenses**
Nothing to report.
- **Other Products**
A method to culture certain BCG vaccine sub-strains that maximizes their production of immunogenic cell-surface components and enhances their utility as TB vaccines.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

No change - Jeffrey Chen PhD (Principal investigator) and Ze Lim MSc (Research technician).

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report.

What other organizations were involved as partners?

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

Not applicable.

QUAD CHARTS:

Not applicable.

9. APPENDICES:

References

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