MRC London Intercollegiate DTP

i. Aims and objectives

This project aims to accelerate the development of effective mucosal vaccines for protection against *Mycobacterium tuberculosis* (MTB) by addressing the following objectives:
1. Develop an *ex vivo* pre-clinical method to evaluate the efficacy of mucosal vaccines in 18 diverse clinical isolates of MTB.
2. Perform subsequent analysis of host transcription and biomarker levels to identify the molecular mechanisms by which
MTB growth is affected by the host immune response, and what constitutes protective immunity during MTB infection.
3. Sequence the transcriptome of MTB clinical isolates able to evade immunity to improve understanding of the mechanisms
involved in evasion and to identify novel vaccine targets.

ii. **Background**

According to the World Health Organization (WHO) global tuberculosis mortality has decreased by 47% since 1990. Despite this significant decrease, tuberculosis remains a major cause of global mortality, killing one person every 21 seconds in 2014, largely due to lack of diagnosis or quality care, and drug resistance. Drug and diagnostic test improvements are an important panacea to tuberculosis; however the only cost-effective solution to long-term control and future eradication of tuberculosis will be the development of an effective tuberculosis vaccine.

Tuberculosis is an airborne bacterial infection caused by MTB. One-third of the world's population is estimated to be infected with MTB, with 10% predicted to develop active tuberculosis. The incidence and mortality of active disease in immunocompromised individuals, such as those with HIV, is higher. In 2014, 12% of the 9.6 million new tuberculosis cases were HIV-positive. In the absence of treatment, the death rate in individuals infected with tuberculosis is high; however treatment success rates have been reported to exceed 85% where effective detection and treatment strategies have been implemented.

Despite considerable advances in the detection and treatment of tuberculosis, multidrug-resistant (MDR) tuberculosis and extensively drug-resistant (XDR) tuberculosis remain a global problem. Both MDR and XDR tuberculosis are associated with long treatment duration and poor outcome compared with drug-susceptible tuberculosis, and are generally considered to arise from a combination of physician error and non-compliance during treatment of drug-susceptible tuberculosis. In 2014, 5% of tuberculosis cases were reported to be MDR, with 9.7% of MDR cases identified as XDR.

At present, the bacille Calmette–Guérin (BCG) vaccine, developed in 1921, remains the only clinically approved vaccine against tuberculosis. The vaccine is administered at birth, with a 60–80% protective efficacy against serious forms of tuberculosis in children. BCG-induced immunity has been reported to decrease over time, so that by adulthood most people are no longer protected. In addition to this, BCG has not shown efficacy in individuals already infected or exposed to tuberculosis.

In 2015, the WHO communicated 'the need for prioritization and additional funding for tuberculosis vaccine research to develop a more effective vaccine to contribute to tuberculosis control' which could i) replace the BCG vaccine, ii) improve the efficacy of BCG and/or iii)

shorten tuberculosis treatment. At present, approximately $8 billion is spent on TB diagnosis and treatment. It was recently reported that the development of a new TB vaccine in the next 10–15 years would cost $0.8–1 billion; just 1% of the annual cost of TB diagnosis and treatment. In the same modelling study it was estimated that delivery of an adolescent and adult vaccine with 60% efficacy to 20% of the at-risk population could prevent 30-50 million new cases of tuberculosis by 2050.

To date, the only new candidate vaccine against tuberculosis to enter clinical trial has been MVA85A. Despite demonstrating no efficacy against tuberculosis in adults or infants, a vast amount of data and experience was generated from the MVA85A trials, and it is important that the direction of future vaccine development and study design is influenced by lessons learnt from this work. At present there are 16 vaccines in active clinical development, with the research community in general agreement that the optimal vaccine candidate must have efficacy both before and after tuberculosis infection. In addition, it has also been largely agreed that a prime-boost strategy in adolescents or young adults is likely to generate the most significant reduction in tuberculosis burden and meet the WHO aim to globally eradicate tuberculosis infection by 2050.

Identification of an accurate surrogate marker of a protective immune response to MTB would facilitate the development of a tuberculosis vaccine substantially. Since T-cell proliferation in response to antigen is a well-known phenomenon, the analysis of the proliferation and cytokine expression profile of T cells is the current mainstay method for vaccine validation, in particular analysis of interferon-γ (IFN-γ). MTB growth has also been investigated as a marker of immune response; based on the principle that host cells will inhibit MTB growth if an effective immune response has been induced. A number of research groups have developed *ex vivo* mycobacterial growth inhibition assays (MGIAs). In the past, use of these assays was met with concerns regarding reproducibility, however, more recent research recommending that a degree of caution should be applied when utilising T-cell immune markers to validate tuberculosis vaccine candidates, has led to the resurrection of MGIAs as tools to evaluate the efficacy of anti-mycobacterial agents.

Fletcher and colleagues recently published an *ex vivo* MGIA protocol developed for tuberculosis vaccine testing. The assay can be used to determine overall change in bacterial count using the BACTEC MGIT system; a fluorescence quenching system based on oxygen concentration. The protocol describes analysis of splenocytes obtained from vaccinated mice but is currently of validating tuberculosis vaccines at a pre-clinical stage, enabling researchers to refine, reject and develop candidates in a more time- and cost-effective manner. The validation of vaccine candidates at this stage is also in line with the guiding principles for use of animals in testing; the Three Rs – replace, reduce, refine. In the current climate where funding for tuberculosis research is scarce and the need for a novel vaccine is crucial, the development of pre-clinical validation methods is desperately needed to ‘de-risk’ vaccine development, and rapidly and effectively identify promising new candidate vaccines.

In the same vein as the validation of vaccine candidates at a pre-clinical stage, a significant movement towards basic research into the tuberculosis–host interaction has occurred of late, with research and funding bodies looking to refine and revise existing models. The tuberculosis research community is now recognising that, in the past, while research concentrated its efforts largely at the clinical level, the tools and technologies of basic research have advanced profoundly. The utilisation and further development of these advances to improve our understanding of tuberculosis may be the method by which an effective vaccine candidate is identified. An example of the ‘single track’ in which tuberculosis vaccination research currently

finds itself can be exemplified by the range of targets within the 16 vaccines currently in active clinical development: these 16 vaccines utilise just 12 of 4,500 (0.27%) targetable antigens identified in the MTB genome.

This change in innovation is exemplified by the GC6 consortium. The group aim to 'define biomarkers of protection and disease for clinical testing of novel drugs, vaccines and diagnostics for tuberculosis as well as for the design of novel vaccine candidates and diagnostics'. This aim is largely being addressed via gene expression profiling and molecular analysis of tuberculosis strains and blood samples obtained from various patient cohorts.

Following the MVA85A vaccine trials, the immune response to vaccination was studied by Fletcher and colleagues using gene expression profiling and molecular analysis. IFN-γ ELISpot assay, microarrays and RNA-seq uncovered considerable variations in both individual and geographical gene signatures, indicative of a highly complex and variable immune response to vaccination.

Gene expression profiling and molecular analysis of MTB isolates and the host response represent a pivotal method by which we can identify novel vaccine candidates and targets. It is becoming increasingly apparent that a multidisciplinary, pre-clinical strategy must be developed in order to understand the vastly complex nature of the immune response to tuberculosis vaccination; ultimately enabling us to develop a long-term vaccination strategy able to tackle the significant global burden of tuberculosis that remains today.

iii. Methodology

1. An MGIA, developed by the Fletcher group, will be adapted to evaluate the efficacy of a vaccine-induced immune response to control growth of 18 diverse clinical isolates of MTB. Mucosal vaccine efficacy will be investigated by intranasal immunisation of mice with the candidate vaccine. Mice will be sacrificed at peak immune response and splenocytes isolated. Whole blood samples will also be obtained for future analysis of peripheral blood mononuclear cell (PBMCs). Splenocytes and each MTB isolate will be co-cultured to determine overall change in bacterial count using the BACTEC MGIT system; a fluorescence quenching system based on oxygen concentration - the lower the oxygen levels, i.e. in the presence of metabolising bacteria, the higher the fluorescence. A 'time to detection' value is generated which can be used to calculate bacterial numbers (CFU). The generation of normalised bacterial numbers will enable direct comparison of vaccine efficacy in the clinical isolates (control vs vaccine), as well as efficacy between the vaccines themselves (vaccine vs vaccine).

2. Analysis of transcription and biomarker levels will also be performed in the splenocytes obtained from the control and intranasally immunised mice. Samples will be obtained at various time points during *in vitro* co-culture of the splenocytes with a clinical isolate of MTB. Flow cytometry, ELISpot and ELISA will be performed to measure cytokines and cell surface markers. Microarray will be performed to determine gene expression levels following MTB exposure in vaccinated mice; as well as identifying the up-/down-regulation of specific genes, data from the microarray will be used to carry out pathway analysis and identify functional clusters of gene expression using databases such as KEGG. Interesting candidate genes identified by microarray will be validated and quantified using qPCR. Transcriptional and biomarker data will be aligned with growth response data generated from the MGIA to correlate known markers of immune response to vaccination, and identify novel mechanisms of growth inhibition and predictors of vaccine efficacy.

3. The transcriptome of the MTB clinical isolates will be sequenced via whole-genome gene expression microarrays or RNAseq to improve understanding of the mechanisms by which it evades the immune system and to identify novel vaccine targets. The method of transcriptome analysis would largely depend on the availability of a reference genome for each isolate; microarrays require *a priori* knowledge of the genome to generate a transcription map whereas sequence reads generated by RNA-seq can be assembled *de novo*. Transcriptomics analyses the set of RNA transcripts produced by the genome under specific conditions, MTB isolates would therefore be analysed following co-culture with splenocytes from intranasally vaccinated mice and transcription quantified for comparison with splenocytes from control mice. This will provide insight into the molecular mechanisms by which MTB isolates evade immunity through the up-/down-regulation of gene expression.

iv. Expected outcomes

1. With seven major lineages of MTB worldwide it is vital that vaccine candidate efficacy is evaluated in diverse clinical isolates of MTB from an early stage in vaccine development. The development of a system which enables direct comparison of vaccine efficacy in clinical isolates, as well as efficacy between the vaccines themselves will generate a more time- and cost-effective method of identifying promising candidates and eliminating unsuitable designs at an earlier stage of development. The development of a 'gold-standard' system of evaluation will not only provide the opportunity for rapid insight into candidate efficacy but it will also generate a standardised method of evaluation for direct comparison of vaccines between research groups, with the potential to create a 'database' of results which will be invaluable for future vaccine design.

2. Gene expression and biomarker analysis of isolated splenocytes co-cultured with clinical isolates of MTB will provide key molecular insight into the correlation between MTB growth and the host immune response. Transcriptional or biomarker correlates of reduced mycobacterial growth will be further validated as novel indicators of protective immunity which could be used to reinforce growth data when evaluating the efficacy of mucosal vaccines. In addition, data may also improve our understanding of the molecular mechanisms involved in protective immunity during MTB infection and aid future drug design or refinement.

3. Transcriptional analysis of MTB clinical isolates able to evade vaccine-induced immunity will provide indispensable direction for the future design and refinement of vaccines. The identification of genes and pathways activated in MTB which facilitate immune evasion will be valuable to both the clinical and basic research community. Candidate genes identified by this analysis should be validated by qPCR and protein analysis performed. Future investigations could employ genetic techniques such as gene knockout to validate the importance of the gene to MTB during evasion.

v. Previous research experience

During my 12-month industrial placement in [removed] I gained experience and expertise in numerous techniques relevant to the work outlined in this proposal. My project focused on the role of decorin in tumour angiogenesis, and included an extensive *in vivo* Matrigel plug assay in mice, western blotting, dot blot and slot blot, immunohistochemistry and qPCR. Other techniques involved in the project included cell culture work with both endothelial and tumour cell lines, migration assays and PCR. Over the year I was able to develop my technique and knowledge of qPCR, and I took over the majority of the qPCR analysis carried out in the laboratory. This included the verification of two arrays, including an important discovery phase

[removed] array carried out for a grant, and an RT2 Profiler PCR Array. In my final year research project on diabetes, experience from my placement was particularly useful in reducing the time to start up this short project and generate data.

My experience in animal research and transcriptional analysis are directly relevant to this research project. In addition, my placement provided me with an important insight into academic research: the day-to-day routine of the lab, weekly data presentation, seminars, poster presentations and grant preparation. As second author of an article published in the *Journal of Biological Chemistry* I also have first-hand experience in the preparation and publication of data in a peer-reviewed journal. As part of my role as the editor of two medical journals I also have considerable experience in figure generation using Adobe Photoshop and Illustrator, which I consider to be invaluable for the communication of data.

Key references

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